Biochemistry and Regulation of Streptomycin and Mannosidostreptomycinase (α-D-Mannosidase) Formation

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"A new field of research has been opened to us; the possibility of elucidating many complex natural processes heretofore not sufficiently understood and of discovering new agents that may help man in controlling diseases . . . is certainly inviting."

Selman A. Waksman, 1943

INTRODUCTION

Since its discovery in 1944 (104), streptomycin has admirably fulfilled Waksman's prophecy. Its extension of chemotherapy to many gram-negative bacteria and to *Mycobacterium tuberculosis* had a major impact on medicine, recognized in the award of the Nobel prize to Waksman in 1952. Moreover, as the first commercially successful antibiotic produced by an actinomycete, it led the way to the recognition of these organisms as the most prolific producers of antibiotics. Finally, streptomycin has provided a valuable tool for studying cell function. After a period of time during which it was thought to act by altering permeability, its interference with protein synthesis was recognized as its primary effect. Its interaction with ribosomes has provided much information on their structure and function; it not only inhibits their action but also causes misreading of the genetic code and is required for the function of ribosomes from dependent mutants. Yet, despite all these advances, the mechanism of the bactericidal action of streptomycin still has not been definitively settled.

Of possible interest to the reader are some previous reviews on the microbial formation of streptomycin, written by Woodruff and McDaniel (143), Hunter (57), Hockenhull (49), Waksman and Lechevalier (126), Walker (129), Mendecino and Picken (78), and Horner (55). The last survey (55) was concluded over 3 years ago. The present review will bring the field up to date as well as emphasize to a greater degree the significance of *Streptomyces* mannosidase in the streptomycin fermentation.

STREPTOMYCIN FERMENTATION— STATE OF THE ART

The first details of a fermentation process for the production of streptomycin appeared in 1946.

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Waksman et al. (127) described production in media containing meat extract or corn-steep liquor. In that same year, Rake and Donovick (99) developed a medium which has become the classical fermentation medium for streptomycin production, containing D-glucose as carbon source, soybean meal as nitrogen source, and sodium chloride. NaCl might be involved in release of the antibiotic from the mycelia. In this type of medium, production of 5 to 10 g/liter is being achieved commercially (55). Many chemically defined media have been devised, some of which are capable of supporting production of almost 4 g/liter with noncommercial strains (29, 37, 44, 103, 105, 112, 122). It appears that complex media contain no unique stimulatory compounds and that with any particular strain, a defined medium can be devised which approximates complex media with respect to antibiotic production (93). Streptomycin fermentations are usually conducted at a poststerilization pH of 7.0 to 7.5 (29, 122), which is also the optimum pH for utilization of glucose and inorganic phosphate (51).

Streptomyces griseus, the most widely used organism for the biosynthesis of streptomycin, has been thought to use glucose mainly by the Embden-Meyerhof pathway and the tricarboxylic acid cycle, with only a minor portion being metabolized via the hexose monophosphate pathway (141). More-recent labeling studies, however, suggest that the hexose monophosphate pathway is more important than previously considered (114). The rate of glucose catabolism is regulated by the degree of oxygen transfer and by the level of inorganic phosphate. As will be discussed below, high phosphate levels accelerate glucose utilization and inhibit streptomycin production. Similarly, restricted aeration increases the rate of glucose breakdown and results in the accumulation of lactate and pyruvate in the medium (51). Streptomycin fermentations are thus conducted with high levels of oxygen transfer (49) and low concentrations of inorganic phosphate. The optimal temperature lies near 28 C. Above 30 C, there is a sharp cutoff of antibiotic production, although good growth generally is obtained at temperatures as high as 37 C (122).

Glucose is the carbohydrate of choice with most strains and in most media, but other compounds support production of streptomycin (30, 96); these include mannose, maltose, glycerol, and mannitol. Starch, dextrin, and fructose have been found to be satisfactory under certain circumstances but poor under others. As sole carbon source, sucrose, pentoses, sorbose, sorbitol, and organic acids do not support growth and in complex media are poor for streptomycin production. In certain media, the addition of lactate, acetate, or citrate to glucose enhances production. Various lipids can replace glucose with excellent results (97). These include corn oil, soybean oil, cottonseed oil, linseed oil, olive oil, peanut oil, coconut oil, lard oil, sesame oil, sperm oil, and more-defined lipids such as stearic acid, tristearin, trilaurin, oleic acid and its glycerides, and palmitic acid and its glycerides; lauric acid, however, is toxic to the growth of *S. griseus*.

The finding that soybean meal is an effective source of nitrogen for streptomycin formation was made over 20 years ago (99); no better crude preparation has been found. Materials such as corn-steep liquor, enzyme-hydrolyzed casein, yeast preparations (70), and distiller's solubles are definitely inferior, although they are occasionally added to soybean meal for specific purposes, e.g., the use of yeast or distiller's solubles to stimulate production of mannosidase. The value of soybean meal is probably due to its slow breakdown continuously yielding ammonia over the course of the long fermentation cycle. In support of this concept, one of the best-defined compounds used as sole nitrogen source is proline (29, 108), which is slowly utilized. Streptomycin can be formed when an ammonium salt [e.g., $(NH_4)_2HPO_4$, NH_4Cl, $(NH_4)_2SO_4$] is the only source of nitrogen, but production is poorer than with organic nitrogen; nitrate or nitrite are ineffective. Amino acids other than proline which can be used as sole nitrogen sources are glycine, alanine, β-alanine, histidine, arginine, asparagine, and glutamate (29, 103). Various workers have found the combination of an ammonium compound and amino acids to be beneficial in defined media. An interesting finding is the ability of ammonium citrate to replace aspartate in a defined medium containing $(NH_4)_2SO_4$ (112).

The various inorganic ions necessary to insure good growth and streptomycin synthesis are Na^+, Mg^{++}, Fe^{++}, Zn^{++}, Cu^{++}, K^+, Ca^{++}, and PO_4^{3-}. Generally, all of these can be omitted from a complex medium such as a soybean meal medium, with the exception of Na^+. Amounts of ferrous iron (25) and magnesium (46) over and above those required for optimum growth must be added to chemically defined media for maximal synthesis of the antibiotic. The level of phosphate is very critical; an excess inhibits streptomycin synthesis, although it stimulates growth and sugar utilization (17, 29, 32, 46, 144). Generally speaking, chemically defined glucose media contain 10^{-3} to 10^{-2} M phosphate. Inhibition is evident at phosphate levels as low as 1.5×10^{-2} M. In complex media containing glucose, phosphate is generally not added to the medium, although in certain cases 10^{-3} to 2×10^{-3} M phosphate has been beneficial. Inhibition may occur at levels of

3×10^{-3} M. The actual concentration of phosphate which causes inhibition varies with the carbohydrate used. Streptomycin production in a complex medium with fructose is more sensitive to phosphate inhibition than is production from glucose or maltose (113). In one study (118), it was noted that addition of 4×10^{-3} M potassium phosphate to a complex medium stimulated streptomycin production from starch but inhibited its formation from glucose. The effect of phosphate appears to be related to pyruvate accumulation in the fermentation medium. Fermentations with starch were characterized by high pyruvate accumulation and low streptomycin yields; the addition of potassium phosphate lowered the buildup of pyruvate and increased streptomycin yields. The use of glucose led to lower pyruvate accumulation and better antibiotic synthesis; however, addition of the same concentration of phosphate increased pyruvate accumulation and lowered streptomycin production. For many years, it was thought that the inhibiting effect of phosphate was due to its known stimulation of glucose utilization. However, Perlman and Wagman (97) showed that phosphate did not markedly affect the rate of substrate usage when oil was used as energy source, although inhibition of antibiotic production was severe. Similarly, Shirato and Nagatsu (113) observed that maltose utilization is not increased by phosphate at levels which markedly inhibit streptomycin formation.

The role of calcium in stimulating streptomycin production could be due to reversal of the effects of high phosphate concentrations introduced in complex additives (45). Calcium has been reported to increase the reproducibility of experiments, presumably by retarding lysis of mycelia (49, 85). The need for Mn^{++} is controversial. Although this cation is not required for growth in defined media, there is disagreement concerning its need for streptomycin synthesis (25, 122).

Although lipid derivatives have not been reported to stimulate the production of streptomycin in carbohydrate-based defined media, the extraction of oil from soybean meal reduces antibiotic synthesis in a complex medium by 40 to 70%. Palmitic acid is the best replacement for soybean oil (65).

The streptomycin fermentation shows two-phase dynamics which are characteristic of many antibiotic fermentations. The first stage (trophophase) is characterized by the growth of the culture; it is followed by a distinct antibiotic production phase (idiophase). In the early days of streptomycin development, autolysis was common during the idiophase (32, 46). As better strains and media were developed, mycelial lysis was delayed more and more so that in fermentations of today, an idiophase of several days duration is common before lysis occurs. The onset of lysis terminates streptomycin formation. The reported activity of barbital as a stimulator of streptomycin production in synthetic medium is evidently due to its reported ability to retard lysis (38).

In addition to complete fermentations, streptomycin can be synthesized by "resting" mycelial suspensions (85, 103, 105). In such systems, the optimum pH for streptomycin production is 6.5 to 7.0 (105). Glucose stimulates production and retards lysis of such suspensions. Like the complete fermentation, resting-cell synthesis is susceptible to inhibition by inorganic phosphate (75, 103), although a low level such as 5×10^{-4} M stimulates production (85).

During fermentation, streptomycin can be found both in the culture fluid and bound to the mycelia. The bound antibiotic is released from the cell walls by treatment with acid, alkali, or ionizable salts or by sonic oscillation. The cell wall of S. griseus is of the bacterial type, no chitin or cellulose being present (6). The structure is susceptible to the action of lysozyme (19) and it contains ribitol teichoic acid (13). [Glycerol teichoic acid also occurs in S. griseus but not in the wall (13, 84).] The amino acids present in walls include LL-diaminopimelic acid, glutamic acid, alanine, and glycine (12, 28, 145). There is some discrepancy as to the presence of other amino acids such as valine, cysteine, aspartic acid, serine, and lysine (9, 117). The major class of carbohydrates present in the wall are hexosamines (101), i.e., muramic acid and glucosamine (12, 145). The minor component is predominantly hexose in nature (101), but whether this is mainly glucose or galactose is controversial (28, 101, 145). Although some investigators report the absence of pentose (101, 145), ribose, arabinose, and the methyl pentose, rhamnose, have been observed by others (9, 28). After an early suggestion that streptomycin might be part of the cell wall (126), Barabás and Szábo (10) and Szábo et al. (120) reported the presence of streptidine in an acid-hydrolysate of the cell wall of a nonproducing mutant of S. griseus. Recently, this group reported that lysozyme treatment of this culture liberates streptomycin (11). These interesting and provocative claims await confirmation.

Culture degeneration is a major problem of streptomycin manufacture. Repeated transfer causes a progressive change in the population to a "nocardial" type of morphology, i.e., an increased tendency for mycelial fragmentation and loss of conidia formation (94, 142). Such asporogenous cultures do not produce the antibiotic. However, significant decreases in production ability can oc-

cur before decreased sporulation is noted. Iron metabolism appears to play some unexplained role in culture degeneration. Degeneration is accompanied by an inhibition of cytochrome synthesis and production of a coproporphyrin-type pigment bound to the mycelium (81, 83). Although production of the pigment is normally favored by the absence of iron, transfer of S. griseus in the absence of iron retards degeneration (82). Some of the reported changes accompanying the shift to nonproducing cultures include increases in growth rate and sugar utilization rate (103), a gain of ability to use arabinose for growth (121), a decrease in hexose content and an increase in pentose and glucosamine content of cell walls (9, 119), an increase in susceptibility to lysozyme, and an increase in resistance to disruption by sonic energy (9). Degenerated cultures have also been reported to appear more wrinkled on agar and to produce less of a soluble yellow pigment (86).

The major factor contributing to the dramatic rise in streptomycin yields from the original 100 to 200 μg/ml to the current 5,000 to 10,000 μg/ml is the selection of high-producing mutants (31, 33, 102).

BIOSYNTHESIS OF STREPTOMYCIN

The structure of streptomycin is shown in Fig. 1. It can be thought of as a trisaccharide formed from streptidine (an inositol derivative), L-strep-

FIG. 1. *Structure of streptomycin.*

tose (a branched chain sugar), and N-methyl-L-glucosamine. All three glycosidic bonds have the alpha configuration. The bond most easily hydrolyzed by mild acid is that between streptidine and streptose, yielding streptidine and streptobiosamine. Streptidine less its amidine [$H_2NC(:NH)$] groups is called streptamine. Other forms of streptomycin found in nature are shown in Table 1.

Most of the important information on the formation of the three streptomycin moieties was derived first from studies on the incorporation of radioactive compounds and later from enzymatic studies. Only a limited amount of information has been obtained from studies involving feeding of suspected precursors, the results varying from strain to strain and from medium to medium. As will be seen below, the firmest data are on the biosynthesis of the streptidine moiety. It should be said at the outset that no information is available on the enzymatic mechanisms by which the three moieties are joined.

Biosynthesis of the Streptidine Moiety

Experiments on the incorporation of labeled compounds and on enzymatic reactions, to be described below, have shown that the carbon skeleton of the streptamine portion of streptidine is derived from glucose via *myo*-inositol, whereas the two amino groups of streptamine arise by transamination. The two amidino groups attached to the streptamine nitrogens are derived by transamidination from arginine. With some strains, addition of arginine and inositol to fermentation media increases streptomycin yields (35, 42, 72, 75, 86, 111); streptidine has also been reported to stimulate production (86). Synthesis of streptomycin by washed suspensions may also be stimulated by arginine and inositol and by streptidine or streptobiosamine (75, 85). Reported stimulation by glutamine and asparagine (106) could be due to increased transamination, although glutamine probably also plays a role in the synthesis of the N-methyl-L-glucosamine moiety.

The early findings on the pathway of streptidine biosynthesis came from studies in which labeled compounds were added to fermentations. After Karow et al. (62) showed that U-^{14}C-glucose was incorporated into streptomycin in a complex medium, Numerof and co-workers (91), working with a chemically defined medium, found that labeled acetate and glycine were much poorer precursors and, for the most part, were converted into CO_2. The small amount of radioactivity that did enter streptomycin from the labeled two-carbon compounds was localized in the guanidino groups. This agreed with the finding of Hunter et al. (58) that $^{14}CO_2$ was incorporated exclusively
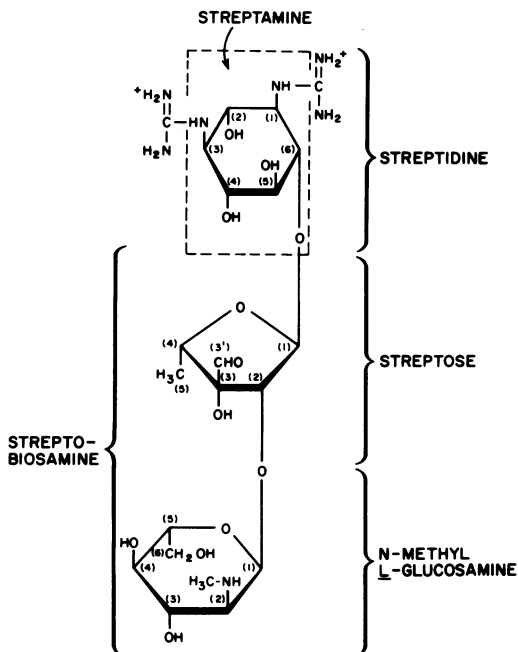
TABLE 1. *Biosynthetic streptomycins*

Antibiotic	Organism	Modification	In vitro activity	Reference
Streptomycin (streptomycin A)	Streptomyces griseus, S. bikiniensis; S. griseus			
Mannosidostreptomycin (streptomycin B)	S. griseocarneus, S. subrutilis, S. sp. 86	Mannose in α-D linkage from C-1 to C-4 of N-methyl-L-glucosamine	Less active than streptomycin	39, 100, 41
Hydroxystreptomycin	S. sp. 86	$-CH_2OH$ instead of $-CH_3$ at position 5 of streptose	Activity similar to streptomycin and dihydrostreptomycin	14, 5, 4
Mannosidohydroxy-streptomycin		Mannose in α-D linkage from C-1 to C-4 of N-methyl-L-glucosamine; $-CH_2OH$ instead of $-CH_3$ at position 5 of streptose	Less active than streptomycin and hydroxystreptomycin	5
N-demethyl-streptomycin	S. griseus (with ethionine)	$-H$ instead of $-CH_3$ in N-methyl-L-glucosamine	10% as active as streptomycin	48
Dihydrostreptomycin	S. humidus, S. sp.	$-CH_2OH$ instead of $-CHO$ at 3' position of streptose	Activity similar to streptomycin	64
Bluensomycin	S. bluensis	$-OCONH_2$ instead of one of the $NH-C$ (:NH) NH_2 groups of streptidine; $-CH_2OH$ instead of $-CHO$ at 3' position of streptose		8

into the guanidino carbon atoms. An important additional discovery was that unlabeled L-arginine diluted out the incorporation of $^{14}CO_2$, suggesting that the amino acid was the immediate precursor of the streptidine guanidino groups and that CO_2 entered after being incorporated into arginine. The conversion by a *S. griseus* suspension of $^{14}CO_2$ to arginine labeled in the guanidino group was demonstrated a few years later (111). In 1955, Hunter and Hockenhull (59) reported that ^{14}C-glucose entered each of the three moieties of streptomycin to an equal extent but that the guanidino carbons were only poorly labeled. They further found that streptamine was not a true precursor since labeled streptamine entered all three moieties of the streptomycin molecule, the streptidine portion becoming no more radioactive than the other portions. Streptamine was presumably degraded before being incorporated into the antibiotic. Incorporation of ^{14}C from guanidino-labeled L-arginine into streptomycin was demonstrated in 1964 by Horner (52); essentially all radioactivity was localized in the streptidine guanidino groups. Tovarova et al. (123) recently showed that arginine donated only the amidino group, whereas the other nitrogen atom of each guanidino group came from another source of nitrogen. As will be described below, these two extra nitrogen atoms arise by transamination, one from glutamine and the second from alanine.

In 1962, Majumdar and Kutzner (75) observed that unlabeled inositol depressed the incorporation of ^{14}C-glucose into streptomycin. Later, Horner (53) found an almost exclusive incorporation of ^{14}C-*myo*-inositol into the streptamine ring carbon atoms. Labeled inositol was a better precursor than glucose. Also, in 1964, similar findings were obtained by Heding (47) with ^{3}H- and ^{14}C-labeled *myo*-inositol. Bruce et al. (18) recently confirmed that the conversion of glucose to streptidine involves inositol as an intermediate by the use of specifically labeled glucose molecules.

The above incorporation studies definitely established the participation of inositol and arginine in the biosynthesis of streptidine. The remainder of this section will outline the information on the actual steps involved in the conversion of glucose to the streptidine moiety. A summary of the probable pathway is shown in Fig. 2. It should be noted at the outset that the structures presented in Fig. 2 are only suggestions, since the true position of the phosphate group is not known for any of the compounds; furthermore, it is not known which group of streptamine is the first to be transamidinated.

Conversion of glucose to myo-inositol. Little has been established about the very early reactions of streptidine biosynthesis in *S. griseus* except that

labeled D-glucose is converted to labeled *myo*-inositol (20). However, there is no reason to believe that the pathway is different from that in other tissues. Reactions 1, 2, and 3 of Fig. 2 illustrate the sequence which occurs in yeast (24), *Neurospora crassa* (98), mammalian tissue (36), and probably also in *S. griseus*. Reaction 1 is the phosphorylation of glucose by hexokinase. Glucose-6-phosphate is then converted to *myo*-inositol-1-phosphate by a cyclase which requires NAD^+ and is stimulated by ammonium ions (reaction 2). The phosphatase of reaction 3 is fairly specific; although a few other phosphate esters are cleaved, the enzyme acts most rapidly on inositol phosphate. Like some other phosphatases, the enzyme is inhibited by inorganic phosphate.

Conversion of myo-inositol to 2-keto-myo-inositol. Reaction 4, the conversion of *myo*-inositol to 2-keto-*myo*-inositol (*myo*-inosose-2; *scyllo*-inosose) has not yet been demonstrated in a *S. griseus* cell-free reaction, but Horner and Thaker (56) have shown that intact mycelia can convert (2-^{14}C)-*myo*-inositol to labeled 2-keto-*myo*-inositol. The oxidation is probably catalyzed by *myo*-inositol dehydrogenase as in *Aerobacter aerogenes* (15). *Scyllo*-inositol is also found as a labeled product. At present, it is not possible to decide whether *scyllo*-inositol is an intermediate or a side-product as depicted in Fig. 2. At any rate, it appears that 2-keto-*myo*-inositol is the compound through which *myo*- and *scyllo*-inositols are epimerized in *S. griseus*. The reversibility of reaction 5a would account for the additional findings that (^{3}H)-*scyllo*-inositol is converted to labeled 2-keto-*myo*-inositol and to *myo*-inositol, that (^{14}C)-2-keto-*myo*-inositol is metabolized to both *scyllo* and *myo*-inositols (56), and that both labeled *scyllo*-inositol and *myo*-inositol are incorporated into the streptidine moiety of streptomycin to the extent of 7 to 8% by intact mycelia (20).

Conversion of 2-keto-myo-inositol to streptidine. The elucidation of steps 5–13 is the result of the extensive studies of Walker which began in 1959 (128). All nine steps have been demonstrated with cell-free extracts.

The conversion of 2-keto-*myo*-inositol to *scyllo*-inosamine (reaction 5) can be demonstrated with a dialyzed sonic extract of *S. bikiniensis* in the presence of L-glutamine, the best amino donor for this transamination reaction (135). Dialysis of the enzyme must be carried out in the presence of pyridoxal phosphate. Although L-alanine and L-glutamate can act as weak amino donors, asparagine, aspartate, glycine, serine, methionine, lysine, and diaminopimelate are inactive. The reaction shifts the inositol derivative to the *scyllo*
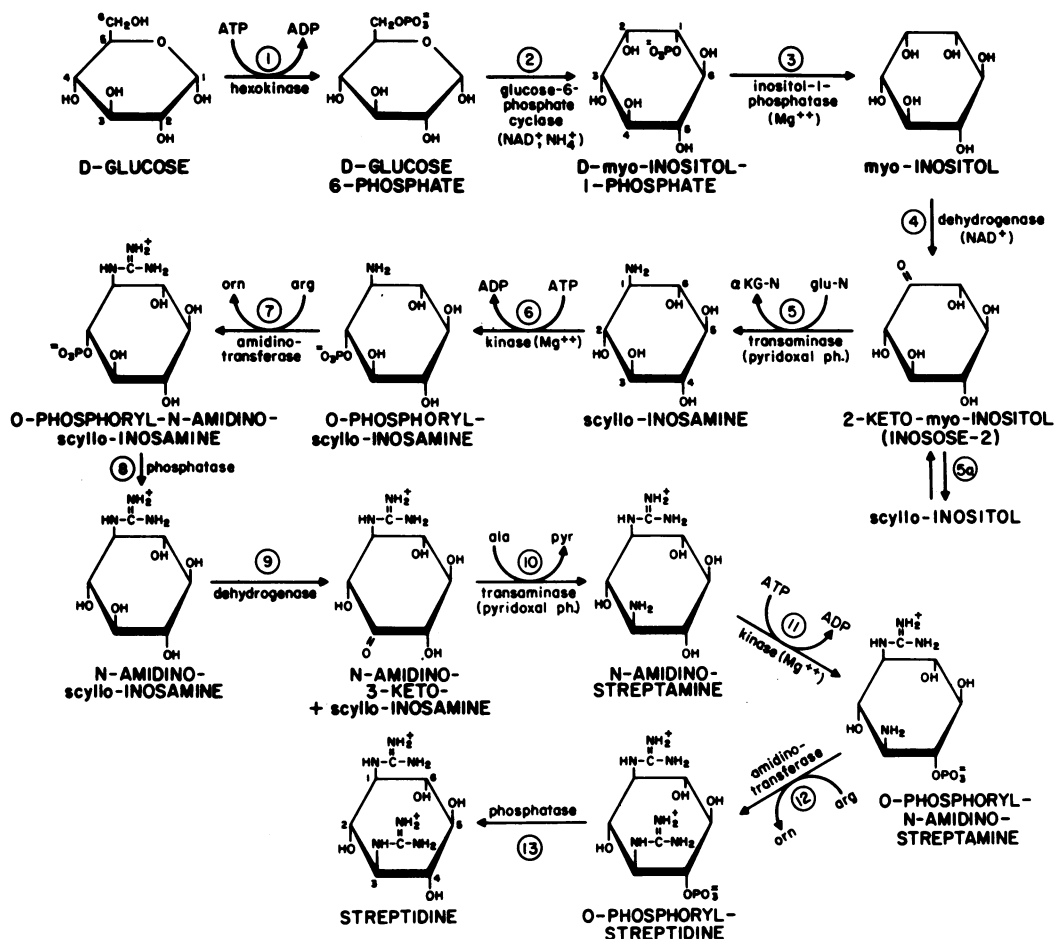
FIG. 2. *Probable pathway of streptidine biosynthesis. Abbreviations: ATP, adenosine-triphosphate; ADP, adenosine diphosphate; NAD+, nicotinamide adenine dinucleotide; ph., phosphate; orn, ornithine; arg, arginine; al, alanine; pyr, pyruvate; glu-N, glutamine; αKG-N, α-ketoglutaramate. Note that the numbering systems for the glucose, inositol, and inosamine-streptidine derivatives are different.*

configuration which is the form present in streptidine. Reactions 4 and 5 account for the observation that when labeled *myo*-inositol is fed to *S. griseus*, radioactive inosamine accumulates in the mycelia (131).

Another compound which accumulates upon exposure of *S. griseus* to labeled *myo*-inositol is *O*-phosphoryl-*scyllo*-inosamine, the product of reaction 6 (140). Inosamine kinase, present in extracts of *S. bikiniensis*, requires adenosine triphosphate (ATP) and magnesium and is relatively specific; i.e., *myo*-inosamine-2, DL-*myo*-inosamine-4, and *neo*-inosamine-2 are not phosphorylated (131).

In reaction 7, the amidino group of arginine is transferred to *O*-phosphoryl-inosamine, producing *O*-phosphoryl-*N*-amidino-*scyllo*-inosamine. Supplementation of a growth medium with *myo*-

inositol leads to increased amounts of *O*-phosphoryl-*N*-amidino-inosamine in mycelia as a result of reactions 4–7. Transamidination reaction 7 can be demonstrated with cell-free extracts from various streptomycin-producing species and also with extracts of *S. bluensis* var. *bluensis* which produces bluensomycin, an analogue of streptomycin containing only one guanidino group (128, 137, 139). Of a large number of compounds tested, only arginine and canavanine are active as amidino donors (130). Although ornithine and hydroxylamine can act as acceptors, many other compounds are inactive (130).

In reaction 8, phosphate is removed from *O*-phosphoryl-*N*-amidino-*scyllo*-inosamine, yielding *N*-amidino-*scyllo*-inosamine. The phosphatase, present in extracts of *S. bikiniensis*, is relatively specific in that it does not attack

O-phosphoryl-N-amidino-neo-2-inosamine (134). The reaction can also be carried out by using E. coli alkaline phosphatase.

The oxidation of N-amidino-scyllo-inosamine to N-amidino-3-keto-scyllo-inosamine (reaction 9) is followed by transamination to N-amidino-streptamine (reaction 10) by using extracts of S. bikiniensis (131). The transaminase is most active with alanine and is stimulated by pyridoxal phosphate. Glutamate and glutamine are poorer amino donors than alanine. The transaminase appears to be relatively specific with respect to the amino group acceptor since N-amidino-neo-2-inosamine is not aminated (134). Both the oxidizing and transaminating activities are lost upon dialysis; reactivation has not yet been achieved.

Reaction 11 is another phosphorylation reaction, the conversion of N-amidinostreptamine to O-phosphoryl-N-amidino-streptamine; ATP and Mg^{++} are required (134). The kinase is relatively specific in that N'-amidino-streptamine (i.e., amidino group on the other N) and various N-amidino-inosamines are not phosphorylated. Activity of the kinase is present in cell-free extracts of S. bikiniensis. Feeding of $(2-^{14}C)$-myoinositol to S. griseus or L-(guanidino-^{14}C)-arginine to S. bikiniensis leads to accumulation of labeled O-phosphoryl-N-amidino-streptamine (131, 140), as would be expected from reactions 4 through 11.

The amidinotransferase which catalyzes reaction 7 acts again in reaction 12 converting O-phosphoryl-N-amidino-streptamine to O-phosphoryl-streptidine. The transamidination reaction can be demonstrated with extracts of S. griseus and S. bikiniensis. Evidence for the identity of the two amidinotransferase activities is the constancy of the relative rates of activity during enzyme purification (129) and the inhibition of formation of both transamidination products by ornithine or cystamine (137). Furthermore, extracts of S. bluensis var. bluensis, an organism which normally carries out transamidination reaction 7 but not reaction 12 in producing the monoguanidino antibiotic bluensomycin, can catalyze reaction 12 if presented with O-phosphoryl-N-amidino-streptamine and arginine (140). As expected from reactions 4–12, the feeding of labeled myo-inositol or arginine to intact mycelia of S. griseus and S. bikiniensis results in the accumulation of radioactive O-phosphoryl-streptidine (131, 140).

The final step (reaction 13) of streptidine formation involves the removal of the phosphate group from O-phosphoryl-streptidine. The reaction can be carried out by cell-free extracts of S. griseus as well as by Escherichia coli alkaline phosphatase (137, 138, 139). It is not known, however, whether streptidine is a true intermedi-

iate of streptomycin synthesis or whether O-phosphoryl-streptidine participates in the reaction joining the streptidine and streptose moieties (133).

Examination of Fig. 2 reveals several interesting facts. The 10 steps (reactions 4–13) between myo-inositol and streptidine can be divided into two analogous series involving a dehydrogenation, a transamination, a phosphorylation, a transamidination, and a dephosphorylation. Each set of five steps is responsible for the synthesis of one guanidino group from a cyclitol OH group, the formation of the first being completed before the synthesis of the second begins. Although the same amidinotransferase appears to function in both sets of reactions, two distinct transaminases are used (136). It is not known whether the other three reaction types are catalyzed by one or two enzymes each. Although it has been stated by Walker and Walker (139) that alkaline phosphatase from S. griseus can act on all the phosphorylated compounds in the scheme, it is doubtful that the enzyme was purified.

Nowhere in the scheme does free streptamine appear. This is in agreement with the facts that streptamine is not incorporated preferentially into the streptidine moiety (59) and is not transamidinated by cell-free amidinotransferase (130).

One disturbing feature of the sequence depicted in Fig. 2 is that it does not agree with Heding's (47) earlier report that myo-(2-^{3}H)-inositol is efficiently incorporated into streptidine. According to the scheme, ^{3}H should be lost during the conversion of myo-inositol to inosose-2. Confirmation of Heding's incorporation data would eliminate consideration of inosose-2 as an obligatory intermediate.

Salvage synthesis of O-phosphorylstreptidine. Intact mycelia of many Streptomyces species, including some that do not produce streptomycin, have the ability to convert exogenous streptidine to O-phosphoryl streptidine (139). Oddly, Walker and Walker (133) found that only the streptomycin producers yield active cell-free extracts. The only compound, other than streptidine, which has been found to be a substrate of streptidine kinase is 2-deoxystreptidine. There is an absolute requirement for Mg^{++} and ATP. Although ATP can be replaced by deoxy-ATP, the triphosphates of guanosine, uridine, cytidine, and thymidine are inactive. Mn^{++} can substitute for Mg^{++} in the reaction. Although the significance of the reaction is unknown, an important role in streptomycin synthesis is suggested by the observations that streptidine kinase is strongly inhibited by streptomycin and that its specific activity markedly increases during idiophase. The enzyme is distinct

from the *N*-amidino-streptamine kinase discussed above (reaction 11 of Fig. 2), which is not inhibited by streptomycin.

Transamidination of O-phosphorylstreptamine. Another reaction of unknown significance is the conversion of *O*-phosphoryl-streptamine to *O*-phosphoryl-*N*-amidino-streptamine by the cell-free amidinotransferase discussed above; arginine is the amidine donor (129, 139). Although the product is an intermediate in the scheme described in Fig. 2, the substrate *O*-phosphoryl-streptamine has never been observed in mycelia of streptomycin-producing streptomycetes.

Biosynthesis of the Streptose Moiety

Glucose is the source of the streptose moiety (59). Radioactive incorporation studies by Candy et al. (21, 23) and Bruton and Horner (19) showed that C-1, C-2, C-4, and C-6 of glucose become C-1, C-2, C-3, and C-5 of streptose, respectively, whereas C-3 of glucose is incorporated into the C-3' branch carbon atom of streptose. Thus, although the whole glucose molecule is incorporated as a unit, there is a rearrangement whereby C-3 is extruded as a branch and C-2 is joined to C-4 of the hexose molecule. That the C-3' branch carbon atom does not arise by one-carbon transfer reactions has been further shown by the lack of selective incorporation into the streptose C-3' of radioactivity from serine-*3*-^{14}C (19) or from $^{14}CH_3$-methionine (74).

Before the above radioactivity studies with selectively labeled glucose molecules were carried out, Blumson and Baddiley isolated thymidine diphosphate (TDP)-rhamnose from *S. griseus* (16), found that a cell-free preparation could convert TDP-glucose to TDP-rhamnose, and suggested that a proposed intermediate between glucose and TDP-rhamnose could be a precursor of streptose. Their postulated mechanism, shown in Fig. 3, would fit in with the radioactive incorporation data described in the previous paragraph. Of interest in this regard are the claims of Majer et al. (74) that rhamnose stimulates production of streptomycin. Furthermore, unlabeled rhamnose was found to depress the incorporation of radioactivity from *1*-^{14}C-glucose into the streptose moiety but had no effect on incorporation into the other two moieties of the antibiotic. These data, however, are clouded by the fact that *1*-^{14}C-rhamnose was only incorporated into streptomycin to the extent of 0.01% and, of this, only 5 to 15% was localized in the streptose fraction. Horner (55) made the interesting suggestion that the rearrangement of glucose or a derivative might precede the removal of oxygen at carbon atom 6 of glucose in view of the existence of hydroxy-
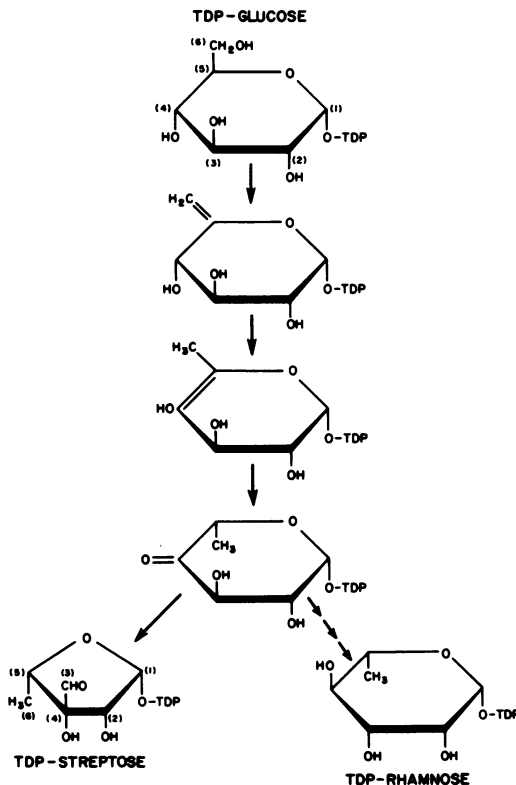

FIG. 3. *Suggested mechanism of TDP-streptose formation from TDP-glucose via a postulated intermediate of TDP-rhamnose formation (19, 21, 23). Numbers in parentheses refer to the C atoms in D-glucose.*

streptomycin, a molecule which retains the hydroxymethyl group of glucose at C-5 of the streptose moiety.

Biosynthesis of the N-methyl-L-Glucosamine Moiety

Like the other two moieties of streptomycin, *N*-methyl-L-glucosamine arises from glucose (59). As will be described below, the source of the methyl group is L-methionine. In several cases, supplementation of chemically defined media with methionine has been observed to stimulate the production of the antibiotic (42, 75); however, inhibition was noted with resting mycelia (75). With some strains, vitamin B_{12} is necessary to demonstrate the stimulation by methionine (42); *p*-aminobenzoic acid is reported to replace B_{12} for this activity (43).

The earliest labeling studies showed that *N*-methyl-L-glucosamine-^{14}C entered predominantly into the methyl-glucosamine moiety of the antibiotic (59). Later studies (20, 114) with specifically

labeled glucose molecules showed that the C-1, C-2, C-3, C-4, and C-6 of D-glucose become C-1, C-2, C-3, C-4, and C-6 of the N-methyl-L-glucosamine moiety, respectively. D-Glucosamine-1-^{14}C also mainly enters this moiety and predominantly labels C-1 of N-methyl-L-glucosamine (2, 20).

Labeling of the methyl group by $^{14}CH_3$-L-methionine was shown by Candy et al. (23) and confirmed by Horner (54). Both ^{14}C-formate and ^{14}C-bicarbonate are not incorporated in a similar fashion.

Although extracts of a streptomycin producer convert glucose-6-phosphate or fructose-6-phosphate to glucosamine (107) and uridine diphosphate-N-acetyl-D-glucosamine has been isolated from mycelial extracts of S. griseus (1), little is known about the conversion of glucose to N-methyl-L-glucosamine. The biosynthetic pathway of N-methyl-L-glucosamine probably involves the conversion D-glucose → D-glucose-6-phosphate → D-fructose-6-phosphate → D-glucosamine-6-phosphate, as in other organisms. The amino group is presumably derived from glutamate. Glutamine (but also asparagine) has been reported to stimulate streptomycin production (106), although this could be due to stimulation of the transamination reactions of streptidine biosynthesis. Presumably, what follows is a series of steps from D-glucosamine-6-phosphate involving epimerization of the four asymmetric carbon atoms, the removal of phosphate, and N-methylation by S-adenosylmethionine. Such multiple epimerizations are known to occur in the conversion of D-glucose to L-rhamnose. Akamatsu and Arai (2) suggested an alternate mechanism in which hexose monophosphate is converted via the Embden-Meyerhof scheme to D-glyceraldehyde-3-phosphate which is epimerized to the L-form and is then recombined to the 6-carbon stage by enzymes of the hexose monophosphate pathway. However, this certainly could not involve L-glucose or a close derivative since ^{14}C-L-glucose is not incorporated into streptomycin despite its uptake by S. griseus (20). The ability of the methylation inhibitor ethionine to inhibit the formation of streptomycin and to cause the accumulation of N-demethyl-streptomycin (48) suggests that the methylation step may be the final one in formation of the N-methyl-L-glucosamine moiety.

Possible Unidentified Intermediates of Streptomycin Biosynthesis

Several groups have reported on the excretion of possible intermediates of streptomycin biosynthesis, but the chemical nature of these compounds is yet to be established. In 1962, Alikhan-yan and Teteryatnik (3) reported that young (24 hr) mycelia of a nonproducing mutant produced streptomycin when added to the 24-hr culture filtrate of a producing culture. This may be related to the observations made by Nomi (85) in 1963 that when mycelial suspensions of a producing strain were incubated for 24 hr in glucose and then centrifuged, the supernatant fluid was capable of antibiotic synthesis but the mycelial mass produced none. This soluble antibiotic-synthesizing system was inactivated by heat or acid and was inhibited by 4 mM K_2HPO_4 or 0.027 M ethylenediaminetetraacetic acid; its optimal pH was 8.5 (88, 90). It was found to contain a low-molecular-weight cationic precursor (less basic than streptomycin) and an anionic heat-labile and acid-labile enzyme system (89). Improved yields of the enzyme could be obtained from extracts of sonically treated mycelia. High concentrations of the precursor were found to be excreted during fermentations conducted in a high glucose medium with pH held between 6 and 7; under these conditions, streptomycin synthesis was inhibited, whereas as much as 0.5 g of the precursor per liter accumulated (87). The identity of the precursor is not yet known, although it apparently is a phosphate ester of streptomycin. The degree of phosphorylation is unclear since the action of the normal enzyme on the precursor yields 0.5 mole of phosphate per mole of streptomycin, whereas with intestinal alkaline phosphatase, 2 moles of inorganic phosphate are produced per mole of streptomycin.

Another factor of unknown structure has been reported by Khokhlov et al. (66). This factor is excreted by a low streptomycin producer and used by a nonproducer so that high yields (2 to 3 g/liter) of streptomycin are made by the combination of the two strains. The active material is of low molecular weight, stable to heat and to low and high pH, acidic, and extractable from culture filtrates by ether, chloroform, and butanol. It has no absorption at 215 or 280 nm. Streptidine, O-phosphoryl-streptidine, streptamine, inositol, and glucosamine failed to replace the factor. It is claimed that 1 part by weight of the factor yields 3,000 parts by weight of streptomycin, suggesting that it is some type of cofactor rather than an intermediate. Confirmation of this work and identification of the compound are clearly needed.

REGULATORY MECHANISMS AND STREPTOMYCIN SYNTHESIS

The presence of a trophophase (growth stage) followed by an idiophase (production stage) in the streptomycin fermentation is indicative that induction or repression, or both, plays a regulatory role; i.e., induction (or derepression) of en-

zymes involved in antibiotic synthesis must occur at the end of trophophase. For evidence of such controls, one can examine Walker's studies on the amidinotransferase which acts at two steps of the streptidine pathway. The importance of this enzyme as one specific for antibiotic synthesis is very firm; it is found only in those *Streptomyces* strains which produce one or another type of streptomycin (130). Walker and Hnilica (130) noted a dramatic increase in the specific activity of the enzyme at the start of streptomycin synthesis by *S. griseus, S. bikiniensis,* and *S. griseocarneus.* This has been confirmed by Horner (52) and by Li et al. (72). Furthermore, in a medium not supporting streptomycin production, no such increase in activity occurs (52). The sudden appearance of the enzyme is due to de novo protein synthesis, not to activation of a precursor; i.e., enzyme appearance can be inhibited by chloramphenicol or puromycin (130). Other enzymes of the streptidine pathway must also be derepressed during early idiophase, since the two substrates of the amidinotransferase cannot be found in young, growing cells (137). Walker and Walker (132) claim that all eight enzymes acting between *scyllo*-inosamine and streptidine undergo derepression after growth, although the data have not yet been published.

A second regulatory mechanism may be responsible for the inhibitory effect of inorganic phosphate on streptomycin formation. As stated earlier, the original hypothesis that excess phosphate ("excess" in terms of streptomycin synthesis, not growth) inhibits by accelerating substrate usage was rendered inadequate by the finding that when lipids are the carbon and energy source, phosphate inhibits antibiotic synthesis without affecting the rate of lipid utilization. Another possibility is that high phosphate interferes with release of streptomycin into the medium. In view of the known repressibility and inhibition of some microbial phosphatases, an alternative hypothesis is that excess phosphate interferes with the formation or action, or both, of one or more phosphatases involved in streptomycin biosynthesis, as suggested by Waksman (125). As discussed earlier in this paper, there are three phosphate-cleavage steps in the biosynthesis of the streptidine moiety, and Nomi's work indicates that a phosphate ester of streptomycin may be a late intermediate. It is known that inositol-1-phosphatase from mammalian tissue is inhibited by phosphate. No work has been done yet on the separation of the phosphatases of *S. griseus.* It is clear that the time is ripe for separation, purification, and characterization of these phosphatases to examine the regulation of these enzymes by inorganic phosphate. Activity has been found both in mycelia and in broth capable of hydrolyzing glycerophosphate and phenylphosphate at an optimal pH of 9.0 (92). Waksman (125) reported that elaboration of phosphatase activity paralleled streptomycin production and that excess phosphate repressed and inhibited phosphatase as well as inhibiting streptomycin formation. Walker and Walker (139) found that the developmental time-course of *S. griseus* alkaline phosphatase is that of an idiophase enzyme and that it has activity on all the phosphorylated intermediates of the streptidine pathway. The enzyme of Nomi et al. which converts their unknown phosphorylated intermediate to streptomycin has an optimal pH of 8.5 and is inhibited by phosphate (90).

Another possible regulatory site concerned with phosphate inhibition is the synthesis of the amidinotransferase, the key enzyme of streptidine biosynthesis. Walker and Hnilica (130) found that growth in high phosphate severely repressed formation of this enzyme.

A third possibility for regulation is the streptidine kinase reaction in which exogenous streptidine is phosphorylated to O-phosphoryl-streptidine (139). Although the importance of this "salvage" pathway is not clear, the reaction is completely inhibited by streptomycin. Examination of this feedback-inhibitory reaction could tell us much about the significance of salvage synthesis in streptomycin formation.

STREPTOMYCES α-D-MANNOSIDASE (MANNOSIDOSTREPTOMYCINASE)

General

The occurrence of mannosidostreptomycin (streptomycin B) along with streptomycin in broths of *S. griseus* was first reported by Fried and Titus (40). Chemical studies subsequently showed that in mannosidostreptomycin, the C-1 of D-mannose is joined by an α-glycosidic linkage to C-4 of the N-methyl-L-glucosamine portion of streptomycin (39).

The ability of streptomycin-producing strains of *S. griseus* to hydrolyze the α-D-mannoside derivative to streptomycin and mannose was demonstrated many years ago by Perlman and Langlykke (95). During a fermentation, mannosidostreptomycin and streptomycin are produced concurrently; under proper conditions, α-D-mannosidase (mannosidostreptomycinase) is produced late in the fermentation cycle and the mannoside is hydrolyzed to streptomycin (50, 67).

There has been much discussion concerning the possibility that mannosidostreptomycin is an obligate intermediate in streptomycin formation. The early finding that the mannosidase is formed by streptomycin producers but not by *S. griseus*

strains which fail to produce streptomycin (95) is compatible with the possibility that the mannoside is formed first and then induces the formation of the enzyme. Indeed, Kollár (68) concluded that mannosidostreptomycin is an obligatory intermediate. However, the recent studies of Inamine, Lago, and Demain (60) indicate that such is not the case. Mannosidase-negative mutants of *S. griseus* were shown to produce the same amounts of the two antibiotics as does the parent strain. The accumulation of only mannosidostreptomycin would be expected if this compound were an essential intermediate in the biosynthesis of streptomycin. These results suggest that mannosidostreptomycin is a shunt product formed from streptomycin (or from a precursor of streptomycin), as originally suggested by Hockenhull (49).

Mannosidostreptomycin can account for up to 40% of the total streptomycins produced in a fermentation (49). Since the biological activity of the α-D-mannoside derivative is only 20 to 25% that of streptomycin against most microorganisms, the enzymatic hydrolysis of mannosidostreptomycin is of considerable economic importance.

Properties of the Enzyme

Streptomyces mannosidase is bound to the mycelium until lysis occurs. All the data available on the properties of the enzyme are derived from studies done with crude preparations. The enzyme hydrolyzes phenyl-α-D-mannoside, p-nitrophenyl-α-D-mannoside, and mannosidodihydrostreptomycin, in addition to mannosidostreptomycin (50, 60, 95). Phosphate has no effect on the hydrolysis of phenyl-α-D-mannoside, whereas arsenate is reported to stimulate hydrolysis (50, 67). Adequate aeration is required for enzyme activity (50, 67, 90).

The enzyme appears to require a divalent cation for activity since the loss of activity, observed on dialysis or on ethylenediaminetetraacetic acid treatment, can be reversed by Ca^{++}, Mn^{++}, or Zn^{++} (60). Metals such as Cu^{++}, Hg^{++}, and Fe^{++} inhibit the enzyme, whereas K^+, Mg^{++}, and Ba^{++} are without effect (50, 95). Perhaps reflecting their diverse metabolic roles, the α-D-mannosidases from other organisms exhibit varying cation effects. For example, the enzyme from an *Arthrobacter* species is stimulated by Ca^{++} but is inhibited by Mg^{++}, Cu^{++}, or Zn^{++} (61). The enzyme from jack-bean meal is activated by Zn^{++} and inhibited by Ag^+ and Hg^{++}, whereas Mn^{++}, Fe^{++}, Cu^{++}, and Hg^{++} have no effect (115, 116). The enzyme from rat epididymis is stimulated by Zn^{++} (115). A marine gastropod, *Charonia lampas*, has a liver enzyme that is stimulated by Zn^{++} and is inhibited by Ca^{++} and Mg^{++} (79).

In contrast, another marine gastropod, *Turbo cortunus*, possesses an enzyme that is stimulated by Ca^{++}, Mg^{++}, and Zn^{++} (80). The enzyme in bovine milk is stimulated by Zn^{++} and Mn^{++} and is inhibited by Cu^{++}, Fe^{++}, and Ca^{++} (77).

The pH of optimal activity for *Streptomyces* mannosidase is in the range of 7.0 to 8.0 (50, 67, 95). This compares to an optimal pH of 7.0 for the enzyme from *Arthrobacter* (61) and a range of 3.0 to 5.0 for the enzymes from jack-bean meal (71, 116), rat epididymis (26), bovine milk (77), and marine gastropod (27, 79). The optimal temperature for the streptomycete enzyme is 40 C and rapid inactivation is observed at 50 C (50, 67).

Cyanide, fluoride, bisulfite, azide, and cysteine are reported to inhibit the enzyme to various degrees (50, 67). Sugars such as methyl-α-D-mannoside, mannose, cellobiose, and maltose at 0.05 M are inhibitory to the hydrolysis of phenyl-α-D-mannoside (50). Although glucose was reported to not inhibit the hydrolysis of the phenyl-α-D-mannoside, the enzyme was inhibited completely by glucose as well as by mannose at 0.05 M when mannosidostreptomycin was used as the substrate (49).

Regulation of α-D-Mannosidase Biosynthesis

Studies on the dynamics of α-D-mannosidase production in streptomycin fermentations show the enzyme to be synthesized late in the cycle, at a time when glucose is nearing depletion (50, 67). The induction of α-D-mannosidase by yeast mannan was first reported by Hockenhull (49). The favorable effect on mannosidase synthesis of supplementing fermentation media with whole yeast or distiller's solubles is evidently due to their content of yeast mannan. Shaw (109), in fact, devised a simple medium containing only distiller's solubles and NaCl which supports high-level synthesis of α-D-mannosidase at the expense of antibiotic production. Methyl-α-D-mannoside, phenyl-α-D-mannoside, and an oligomannoside mixture chemically derived from yeast mannan have also been reported to induce the α-D-mannosidase (49, 60, 68); yeast mannan, however, is the most active inducer known. Mannosidostreptomycin fails to induce the enzyme, but Kollár considers impermeability to be a factor in this case (68). Likewise, yeast phosphomannans with mannose-to-phosphorus ratios ranging from 2.5 to 20 are not inducers (60).

The use of a washed-cell induction system with yeast mannan as the inducer has shown that orthophosphate and Mg^{++} are absolute requirements for induction (60). Zn^{++} and Ca^{++} are stimulatory but only if they are added with Mg^{++}. Earlier studies in complex fermentation media

showed that Fe^{++} is inhibitory to the formation of the enzyme but that the effect can be negated by Ca^{++} (110). A pH of 7.0 to 7.5 and a temperature of 28 C are optimum for enzyme synthesis (60, 68). Aeration is also required for enzyme induction (68).

The extreme importance of glucose depletion for enzyme formation in streptomycin fermentations (49) suggests that catabolite repression is an important factor. Recent studies by Inamine, Lago, and Demain (60) have shown that catabolite repression is easily observable when various metabolizable carbon sources are added to a simple mannan-orthophosphate-salts induction medium (Table 2). Repressible carbon sources include glucose, galactose, inositol, mannose, maltose, lactose, citrate, fumarate, and malate. As expected, L-rhamnose, which is not utilized as a sole carbon source by the organism, has no effect.

In the mannan-orthophosphate-salts induction system, the addition of certain nitrogen-containing compounds, such as ammonium chloride, D-glucosamine, or certain amino acids was found to stimulate enzyme synthesis to greater than 150% of the control (60). This indicated that the endogenous nitrogen supply was limiting. Other amino acids, such as L-valine, L-isoleucine, L-methionine, or L-cystine, were found to be repressors when tested at 0.01% (Table 3). The sulfur-containing amino acids were such severe repressors that they virtually eliminated enzyme production; they did not, however, inhibit the action of the enzyme. Earlier, Kollár (68) reported that the induction of α-D-mannosidase by phenyl-α-D-mannoside in a washed-cell system was repressed by 0.01 M valine, ornithine, glutamate, or methionine.

By using the mannan-orthophosphate-salts induction system, it was found that metabolizable carbon sources caused less catabolite repression in a nitrogen-supplemented system than in the absence of added nitrogen (60). This finding is in

TABLE 2. *Catabolite repression by carbon sources*[a]

Compound added	α-Mannosidase (units per ml) carbon sources added		
	None	0.1%	1.0%
None.............	1,320		
Dextrin...........		121	66
Galactose.........		225	81
Glucose...........		150	40
Inositol..........		970	650
Mannose..........		170	40
L-Rhamnose.......		1,225	1,350

[a] Basal induction medium contained 0.2% mannan. Carbon sources were tested at 0.1 and 1.0%. Enzyme activity was assayed at 24 hr.

TABLE 3. *Amino acids which severely repress mannan-induced enzyme synthesis*

Compound added	α-Mannosidase (units per ml) amino acids added		
	None	0.01%	0.1%
None..............	1,100		
L-Cystine..........		37	33
L-Methionine.......		39	39
L-Isoleucine........		410	255
L-Valine...........		400	240

agreement with Mandelstam's (76) observation that in *E. coli* β-galactosidase is much more susceptible to catabolite repression when the organism is starved for nitrogen. Furthermore, it was found that in the presence of sufficient nitrogen a low level of glucose (0.1%) stimulated, instead of repressing, mannosidase induction, presumably by acting as an energy source (60). Such a dualistic role of glucose has been observed in other inducible enzyme systems (34, 73, 124).

The above studies led to the finding that the α-D-mannosidase of *S. griseus* not only is an inducible enzyme but that it also is semiconstitutive (60). Orthophosphate, salts, glucose (at a low concentration), and ammonium chloride are sufficient for low-level production of the enzyme in the washed-cell system. Mannan, of course, markedly stimulates enzyme synthesis in such a medium (Fig. 4).

The results of Inamine, Lago, and Demain (60) can explain the earlier findings by Kollár (68) that unstarved resting cells responded poorly to inducer, that cells starved for 16 hr responded well, and that cells starved for 24 hr responded to inducer only when glucose was added; also, glucose added to unstarved cells further delayed induction. Apparently, endogenous catabolite repressors in unstarved cells inhibited enzyme induction. When the repressor concentration was lowered by starvation, induction could proceed. Extensive starvation caused depletion of the carbon source to a point at which energy was limiting and glucose had to be added. In the unstarved cells which contain high levels of endogenous carbon sources, further addition of glucose only increased the repressor level.

The effect of antibiotics on the induced synthesis of α-D-mannosidase has been studied (60). Penicillin G has no effect on induction, indicating that enzyme synthesis is not dependent on growth. Enzyme appearance is due to de novo synthesis; i.e., the induction is inhibited by chloramphenicol and by actinomycin D. As one might expect with a streptomycin producer, streptomycin fails to inhibit enzyme induction in *S. griseus* even at extremely high levels.
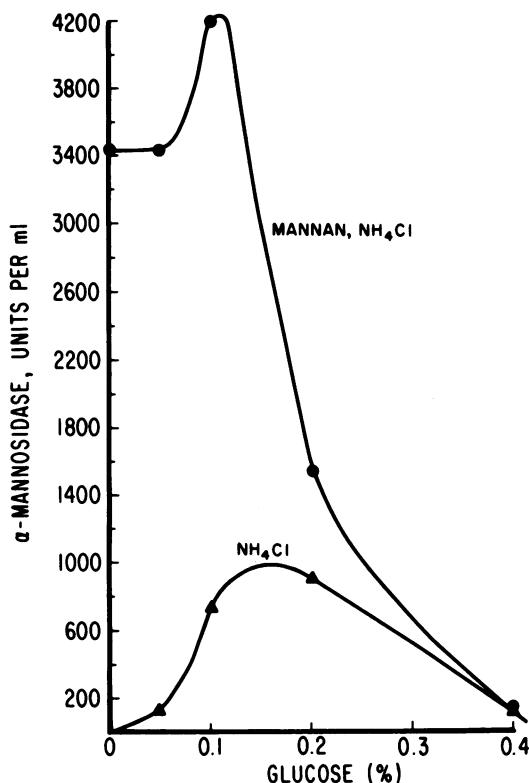
FIG. 4. *Stimulation of mannosidase synthesis by mannan at various concentrations of glucose.*

Mannose-Containing Compounds in S. griseus

Several mannose-containing compounds besides mannosidostreptomycin have been isolated from strains of *S. griseus*. TDP mannose was characterized by Blumson and Baddiley (16), who also obtained a crude enzyme fraction capable of interconverting TDP glucose and TDP mannose (7). They suggested that the nucleotide may be involved in the biosynthesis of mannosidostreptomycin and of polysaccharides. A polymer made up of 3-*O*-methylmannose (22) and mannose-containing heteropolymers (69, 84) is present in this organism. The phospholipid fraction is reported to contain phosphatidyl inositol monomannoside (63). Whether any of these compounds plays a role in the formation of mannosidostreptomycin remains an unanswered question.

SUMMARY AND CONCLUSIONS

Streptomycin is a. typical secondary metabolite; it is a low-molecular-weight natural product produced after growth has ceased, having no apparent function in the growth process. It is produced only by a limited number of closely related organisms; it is a member of a family of antibiotics of similar structure. Production ability is easily lost by mutation (strain degeneration).

Fermentative production of streptomycin is best carried out in a glucose-soybean meal-NaCl medium; about 5 to 10 g/liter is produced by commercial strains of *S. griseus*. Chemically defined media are capable of supporting production of up to 4 g/liter using noncommercial strains. The process operates best at an initial pH near neutrality, requires a high degree of oxygen transfer, and is inhibited by levels of inorganic phosphate which support optimal growth. Although growth occurs above 30 C, there is a sharp cutoff of streptomycin production above 28 C. Although most of the antibiotic is found in the extracellular medium, a fair portion is bound to mycelial walls. The latter is released by acid, alkali, ionizable salts, or sonic oscillation. The selection of high-producing mutants has been the main factor in raising streptomycin titers from the original 100 to 200 μg/ml to the 5,000 to 10,000 μg/ml levels obtained today.

Streptomycin can be synthesized by washed mycelial suspensions, but a cell-free system capable of converting glucose to streptomycin has not yet been devised. The antibiotic contains three moieties: streptidine, streptose, and *N*-methyl-L-glucosamine. All three parts arise from glucose.

In addition to glucose, precursors of the streptidine portion are arginine, glutamine, and alanine. Glucose is apparently first converted to *myo*-inositol-6-phosphate. The next 10 steps (all of which have been demonstrated in cell-free extracts) leading to streptidine involve two sequential sets of reactions as follows: dehydrogenation, transamination, phosphorylation, transamidination, and dephosphorylation. Each set of five steps results in the conversion of a cyclitol hydroxyl group to a guanidino group.

Streptose, the branched chain hexose, is formed entirely from glucose by a rearrangement in which the C-3 of glucose is extruded and C-2 is attached to C-4. Nothing is known about the individual steps of this conversion. Although the C-6 hydroxymethyl group of glucose ends up as a methyl group in streptomycin, the hydroxmethyl group is retained in hydroxystreptomycin produced by *S. griseocarneus* and *S. subrutilis*.

The *N*-methyl-L-glucosamine moiety arises from D-glucose and L-methionine. The actual pathway is unknown, but it does not involve L-glucose as an intermediate. Ethionine inhibits methylation and results in the production of *N*-demethylstreptomycin, which is only about 10% as active as streptomycin.

Derepression of enzymes responsible for strep-

tomycin production after growth appears to be the regulatory mechanism which triggers antibiotic synthesis. Enzymes shown to be formed only during the idiophase include the amidino-transferase and streptidine kinase. The well-known phosphate inhibition of streptomycin formation is possibly due to repression or inhibition, or both, of the numerous phosphatases involved in the biosynthesis of the antibiotic. Work is needed on the purification and regulation of these enzymes. Feedback inhibition may also play a role in streptomycin formation in view of the complete inhibition of streptidine kinase by streptomycin.

Production of the less desirable mannosido-streptomycin generally occurs concurrently with streptomycin until mannosidostreptomycinase is formed very late in the fermentation. At this time, the mannosidostreptomycin is rapidly cleaved to streptomycin. The main reason for its late appearance is catabolite repression by glucose. The enzyme, which is bound to the mycelium, can also hydrolyze phenyl-α-D-mannoside, p-nitrophenyl-α-D-mannoside, and mannosidodihydrostreptomycin. A divalent cation such as Ca^{++}, Mn^{++}, or Zn^{++} is necessary for its action which is optimal at pH 7.0 to 8.0 at 40 C. Although the enzyme is inducible by yeast mannan, a small amount can be formed under special conditions without inducer. These conditions include a low, nonrepressive concentration of glucose, NH_4^+, and salts. Orthophosphate and Mg^{++} are absolute requirements for enzyme production, whereas Zn^{++} and Ca^{++} stimulate. Although yeast mannan is the most active inducer known, phosphorylated mannans from yeast are inactive. Aeration, pH values of 7.0 to 7.5, and a temperature of 28 C are optimal for enzyme synthesis.

Catabolite repression of enzyme synthesis is also caused by galactose, inositol, mannose, lactose, citrate, fumarate, and malate. Certain nitrogen-containing compounds, i.e., some amino acids, are markedly repressive in their own right. Enzyme formation is not inhibited by penicillin G, but chloramphenicol and actinomycin D are effective inhibitors. Streptomycin itself is inert. The observation that mutants lacking mannosidostreptomycinase, like the parent culture, produce both antibiotics suggests that mannosidostreptomycin is not an obligatory intermediate of streptomycin formation but rather is a shunt product.

ADDENDUM IN PROOF

Idiophase mycelial extracts containing streptidine kinase activity also catalyze phosphorylation of streptomycin at the 5 or 6 hydroxyl group of the streptidine

moiety (A. L. Miller and J. B. Walker, *J. Bacteriol* 99:401, 1969). Column chromatography failed to separate the two activities, indicating their probable identity. ATP and Mg^{++} are required for phosphorylation. The physiological role of streptomycin phosphate is unknown, but it might be identical with Nomi's "precursor" (*see* section "Possible Unidentified Intermediates of Streptomycin Biosynthesis"). The finding that streptomycin can be phosphorylated appears to explain the earlier observation of "feedback inhibition" of streptidine kinase by streptomycin (*see* section "REGULATORY MECHANISMS AND STREPTOMYCIN SYNTHESIS").

Further isotopic evidence has been provided using D-[3-^{14}C]-glucose that the C-3' branch carbon atom of streptose is derived from C-3 of D-glucose (J. Bruton and W. H. Horner, *Biochim. Biophys. Acta* 184:641, 1969).

The action and location of *S. griseus* α-D-mannosidase has been further studied (E. Inamine and A. L. Demain, Biotech. Bioeng., *in press*). The enzyme is bound at or near the cell surface and is inactivated by sonic oscillation. Small particulate matter containing most of the activity can be released from the cells into water, such release being inhibited by phosphate, tris(hydroxymethyl)aminomethane, or sodium chloride.

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