Radioactive Myoinositol: Incorporation into Streptomycin

Abstract. *Radioactive myoinositol was rapidly incorporated into streptomycin by* Streptomyces griseus. *All the incorporated radioactivity was found in the streptidine moiety of the antibiotic, as shown by chemical degradation of the streptomycin. To a certain extent inositol reversed the depressing effect of phosphate on the production of the antibiotic.*

Myoinositol exerts a certain stimulatory effect on the production of streptomycin by *Streptomyces griseus*. Majumdar and Kutzner (*1*) found that this effect was greatly increased by simultaneously adding arginine. Arginine itself stimulated streptomycin production to approximately the same extent as myoinositol, but the yields obtained were invariably highest when the two compounds were added together to the media tested. This is in agreement with a theory by Hockenhull (*2*) according to which a glucose derivative is assumed to undergo a ring closure to form a cyclitol that may serve as a precursor for the streptidine. The increased stimulation produced by adding arginine to myoinositol may be explained by the fact that the former most likely functions as the donor of the guanido groups in the streptidine (*3*). That such a transfer of amidine groups actually can take place in *S. griseus* is known from the studies of Walker (*4*) and Shan Chiung Shen *et al.* (*5*). The best evidence for a direct incorporation of the myoinositol in the streptomycin molecule was presented by Majumdar and Kutzner based on the isotope-dilution method. They found that the incorporation of uniformly labeled glucose in the streptomycin was depressed in the presence of myoinositol under growing-culture conditions. The distribution of the labeling in the different parts of the isolated streptomycin showed that the depressing effect on the incorporation was 41 percent for the streptidine moiety and 22 percent for the streptobiosamine. The investigation reported here was undertaken to confirm these results.

Streptomyces griseus strain 107 (*6*), isolated from *S. griseus* strain Z 38, was grown in a medium containing, in grams per liter, glucose, 10.0; yeast extract, 10.0; NaCl, 5.0; $MgSO_4 \cdot 7H_2O$, 0.25; and $FeSO_4 \cdot 7H_2O$, 0.01; the *p*H was adjusted to 7.0 with NaOH before the solution was sterilized. Four 250-ml erlenmeyer flasks, each containing 50 ml of broth, were inoculated with 0.5 ml of a vegetative culture and shaken for 23 hours at 28°C on a reciprocal shaker. At that time the production of streptomycin had reached 40 μg/ml according to assay by the usual biological methods with *Bacillus subtilis* as test organism. A 1-ml portion of a solution of myoinositol-2-H^3 containing 0.4 mg/ml (31 μc) was then added to two of the flasks. After shaking the four flasks for an additional 49 hours (activity 240 μg/ml), the streptomycin was recovered from the two flasks containing the radioactive material by acidifying and filtering the broth and extracting with an organic solvent; the streptomycin was finally obtained as an aqueous solution of the sulfate. To this solution was added 1.500 g of streptomycin sulfate as carrier, diluting the extracted material 46 times. The antibiotic was crystallized from this solution as the reineckate, which was thereafter recrystallized twice from water. The reineckate was then transformed to the sulfate by acidification with dilute H_2SO_4 and the Reinecke's acid was removed by extraction with ethyl acetate three times. The remaining colorless solution was neutralized to *p*H 6.5 with triethylamine, and the streptomycin sulfate was precipitated by slowly adding the aqueous solution to ten volumes of methanol at 35°C while stirring vigorously. A sample of the product thus obtained was dissolved in a small volume of water and after further purification (decolorization with activated carbon and filtration) it was reprecipitated with methanol and dried in a vacuum at 40°C for 48 hours and used immediately for testing. The remaining material was hydrolyzed with sulfuric acid (*7*) and the crystallized streptidine sulfate was recrystallized several times from boiling water and finally dried at 56°C for 6 hours. Radioactive measurements on the streptomycin sulfate and the streptidine sulfate were made with a liquid scintillation counter. The data are shown in Table 1.

It was further found that the streptomycin sulfate showed 8700 count min^{-1} $\mu mole^{-1}$; the streptidine showed 8460 count min^{-1} $\mu mole^{-1}$.

The experiments described were repeated with myoinositol-2-C^{14} (Table 1). The streptomycin isolated showed 992 count min^{-1} $\mu mole^{-1}$ and the streptidine 988 count min^{-1} $\mu mole^{-1}$. This experiment was done in order to exclude the possibility of formation of nonradioactive myoinososе-2 from the tritium-labeled inositol. If this were the case and the inosose thereafter were undetected, it could serve as a precursor for parts of the streptomycin molecule other than the streptidine, though such reactions were not considered likely to occur.

The results (Table 1) show that myoinositol can function as a precursor for streptomycin. Furthermore, they show that the myoinositol is incorporated exclusively into the streptidine moiety of the molecule, since all radioactivity in the isolated streptomycin can be recovered from the streptidine. These results exclude the possibility that the L-glucosamine moiety of the streptomycin is formed by an opening of the myoinositol ring between carbon atoms 3 and 4. If this were the case, the specific labeling of the streptidine would have been considerably lower than that of the streptomycin. Almost 30 percent of the myoinositol is incorporated in the acid-insoluble fraction of the mycelium because this amount of radioactivity is lost by the acidification and filtration of the broth. If the extraction of the streptomycin were completely selective the incorporation into the streptomycin would be almost 50 percent. This

Table 1. Distribution of radioactivity in *S. griseus* broth labeled with myoinositol-2-H^3, and in broth labeled with myoinositol-2-C^{14}.

Fraction	Myoinositol-2-H^3		Myoinositol-2-C^{14}	
	Radioactivity per ml (10^4 count/min)	No. of ml	Radioactivity per ml (10^3 count/min)	No. of ml
Broth	44.4	100	56.26	50
Broth after shaking for 48 hours			51.2	53
Filtrated broth			29.6	67
Residue after extraction of streptomycin	28.0	105	10.3	70
Streptomycin sulfate	40.00	40	26.8	50

Table 2. Effect of myoinositol in broths containing varying amounts of inorganic phosphate.

Additional phospate (%)	Additional inositol (%)	Maximum yield (μg/ml)	Stimulation (%)
	*Organic substrate**		
0	0.00	269	
0	.05	279	+ 4
0.1	.00	60	
.1	.05	117	+87
.2	.00	83	
.2	.05	118	+41
	Synthetic substrate†		
0.005	0.000	166	
.005	.050	120	−28
.015‡	.000	288	
.015	.050	350	+ 22
.030	.000	72	
.030	.050	111	+ 54
.050	.000	14	
.050	.050	17	+ 21

* Glucose-yeast extract medium. † Glucose-ammonium nitrate (*8*). ‡ Normal amount of phosphate in this substrate.

would be unusually high. This indicates that the formation of the inositol ring may be a limiting step at least in many cases in the formation of streptomycin.

Further support for the theory that myoinositol is a direct precursor for streptomycin is that myoinositol, to some extent, is capable of overcoming the depressing effect of high concentrations of phosphate. This is shown to be true for the organic substrate already described and for a simple medium of glucose and ammonium nitrate (*8*). The total activity in the high phosphate cultures did not reach that of the controls, but the percentage of stimulation was much higher in the presence of large amounts of phosphate (Table 2). The optimal proportion between inositol and phosphate was not determined. There is still the question of whether myoinositol is the natural precursor for the streptidine, although the indications are that it is the precursor. An interesting fact which some authors seem to have overlooked is that the myoinositol has a steric configuration other than that of the streptidine which has a scyllo configuration. This means that if the myoinositol before or after the attachment of the two guanido groups does not undergo any rearrangements, the glucosidic band connecting it with streptobiosamine must be established; at carbon atom 2 simultaneous inversion of the steric configuration at this atom takes place. Only one author (*9*) has tested scylloinositol which has the same steric configuration as streptidine. He found that scylloinositol did not increase the production of streptomycin; the compound was, however, capable of reversing the inhibitory effect shown by its oxidation product myoinosose.

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References and Notes

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Conversion of Leucoanthocyanins into the Corresponding Anthocyanidins

Abstract. *Purified preparations of leucoanthocyanins from several sources were heated in butanol–hydrochloric acid solutions from 50° to 90°C and the rate of production of cyanidin was measured. From the temperature dependence of this rate, the calculated energy of activation was of the order of magnitude of 20,000 calories. The rate-limiting step in this reaction was similar for two preparations tested, and of lower energy for a third, presumably of lower degree of polymerization.*

Colorless precursors of anthocyanidins occur either as the pesudobase (*1*) or leucoanthocyanin (*2*), also termed anthocyanogens, proanthocyanidins, or flavolans (*3*). Since Rosenheim (*2*) first demonstrated the presence of a colorless precursor of oenidin (cyanidin) in grape leaves, which was converted into the colored oenidin during boiling with 20 percent hydrochloric acid, the existence and widespread distribution of leucoanthocyanins in plant tissue has been demonstrated qualitatively (*4–9*). While the Robinsons (*4*) used solubility and color reactions for qualitative identification, Bate-Smith (*6*) first introduced paper chromatography for the identification of anthocyanidins formed from leucoanthocyanidins, and this technique was extended by Roux (*8, 9*). Cyanidin is the chief anthocyanidin pigment obtained from plant leucoanthocyanins, although delphinidin and others have been reported in some cases. The conversion was obtained by heating aqueous extracts with hydrochloric or sulfuric acid (the Robinsons (*4*) used boiling 10 percent HCl, Bate-Smith (*6, 7*) used hot 2*N* HCl, heating the reaction in a boiling water bath). Quantitative methods for their determination were introduced by Pigman *et al.* (*10*) and modified by others (*11–13*). More empirical modifications were reported by Luh *et al.* and by Nakayama and Chichester (*14*). Pigman *et al.* (*10*) introduced the use of *n*-propanol solutions of hydrochloric acid instead of aqueous or methanol solutions and reported some observations on the kinetics of conversion of spruce leucoanthocyanins to cyanidin. Swain and Hillis (*11*) introduced the use of *n*-butanol in place of the more volatile *n*-propanol, and reported that the conversion was not quantitative even over the range of 50 to 400 μg of leucoanthocyanin. While it is known that the conversion of anthocyanogen into the corresponding anthocyanidin, on heating with alcohol containing HCl, is not complete, yields of 10 percent or less being obtained, the kinetics of the reaction and its mechanism have not been investigated. Pigman *et al.* (*10*) reported that the conversion in *n*-propanol containing HCl was reduced in rate and extent by the presence of water. They obtained a higher yield of anthocyanin in 0.03*N* HCl in the absence of water than in 1.8*N* HCl in the presence of 20 percent of water, by volume. This result was confirmed and extended by Roux and Bill (*15*) who reported yields of about 40 percent with anhydrous 0.03 to 0.02*N* HCl in *n*-propanol in comparison with 3 percent yields in aqueous 3*N* HCl and 20 percent yield with 3*N* HCl in *n*-propanol under the conditions of Pigman *et al.* (*10*). They proposed that the conversion of flavan-3, 4-diols into anthocyanidin occurs by dehydration at the diol group followed by oxidation or disproportionation. Molecular size limited the extent of conversion (*13, 16*). The yield of fise-