STUDIES ON THE BIOSYNTHESIS OF THE ANTIBIOTIC STREPTOZOTOCIN (STREPTOZOCIN) BY *STREPTOMYCES ACHROMOGENES* VAR. *STREPTOZOTICUS*

FEEDING EXPERIMENTS WITH CARBON-14 AND TRITIUM LABELLED PRECURSORS

SARASWATHI SINGARAM, REBECCA S. LAWRENCE and ULFERT HORNEMANN*

Department of Medicinal Chemistry and Pharmacognosy,
School of Pharmacy and Pharmacal Sciences, Purdue University,
West Lafayette, Indiana 47907, U.S.A.

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Feeding experiments and chemical degradations have shown that D-[1-^{14}C,2-^{3}H]- and -[1-^{14}C,6-^{3}H] glucosamine, L-[ureido-^{14}C] citrulline, L-[guanidino-^{14}C] arginine and L-[^{14}CH$_3$] methionine specifically label the glucosamine moiety, the urea carbonyl and the N-methyl group of the antibiotic streptozotocin, respectively. Feeding these precursors in amounts of 5~10 μmoles per 100 ml of culture medium under conditions where the fermentation yielded approximately 20 μmoles of streptozotocin in 24 hours gave incorporation rates which approached 40%. Upon feeding 100 μmoles of either D-[1-^{14}C] glucosamine or L-[ureido-^{14}C] citrulline they were incorporated into newly synthesized streptozotocin essentially without dilution by endogeneous precursors. D-[1-^{14}C, 6-^{3}H] Glucosamine was incorporated without change in T/C ratio while 20% of the tritium was lost from D-[1-^{14}C,2-^{3}H] glucosamine, suggesting the possibility that D-glucosamine can partially equilibrate with D-fructose prior to its incorporation.

Streptozotocin (**I**)[1], the only known naturally occurring N-methyl-N-nitrosourea derivative, is elaborated by *Streptomyces achromogenes* var. *streptozoticus*[2]. Its diabetogenic action, its use as an antiinsulinoma and antileukemia agent as well as its carcinogenicity have recently been reviewed[3]. Our general interest in the unexplored area of biochemical nitrogen-nitrogen bond formation and our specific interest in the biosynthesis of antibiotics that contain a nitrogen-nitrogen bond (*cf* ref. 4) prompted us to undertake feeding experiments to gain information on the streptozotocin biosynthetic pathway.

Compounds which like streptozotocin contain a nitrogen-nitrogen bond are not found in great abundance in natural products yet approximately 20 antibiotics and approximately 15 other microbial metabolites as well as approximately 15 mushroom constituents or plant alkaloids are known to contain such bonds. Many of these compounds are of interest as anticancer agents or as carcinogens but in spite of this interest hardly anything is known about their biosynthesis especially with regard to the establishment of the nitrogen-nitrogen bond[4]. Since many of the producing organisms are unable to fix atmospheric dinitrogen it is highly likely that the nitrogen-nitrogen bonds are established by the joining of two separate nitrogen atoms. One of them, presumably an amino or amido-nitrogen atom, could be provided in many cases in association with a carbon skeleton, and the other could presumably arise from a source for a single nitrogen atom. A particularly attractive candidate to provide a single nitrogen atom would be nitrous acid or a biochemical equivalent of presently unknown

nature. Nitrous acid is attractive because it is generally known to organic chemists to be of great utility in the synthesis of compounds containing linked nitrogens and because enzymes have been described from a variety of sources[5], including *Streptomycetes*[6] which can generate it by oxidizing either aromatic or aliphatic nitrocompounds.

Our working hypothesis on streptozotocin biosynthesis assumes that D-glucosamine is the precursor for the glucosamine moiety, that carbamoylphosphate or a related compound donates part of the urea grouping and that the methyl group of L-methionine donates the N-methyl group of the antibiotic. We report in this paper the results of our biosynthetic experiments which demonstrate that this working hypothesis is valid.

Experimental

Fermentation and Isolation of Streptozotocin

Method A. *S. achromogenes* var. *streptozoticus* (NRRL 3125) was maintained on EMERSON agar on which it sporulates readily, and as a frozen spore suspension in 20% glycerol. A medium[2] composed of (g/liter) refined yellow cornmeal, 40; potato starch (Sigma), 15; Cerelose (glucose × 1 H_2O), 3; peptone (Difco), 3; and ammonium sulfate, 4, served as a seed and production medium. Two ml of mycelial suspension obtained in this medium from the outgrowth of a spore inoculum were used to seed production flasks containing 100 ml sterile medium in 500-ml Erlenmeyer flasks which were incubated at 24°C on a rotary shaker (240 rpm) for 5 days. Radioactive precursors were added after 4 days of growth; 24 hours later the antibiotic was harvested by gradual addition with stirring of 100 ml of acetone followed by removal of precipitated cells and other acetone-insoluble materials by filtration. Evaporation of the filtrate under vacuum to approximately 5 ml followed by removal of a formed precipitate by filtration and subsequent addition to the supernatant of 25 ml of acetone and removal by centrifugation of a further precipitate, afforded an enriched solution of the antibiotic. After concentration under vacuum to approximately 5 ml, one third of this solution was spotted on Whatman No. 3 paper for ascending chromatography in 1-butanol - acetic acid - water (2: 1: 1). The antibiotic usually travels at an Rf between 0.45 and 0.60 and was detected by spraying a portion of the chromatogram with a solution consisting of 20 ml 6 N hydrochloric acid, 50 ml 1% sulphanilic acid in 30% acetic acid, and 50 ml 0.1% N-1-naphthylethylenediamine in 30% acetic acid or by scanning the chromatogram with a radiochromatogram scanner. The band containing the streptozotocin was eluted with water.

Method B. Seed medium[2] consisting of (g/liter) cotton seed meal, 25 and Cerelose, 25, in 25 ml distilled water in 125-ml Erlenmeyer flasks was inoculated with 0.1 ml of spore suspension of *S. achromogenes* and shaken for 40~80 hours at 27°C at 250 rpm. The resulting mycelial suspension (1.5 ml) was transferred into 500-ml Erlenmeyer flasks containing 100 ml of production medium having the composition given under Method A except that 14 g/liter $(NH_4)_2SO_4$ was used instead of only 4 g/liter and that the glucose was reduced to 1 g/liter. The cultures were shaken at 350 rpm at 28°C for 5 days, radioactive precursors and/or other supplements were added 24 hours before isolating streptozotocin by the acetone precipitation procedure. The resulting solution was concentrated to approximately one ml and chromatographed on a column of Sephadex G-10 (10 × 0.5 cm) using water as the eluant. The elution was monitored by spotting small aliquots of the column fractions on paper followed by spraying with the spray reagent given above. The pooled fractions were concentrated to a small volume and the antibiotic was subjected to paper chromatography in acetone - water (85: 15). The streptozotocin zone was eluted with water and further purified by paper chromatography in 1-butanol - acetic acid - water (2: 1: 1) yielding a homogeneous sample of the antibiotic after its elution with water. The aqueous solutions obtained were either freeze dried or kept frozen.

Analytical Procedures

Streptozotocin was assayed by the method of FORIST[7]. For direct determination of the streptozotocin content in broth 3 ml of the culture medium were centrifuged at 10,000 rpm for 15 minutes prior

to the addition of the reagents. Glucose was determined using glucose oxidase from *Aspergillus niger* (Sigma) and horseradish peroxidase[8]. Ammonia was determined by the indophenol blue method[9]. Radioactive samples were analyzed on Beckman LS-100 and LS-250 liquid scintillation counters in BRAY's solution[10]. Counting efficiencies were determined by addition of external standard. Radiochromatograms were analyzed on a Packard model 7201 radiochromatogram scanner.

Precursor incorporation into streptozotocin was determined as follows: The specific radioactivity of the antibiotic was determined after the paper chromatographic purification step in Method A and after the last paper chromatographic purification step in Method B. These values were multiplied by the amount of streptozotocin produced by the respective culture as determined in the centrifuged culture broth. The resulting value for the total radioactivity residing in streptozotocin was divided by the total amount of radioactivity administered and multiplied by 100 to give the incorporation rate.

Degradation of Streptozotocin

To samples of labeled streptozotocin obtained as eluates from paper chromatograms following Method A and to a sample of streptozotocin labelled from L-citrulline obtained by Method B were added separately 300 mg of carrier streptozotocin for triplicate recrystallization to constant specific activity from absolute ethanol by slow evaporation of the solvent. The antibiotic was subsequently degraded by the procedure of KARUNANAYAKE *et al.*[11] Usually 200 mg of the recrystallized streptozotocin were suspended in H_2O (1.4 ml), covered with ether (50 ml) and then treated with 2 N NaOH (1.5 ml) at 0°C for 10 minutes yielding compound II[12] and diazomethane which was trapped with 250 mg of *p*-nitrobenzoic acid dissolved in 60 ml of ether as *p*-nitrobenzoic acid methyl ester whose specific radioactivity was determined. Treatment of compound II remaining in the reaction vessel with 2 N HCl (3 ml) for 45 minutes at room temperature yielded glucosamine III, and CO_2. The latter was trapped in a saturated solution of barium hydroxide. The precipitate of barium carbonate formed was isolated by filtration, washed with water, dried, weighed and the radioactivity was counted in Triton-EDTA by the method of LARSEN[13]. D-Glucosamine remaining in the reaction flask was isolated and purified by preparative paper chromatography in methanol - 1-butanol - water - conc. ammonia (80: 20: 10: 3) Rf=0.65, followed by elution from the paper with water and subsequent recrystallization to constant specific radioactivity from water - acetone.

Materials

S. achromogenes var. *streptozoticus* strains NRRL 2697 and NRRL 3125 were obtained from the Northern Regional Research Laboratory, USDA, Peoria, Ill. Streptozotocin was obtained from the Upjohn Co., Kalamazoo, Mich. Radioactively labeled compounds were obtained from Amersham, Chicago, Ill. except for D-[2-^{3}H]glucosamine which was synthesized[14]. Double-labeled precursors were obtained by mixing the respective labeled species. All other materials used were analytical grade commercial products.

Results

Our initial feeding experiments were carried out with *S. achromogenes* (NRRL 3125) cells grown by Method A which afforded stable antibiotic production at a level of 1~3 mg of streptozotocin per 100 ml medium in 5 days. This level of production was adequate for the biosynthetic experiments with the carbon-14 and tritium labeled precursors listed in Tables 1 and 2 since the experimental design permitted and even demanded the dilution with carrier streptozotocin to perform the required degradations. However, our later feeding experiments required the isolation of undiluted streptozotocin in larger quantity than obtainable by Method A and therefore Method B was developed.

Method B is based on previously reported work[2]. We observed that *S. achromogenes* strain NRRL 3125 gave higher titres than strain NRRL 2697, therefore strain NRRL 3125 was also used in all feeding experiments dependent on Method B. We observed that 1 g/liter glucose rather than 3 g/liter was beneficial for antibiotic production and that 14 g/liter ammonium sulfate, rather than

Table 1. Incorporation of singly and doubly labelled precursors into streptozotocin.

Precursor				Stretozotocin		
	Quantity fed (mg)	Radioactivity fed (dpm)	T/C ratio	Method of cultivation and isolation	Incorporation (carbon-14) %	T/C ratio (T-Retention) %
D-[6-^{3}H]Glucosamine	2.2	7.92×10^{6}		A	1.5*†	
D-[1-^{14}C, 6-^{3}H] Glucosamine	5.4	^{14}C=8.38×10^{6} ^{3}H=7.58×10^{7}	9.05	B	42.0	9.00 (99.9)
D-[1-^{14}C, 2-^{3}H] Glucosamine	7.6	^{14}C=2.16×10^{6} ^{3}H=1.92×10^{7}	8.90	B	34.0	7.16 (80.8)
L-[Guanidino-^{14}C] arginine	2.1	2.11×10^{7}		A	1.5†	
L-[Guanidino-^{14}C] arginine	1.9	1.46×10^{7}		B	33.0	
L-[Ureido-^{14}C] citrulline	1.7	2.51×10^{7}		A	5.4	
L-[Ureido-^{14}C] citrulline	1.7	4.55×10^{7}		B	38.6†	
L-[$^{14}CH_3$] Methionine	1.6	2.66×10^{7}		A	6.1†	

* In this instance the tritium incorporation is given.
† Subjected to chemical degradation, see Table 2.

Table 2. Degradation of streptozotocin.

Precursor	Incorporation into streptozotocin (%)	Specific radioactivity (dpm /mM) [Percentage of streptozotocin specific radioactivity]			
		Streptozotocin	Methyl *p*-nitro benzoate	$BaCO_3$	Glucosamine
D-[6-^{3}H]Glucosamine	1.5	5.32×10^{4}	*	*	4.73×10^{4} [89.0]
L-[Guanidino-^{14}C]arginine	1.5	6.51×10^{4}	*	6.46×10^{4} [99.2]	*
L-[Ureido-^{14}C]citrulline	38.6	3.99×10^{4}	*	3.61×10^{4} [90.5]	*
L-[$^{14}CH_3$]Methionine	6.1	2.43×10^{5}	2.36×10^{5} [97.1]	*	*

* Less than 0.5% of specific radioactivity of streptozotocin detected in fragment.

4 g/liter, was optimal for pH control. Ammonium sulfate was recommended by previous workers[2] to maintain the pH of the fermentation at pH 4~5, the pH range of optimal stability of streptozotocin, and it was assumed that selective utilization of the ammonium by the culture was responsible for the pH controlling effect. We have measured the levels of streptozotocin, glucose and ammonium as well as the pH in the fermentation and find that while the streptozotocin titre rises to a peak of 70 mg in 14 days, which is higher than any previously reported streptozotocin titre, that glucose is utilized completely during the first 24-hour period after inoculation and that the ammonium level and the pH remain essentially constant (data not shown). Attempts to achieve stabilization of the pH at the desired level by substituting Na_2SO_4 for $(NH_4)_2SO_4$ were not successful. The streptozotocin fermentation according to Method B is very stable and highly reproducible from batch to batch. This method afforded very high incorporation rates enabling us to conduct the

studies to determine the dilution factors for the precursors listed in Table 3.

Table 3. Dilution factors for glucosamine and citrulline (Method B).

Precursor		Streptozotocin	
Radioactivity fed: 10 μCi	Amount fed (mg)	Yield (mg)	Dilution factor
D-[1-^{14}C]Glucosamine	25	14.0	2.3
	50	15.5	2.1
	100	10.4	
	300	7.6	
L-[Ureido-^{14}C] citrulline	25	21.5	4.4
	50	10.5	3.3
	100	8.8	2.2
	300	6.3	

It is evident from the results of our feeding experiments with the precursors listed in Table 1 and from the results of the chemical degradations (Table 2), which were carried out by the sequence of reactions shown in Fig. 1, that specific building blocks for streptozotocin have been identified. D-Glucosamine is incorporated into the aminohexose moiety of streptozotocin and since the T/C ratio of the (1-^{14}C, 6-^{3}H) labeled sample did not change upon incorporation it is concluded that the carbon skeleton of D-glucosamine is incorporated intact. The loss of 20% of the tritium from the 2 position, revealed in the feeding experiment with D-[1-^{14}C, 2-^{3}H]glucosamine, suggests that a portion of the aminohexose may equilibrate with D-fructose prior to incorporation. L-[^{14}CH$_3$]Methionine is an efficient and specific donor of the N-methyl group of streptozotocin and it will be of interest to determine at what stage of the pathway it is introduced. Both L - [guanidino - ^{14}C] arginine and L-[ureido-^{14}C]citrulline specifically label the ureido carbon of streptozotocin. A decision as to which of these compounds is the more proximate precursor can not be derived from the available data. A possible direct role of carbamoylphosphate in streptozotocin biosynthesis could not be tested by feeding sodium carbonate, or sodium cyanate, which, at neutral pH would be expected to be precursors of carbamoylphosphate, because the acidic pH of the fermentation precluded such a feeding experiment.

Fig. 1. Degradation of streptozotocin.

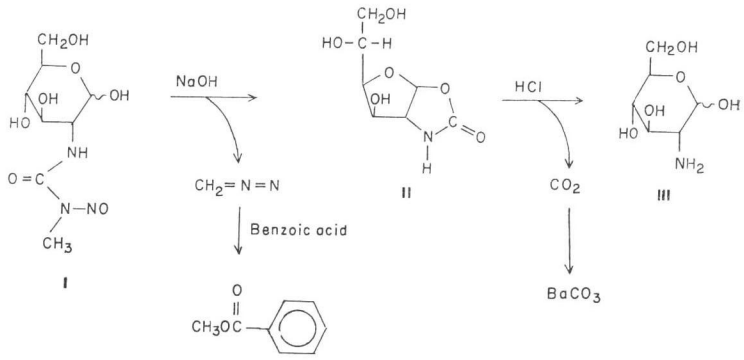
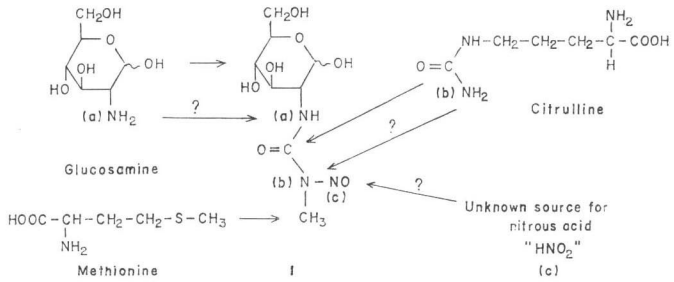


Fig. 2. Precursors of streptozotocin.



The demonstration that D-[1-^{14}C, 6-^{3}H]glucosamine and L-[ureido-^{14}C]citrulline are specific precursors of streptozotocin suggests that N(a) and N(b), respectively, of the antibiotic may be provided by these precursors as outlined in Fig. 2. If this assumption could be substantiated then it should be possible to saturate these two nitrogen atoms by feeding a suitable excess of D-glucosamine and

L-citrulline. The origin of the nitrogen atom of the nitroso group (N(c)) could then be investigated by feeding putative nitrogen-15 labeled precursors for that group in the presence of excess D-glucosamine and L-citrulline. In preparation for experiments to investigate if indeed D-glucosamine and L-citrulline donate N(a) and N(b), we determined dilution factors for these precursors using the carbon-^{14}C labeled compounds. The results of these experiments which are shown in Table 3 demonstrate that there is extremely efficient utilization of these precursors. Given the fact that dilution factors as low as two are observed and assuming that during the 24-hour feeding period approximately 6 mg of streptozotocin were synthesized, it is conceivable that the dilution is due entirely to streptozotocin that had been produced prior to the addition of the precursors. This would mean that the added precursors were incorporated essentially without dilution from endogeneous precursor. It is planned in furture experiments to feed D-[^{15}N]glucosamine and L-[NH_2CO-^{15}N]citrulline to see if under these conditions the expected nitrogens of streptozotocin are labeled. In the case of D-glucosamine some incorporation of nitrogen into nonspecific positions can be predicted since, as reported above, D-[1-^{14}C,2-^{3}H]glucosamine may be in partial equilibrium with D-fructose thus liberating some of the nitrogen for other metabolic reactions.

Discussion

The results of this investigation show that D-glucosamine, L-methionine, L-citrulline and L-arginine are highly efficient and specific precursors of the antibiotic streptozotocin. Free D-glucosamine or a suitable derivative such as a nucleoside derivative, provides the basic skeleton including presumably the nitrogen atom N(a). There are numerous possible intermediate stages of the sreptozotocin pathway where the methyl group of methionine may be introduced, including the possibility that methylamine may be involved in the pathway. These possibilities await study experimentally.

Similarly several possible intermediates can also be envisioned to account for the incorporation of citrulline and arginine. Both compounds have been shown in this laboratory to be efficient precursors of a carbamoyl group which is present in the mitomycin antibiotics, and L-[NH_2CO-^{13}C,^{15}N]-citrulline was demonstrated to donate the intact carbamoyl group in that pathway[15]. In both the streptozotocin and the mitomycin pathway it is possible that the carbamoyl group of the citrulline ureido group is transferred directly to an appropriate acceptor, or that carbamoyl phosphate is involved as an intermediate, which could be formed *via* a reversal of the ornithine transcarbamoylase reaction. Arginine could feed into these pathways after its conversion into citrulline by the action of the enzyme arginine deiminase. Low levels of this enzyme have been demonstrated in mitomycin-producing *Streptomyces verticillatus* from which an arginine deiminase activity has been enriched nearly 50 fold and also *S. achromogenes* var. *streptozoticus* crude-cell-free extracts were shown to have low levels of this activity (J. H. EGGERT, T. J. SMITH, M. R. DHAWALE, and U. HORNEMANN, unpublished observations).

The very efficient utilization of D-glucosamine and of L-citrulline in the dilution experiments reported in Table 3 is of great interest. Two possible explanations for this efficient utilization may be considered: (1) the existence of very efficient uptake systems for both precursors and (2) the possible existence of membrane bound enzymes involved in the streptozotocin pathway or even the existence of exoenzymes. If the latter possibility were used by *S. achromogenes* var. *streptozoticus* then a mechanism would exist to prevent the highly mutagenic antibiotic from damaging the DNA of the producing organism.

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