

Streptozotocin: Depression of Mouse Liver Pyridine Nucleotides¹

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SUMMARY

A single intravenous diabetogenic dose of Streptozotocin, 200 mg/kg, produced a 24-hour depression of oxidized and reduced nicotinamide adenine dinucleotide (NAD and NADH) content in mouse liver. This was followed by a gradual return to normal coenzyme concentrations by the end of 48 hours. Intraperitoneal nicotinamide, 500 mg/kg, administered ten minutes prior to Streptozotocin protected animals against NAD depression for approximately 16 hours. Animals receiving nicotinamide alone showed significantly higher liver NAD values than the combination treatment group.

It was found that 1-methyl-1-nitrosourea, the nondiabetogenic but antileukemic moiety of the Streptozotocin molecule, could produce a similar dose-related depression of NAD, while Alloxan did not.

The serum half-life of Streptozotocin after an intravenous injection, 200 mg/kg, was approximately 5 minutes, with no drug measurable by two hours. Streptozotocin was detectable in the acid-soluble fraction of liver for 20 hours, and it was found that nicotinamide pretreatment did not significantly alter the drug uptake by this organ.

Using prevention of diabetes as the criterion of toxicity modification, a schedule of small serial administrations of nicotinamide over a 24-hour period was shown to be more effective than the injection of the total dose just prior to Streptozotocin.

It is suggested that Streptozotocin is inhibiting synthesis of pyridine nucleotides.

INTRODUCTION

Streptozotocin, an antitumor agent composed of glucose and 1-methyl-1-nitrosourea (MNU), was isolated from a fermentation broth of *Streptomyces achromogenes* (3, 5, 6). In addition to hepatotoxic properties, the compound can produce a permanent diabetic state in rodents, dog, and monkey (18, 21, 23, 24). Recent studies have shown that the acute diabetogenic action of Streptozotocin can be prevented by the simultaneous administration of large single doses of nicotinamide. In the course of study of this protective effect, it was demonstrated that toxicity could be correlated with depression of liver nicotinamide adenine dinucleotide (NAD) concentration, a

phenomenon readily prevented by nicotinamide pretreatment (21).

This communication deals with the changes in liver NAD and reduced nicotinamide adenine dinucleotide (NADH) concentrations over a 48-hour period following the single intravenous administration of Streptozotocin. These effects are correlated with the measurement of drug concentration in serum and the acid-soluble fraction of liver. The possible relationships between the separate phenomena of NAD depression and diabetogenicity and the development of more efficient nicotinamide treatment schedules are presented.

MATERIALS AND METHODS

Male albino mice (Swiss strain) weighing 25-30 grams were used for all studies and were maintained on Purina laboratory chow pellets and water *ad libitum*.

Streptozotocin (Upjohn), NSC-85998, was prepared fresh daily in a 0.005 M citrate buffer, pH 4.0. Nicotinamide (Calbiochem), 1-methyl-1-nitrosourea (NSC-23909), and Alloxan (NSC-7169) were dissolved in distilled water. Drugs were administered at a volume of 1 ml per 100 gram body weight intravenously via the tail vein or intraperitoneally.

The NAD and NADH content of liver was determined as follows: animals were killed by cervical dislocation, and the liver was removed rapidly and blotted to remove excess blood. Samples for NAD analysis were weighed and immediately homogenized 1:5 weight:volume in 0.6 N perchloric acid kept at 0°C. The homogenate was centrifuged for five minutes at 5,000 × g after neutralization with 3 N KOH; the supernate was then assayed for NAD using alcohol dehydrogenase (Sigma) (11). The NADH content was assayed enzymatically by the method of Klingenberg, using lactic dehydrogenase (Sigma) (12).

Blood samples for blood sugar determinations were obtained from the tail veins of mice. An 0.01 ml aliquot of plasma was incubated in 5.4 ml of Glucostat reagent (Worthington) for 1 hour at 37°C. After the addition of 0.6 ml of 12 N sulfuric acid, optical densities were determined at 530 mμ against a blank composed of reagent and sulfuric acid and compared to a standard curve (10).

The concentration of Streptozotocin in serum and liver was measured colorimetrically using the method of Forist (2). Blood samples were obtained by cardiac puncture and centrifuged at 5,000 × g. The plasma was diluted 1:5 with 0.6 N perchloric acid. A 0.1 ml sample of the resultant serum was

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Table 1

Time (hr)	Streptozotocin, 200 mg/kg i.v.		Nicotinamide, 500 mg/kg i.p., and Streptozotocin, 200 mg/kg i.v.		Nicotinamide, 500 mg/kg i.p.	
	Mean ^a	±S.D. (μg/gm liver)	Mean ^a	±S.D. (μg/gm liver)	Mean ^a	±S.D. (μg/gm liver)
0.5	319	4	764	62	656	25
1	272	35	811	118	888	103
2	103	24	1017	166	1445	137
4	104	34	1187	200	1722	88
6	82	35	878	115	1548	92
8	104	18	829	105	1406	99
12	166	23	785	277	1350	38
16	157	26	332	79	582	60
20	151	24	135	18	553	30
24	128	19	224	12	392	27
30	253	21	624	102		
36	356	32	477	88	480	38
48	416	30	410	35	424	24

Nicotinamide adenine dinucleotide concentration in mouse liver after the single injection of Streptozotocin and nicotinamide. Control mean, 434 ± 38 ; 15 determinations.

^a 5 determinations.

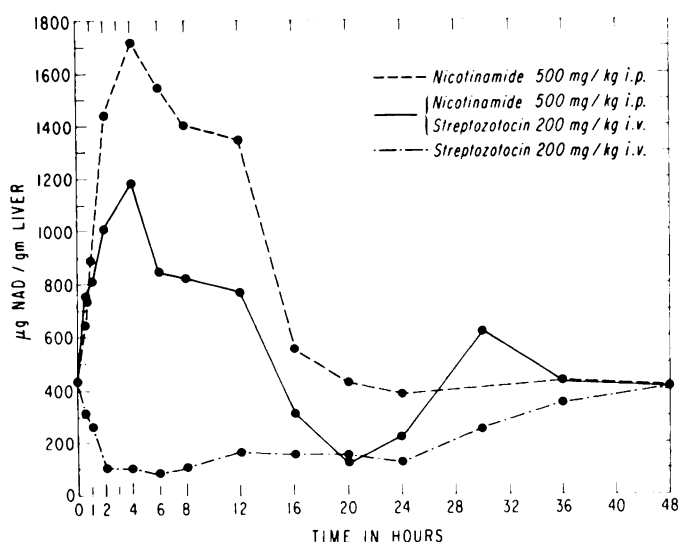


Chart 1. Nicotinamide adenine dinucleotide (NAD) concentration in mouse liver after a single treatment with Streptozotocin, nicotinamide 10 min prior to Streptozotocin, and nicotinamide alone. Each point represents the mean of 5 determinations.

Table 2

Time (hr)	Mean (μg/gm liver) ^a	±S.D.
2	85	22
4	37	13
12	39	11
24	85	16
48	162	32

Reduced nicotinamide adenine dinucleotide concentration in mouse liver after a single intravenous injection of Streptozotocin, 200 mg/kg. Control mean, 188 ± 28 ; 10 determinations.

^a 5 determinations.

added to 0.5 ml of the color reagent (0.5% sulfanilic acid and 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride in 30% acetic acid). To this was added 0.1 ml of 6 N HCl and the solution was incubated at 60°C for 45 minutes. Liver samples were weighed and homogenized 1:5 weight:volume in 0.6 N perchloric acid. After centrifugation 1 ml of supernatant was added to 5 ml of the color reagent and 1 ml of 6 N HCl and incubated at 60°C for 45 minutes. All samples were read at 550 mμ and compared to a standard curve.

RESULTS

The Effect of Streptozotocin on Liver Oxidized and Reduced Nicotinamide Adenine Dinucleotide Concentrations (Chart 1, Tables 1, 2). After a single injection of a diabetogenic dose of Streptozotocin, there occurred an acute fall in coenzyme concentration to approximately one-fourth that of control values. This NAD effect continued for 24 hours, following which time there was a gradual return to control level. A single large dose of nicotinamide, 500 mg/kg administered 10 minutes prior to Streptozotocin, gave significant protection against the NAD depression for approximately 16 hours, while at 20 hours no protective effect was demonstrable. At 24 hours NAD values in this combination treatment group showed a rise, with concentrations significantly higher than control being recorded at the 30-hour determination period. Nicotinamide treatment alone gave statistically higher NAD concentrations throughout the first 24 hours, compared to the combination treatment groups. All three treatment groups returned to control values by 48 hours.

Table 2 shows the effects of Streptozotocin treatment on liver NADH concentration. The results, in general, parallel those found in measurements of NAD level. There is a rapid fall in the reduced coenzyme level by 2 hours, a phenomenon which lasts for at least 24 hours. A return to near normal values was recorded 48 hours after the injection.

Table 3

Agent	Route	Dose (mg/kg)	Mean NAD ($\mu\text{g}/\text{gm}$ liver) ^a	\pm S.D.
1-Methyl-1-nitrosourea	i.v.	50	288	18
		100	170	8
		150	102	9
Nicotinamide ^b 1-Methyl-1-nitrosourea	i.p.	500	1011	136
	i.v.	100		
Alloxan	i.v.	100	488	84
		200	402	13

Nicotinamide adenine dinucleotide (NAD) concentration in mouse liver four hours after the intravenous injection of 1-methyl-1-nitrosourea and Alloxan.

^a Mean of 5 determinations.

^b Administered 10 minutes prior to 1-methyl-1-nitrosourea.

The Effect of Intravenous MNU and Alloxan on Liver Nicotinamide Adenine Dinucleotide Concentration (Table 3). The administration of graded doses of MNU resulted in dose-related decrements of NAD content at four hours. Pretreatment with nicotinamide prevented the NAD depression of 100 mg/kg of MNU and resulted in NAD concentrations greater than control. Alloxan, at doses of 100 and 200 mg/kg, failed to lower liver NAD content.

Table 4

Time (min)	Mean ($\mu\text{g}/\text{ml}$ serum) ^a	\pm S.D.
1	5097	503
5	2338	364
15	763	117
30	83	12
60	31	5
120	<1	
180	<1	

Concentration of Streptozotocin in mouse serum after a single intravenous injection of 200 mg/kg, approximately 6 mg total dose.

^a 5 determinations.

Streptozotocin Concentrations in Serum and Liver (Tables 4 and 5). One minute after the intravenous injection of 200 mg/kg, approximately 80–90% of the total administered dose was present in the serum. Subsequently there occurred a rapid decrease in concentration, with no drug detectable by two hours.

The acid-soluble fraction of liver showed detectable levels of Streptozotocin for 20 hours. Approximately 7% of the total administered dose was measureable in the liver by one hour. Pretreatment with nicotinamide gave slightly lower mean Streptozotocin concentrations for the initial four hours but did not appreciably affect liver uptake or length of time the drug could be detected.

Effect of Various Schedules and Doses of Nicotinamide on the Prevention of Diabetes (Table 6). Single doses of 100, 200, 300, and 400 mg/kg of nicotinamide failed to completely prevent the diabetes of Streptozotocin, though partial protection was afforded. When 200 and 300 mg/kg doses were given in divided doses over a 24-hour period, more effective protection was demonstrated; virtually all animals presented normal blood sugars one week after treatment. Oral administration of nicotinamide also was capable of preventing the diabetes.

Table 5

Time (hr)	Streptozotocin, 200 mg/kg i.v.		Nicotinamide, ^a 500 mg/kg i.p., and Streptozotocin, 200 mg/kg i.v.	
	Mean ^b ($\mu\text{g}/\text{gm}$ liver)	\pm S.D.	Mean ^b ($\mu\text{g}/\text{gm}$ liver)	\pm S.D.
1	436	43	382	54
2	181	19	166	44
4	135	11	107	24
8	84	10	89	8
12	23	3	18	2
16	16	2	13	2
20	8	2	9	1
24	<1		<1	

Concentration of Streptozotocin in the acid-soluble fraction of mouse liver after a single intravenous injection.

^a Administered 10 minutes prior to Streptozotocin.

^b 5 determinations.

Table 6

Agent	Route	Dose (mg/kg)	Schedule	Number of animals	Range (mg %)	Mean (mg %)
Control				15	136-178	157
Streptozotocin	i.v.	200	× 1	15	315-525	484
Nicotinamide	i.p.	100				
Streptozotocin	i.v.	200	× 1	5	339-564	476
Nicotinamide	i.p.	200				
Streptozotocin	i.v.	200	× 1	5	167-412	258
Nicotinamide	i.p.	300				
Streptozotocin	i.v.	200	× 1	5	168-327	227
Nicotinamide	i.p.	400				
Streptozotocin	i.v.	200	× 1	5	168-259	213
Nicotinamide	i.p.	50	q 6 hr × 4 ^a			
Streptozotocin	i.v.	200	× 1	5	89-206	145
Nicotinamide	i.p.	100	q 8 hr × 3 ^b			
Streptozotocin	i.v.	200	× 1	5	88-177	131
Nicotinamide	p.o.	500				
Streptozotocin	i.v.	200	× 1	5	130-189	158

Blood sugars of albino mice (Swiss) feeding *ad libitum*, one week after the various treatments.

^a Every six hours for a total dose of 200 mg/kg.

^b Every eight hours for a total dose of 300 mg/kg.

DISCUSSION

The metabolism of nicotinamide has been found to have important relationships to both the antitumor activity and toxicity of a number of chemotherapeutic agents. Experiments conducted in rodent tumor systems have identified either activity-dependent depression of NAD concentration or the formation of fraudulent analogs of NAD as the principal mechanism of action. In all cases, the antitumor activity was reversed by the concomitant administration of nicotinamide with the chemotherapeutic compound (1, 4, 7-9, 22). Recent studies with Streptozotocin have shown that nicotinamide pretreatment could significantly modify this compound's toxicity, as measured by protection against diabetogenicity and acute lethality, without loss of antileukemic activity in either the L1210 or P388 systems. Streptozotocin was found to cause a rapid reduction of NAD concentrations in mouse liver and L1210 ascites cells, a phenomenon which could be correlated with toxicity but not antitumor activity. Nicotinamide pretreatment protected against the fall in coenzyme level over the full eight-hour study period (21).

The present study demonstrates that the liver concentrations of both the oxidized and reduced coenzyme were lowered for 24 hours after Streptozotocin treatment and that an additional 24 hours were required to reverse this phenomenon. A single large dose of nicotinamide protected against NAD depression for approximately 16 hours, following which time there was complete loss of any protective effect. This 16-hour period of protection corresponds to what has previously been reported as the time period in which significant rodent liver nicotinamide concentrations are recorded after a single intraperitoneal in-

jection (15, 19). Nicotinamide disappeared from the liver with a half-life of 4.4 hours, which roughly corresponded to the peak liver NAD level in one study (15). Two principal mechanisms have been proposed to explain the elevated pyridine nucleotide concentrations measured after nicotinamide administration: *de novo* synthesis, requiring deamidation to nicotinic acid, which then enters the Preiss-Handler pathway, and inhibition of NAD glycohydrolase activity (15-17, 25). Since nicotinamide did not significantly decrease the uptake of Streptozotocin by the mouse liver, this cannot be implicated as a contributing mechanism of protection. The complete loss of protection seen in the nicotinamide-Streptozotocin combination-treated group at 20 hours may represent the withdrawal of a critical nicotinamide concentration which had initially counteracted Streptozotocin interference in pyridine nucleotide biosynthesis. It will be noted that Streptozotocin could be detected in the liver at the 20-hour time period, though in lower but potentially functional concentrations.

The possibility that Streptozotocin may be inhibiting pyridine nucleotide synthesis is suggested by the rapid fall in NAD level, which is reminiscent of the studies with azaserine in mouse liver (14). This compound has been shown to produce an inhibition of NAD synthetase, the enzyme responsible for the conversion of desamido-NAD to NAD (16, 17). Further support for the hypothesis of inhibition of synthesis is added by comparison of the results of the NAD levels achieved by nicotinamide treatment, as contrasted with the combination treatment group. The former regimen produced significantly higher coenzyme concentrations over the first 24-hour period. The difference appears to have its counterpart in the level of

NAD depression produced by Streptozotocin treatment alone.

The depressant effect on liver NAD content has been shown to be directly related to the 1-methyl-1-nitrosourea group on the Streptozotocin molecule. As in the case of Streptozotocin, this phenomenon could be prevented by pretreatment with intraperitoneal nicotinamide. 1-Methyl-1-nitrosourea (NSC-23909) is nondiabetogenic but is active against the L1210 leukemia, and as such accounts for the antitumor activity of Streptozotocin (20). It is probable that the addition of glucose to the nitrosourea moiety conferred, on the resulting molecule, the ability to enter and destroy the pancreatic beta cell, with consistency previously unknown with other existing diabetogenic agents. This potential "carrier function" of glucose should receive strong consideration in the development of compounds to be specifically tested against functioning pancreatic islet cell tumors.

It is of interest that Alloxan (NSC-7169), a diabetogenic compound in animals, but inactive against the L1210 leukemia, failed to lower liver NAD levels, though nicotinamide protects against its diabetes (13). The question arises whether Streptozotocin-induced diabetes and depression of liver and L1210 NAD concentrations are related or distinct phenomenon, particularly since the diabetes is permanent, while the latter effect is reversible. This problem cannot be answered at this time, but it is possible that the lowering of beta cell NAD over a critical period of time might lead to necrosis of this metabolically active tissue. As in the case with protection of liver NAD levels, the large single dose of 100 to 400 mg/kg nicotinamide was found to give incomplete protection against diabetogenicity. A schedule of serial small nicotinamide treatments over a 24-hour period was a more efficient and effective method of administration. This would imply that protective levels of nicotinamide are being maintained at the target organ, in this case the pancreatic beta cell, over the critical period of time required to prevent destruction. These studies also demonstrated that orally administered nicotinamide can adequately protect against Streptozotocin diabetogenicity.

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