Streptozotocin Diabetes CORRELATION WITH EXTENT OF DEPRESSION OF PANCREATIC ISLET NICOTINAMIDE ADENINE DINUCLEOTIDE

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Research Article

The diabetogenic activity of streptozotocin has been correlated with a reduction in pyridine nucleotide synthesis in the mouse pancreatic islet. To determine the specificity of this reduction for diabetogenicity, a comparative study of streptozotocin, its cytotoxic moiety, 1-methyl-1-nitrosourea, and alloxan was performed. Streptozotocin administered intraperitoneally (i.p.) producd a dose-related reduction in islet NAD which was proportional to the degree of diabetogenicity. A diabetogenic dose, 200 mg/kg, attained a peak plasma *N*-nitroso intact streptozotocin concentration of 0.224 µmol/ml and reduced the mean islet NAD from a control of 0.78 to 0.15 pmol. At borderline, 150 mg/kg, and nondiabetogenic, 100 mg/kg, doses, plasma concentrations reached 0.161 and 0.136 µmol/ml, and NAD was 0.36 and 0.86 pmol/islet, respectively. 1-Methyl-1-nitrosourea, 100 mg/kg, attained a maximum *N*-nitroso intact 1-methyl-1-nitrosourea concentration of 0.162 µmol/ml and reduced the mean NAD to 0.58 pmol/islet, and was nondiabetogenic; 200 mg/kg attained a peak plasma concentration of 0.344 µmol/ml and depressed NAD to 0.38 pmol/islet, and was inconsistently diabetogenic. Islet NAD of 0.4 pmol/islet or greater is required for integrity of the beta cell. A diabetogenic dose of alloxan, 500 mg/kg, did not depress NAD, 0.85 pmol/islet, therefore confirming that its mechanism of diabetogenicity differs from that of streptozotocin.

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In vivo uptake of [methyl-^{14}C]streptozotocin by islets was 3.8 times that of [methyl-^{14}C]-1-methyl-1-nitrosourea, whereas uptake by the exocrine pancreas favored 1-methyl-1-nitrosourea over streptozotocin 2.4:1. The decreased islet uptake of 1-methyl-1-nitrosourea correlates with the 3.5 times increased molar dosage required to produce islet NAD depression comparable to that of streptozotocin, 150 mg/kg. These studies indicate that

the glucose carrier of streptozotocin facilitates uptake of its cytotoxic group, 1-methyl-1-nitrosourea, into islets.

INTRODUCTION

Streptozotocin, composed of the cytotoxic moiety, 1-methyl-1-nitrosourea (MNU)[1] attached to the carbon-2 position of glucose, is an antibiotic isolated from cultures of *Streptomyces achromogenes* (1). This compound produces diabetes in laboratory animals through the destruction of the pancreatic beta cell (2) and has demonstrable clinical antitumor activity against pancreatic islet-cell carcinoma (3). The diabetogenicity of streptozotocin has been correlated with a rapid reduction in pancreatic islet pyridine nucleotide concentration and subsequent beta cell necrosis (2, 4). Nicotinamide, but not nicotinic acid, acts as a specific inhibitor of streptozotocin-induced diabetes by preventing the reduction of NAD (2). While both streptozotocin and its cytotoxic moiety, MNU, decrease hepatic NAD concentrations, MNU was previously considered to be nondiabetogenic (5, 6). Alloxan also produces experimental diabetes through the destruction of the pancreatic beta cell (7) and has demonstrated limited clinical antitumor activity against malignant insulinoma (8).

Because the end result of alloxan and streptozotocin treatment is qualitatively similar, the present study was undertaken to determine whether the two agents had a common mechanism of diabetogenic action at the biochemical level. In this study, we compare the NAD content of individual mouse pancreatic islets after in vivo treatment with diabetogenic doses of streptozo-

[1] *Abbreviations used in this paper:* ADH, yeast alcohol dehydrogenase; HBSS, Hank's balanced salt solution; LD, lethal dose; LDH, rabbit muscle lactate dehydrogenase; MNU, 1-methyl-1-nitrosourea.

tocin or alloxan, or after a lethal dose (LD) of MNU, to determine the specificity of the reduction of NAD for the diabetogenicity of streptozotocin.

METHODS

Male Swiss mice, weighing 18–25 g, maintained on a standard laboratory diet (Ralston Purina Co., St. Louis, Mo.) and water ad libitum, were used throughout. All drugs were dissolved immediately before use: streptozotocin (glucopyranose, 2-deoxy-2-[3-methyl-3-nitrosoureido]) (lot no. 2465-EHL-86, The Upjohn Co., Kalamazoo, Mich.) was prepared in 0.005 M citrate buffer, pH 4.5; MNU (NSC 23909) and alloxan (NSC 7169) were prepared in 0.85% sodium chloride solution (Fig. 1). All drugs were administered intraperitoneally in a volume of 0.1 ml/10 g body weight. Controls received appropriate volumes of citrate buffer.

After the administration of each drug to a group of five mice feeding ad libitum, serial blood glucose determinations were performed as follows: 40 μl of tail vein blood were drawn into heparinized microhematocrit tubes (Sherwood Medical Industries, Inc., St. Louis, Mo.) and centrifuged for 3 min in an International model MB hematocrit centrifuge (Arthur H. Thomas Co., Philadelphia, Pa.). A 10-μl aliquot of the plasma supernate was then assayed for glucose by the method of Kingsley and Getchell (9).

Isolation of individual pancreatic islets was performed by a method previously described (4). In brief, 3 h after drug administration, the six animals in each group were sacrificed and the pancreatic duct and parenchyma injected with 1% collagenase (Worthington Biochemical Corp., Freehold, N. J.) in Hank's balanced salt solution (HBSS) before removal. The pancreatica of each group were combined, minced, and agitated in 1% collagenase in HBSS at 37°C for 10 min, then washed five times with HBSS at 4°C. By using a dissecting microscope, islets were serially transferred in HBSS until free of contaminating exocrine tissue. Individual islets, suspended in 5 μl of HBSS, were transferred into 1.5-ml Eppendorf microtubes (Matheson Scientific Div, Will Ross Inc., Elk Grove Village, Ill.) and ruptured with 5 μl of 0.1 N HCl followed by six cycles of freeze-thawing. 5-μl aliquots of the final HBSS rinse served as blanks for each group. All samples were frozen and lyophilized.

The NAD content of each individual islet or HBSS blank was measured by using a radiometric cycling assay as shown in Fig. 2. 25 mCi of tritiated ethanol, sp act 25 mCi/ mmol, (New England Nuclear, Boston, Mass.) were further purified by adding 1 ml of distilled water and sublimating twice. The product, containing only 0.0016% nonvolatile impurities, was then diluted to 20 ml with distilled water and frozen in 1-ml aliquots. Yeast alcohol dehydrogenase (ADH), sp act 200 U/mg, and rabbit muscle
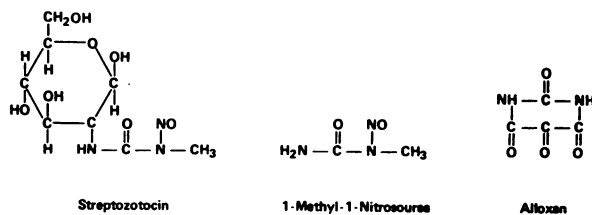

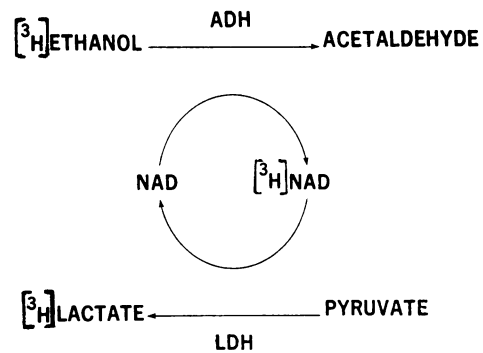FIGURE 1 Graphic formulae of drugs investigated in this study.

FIGURE 2 Schematic representation of radiometric cycling assay for the measurement of NAD. The labeled hydrogen from [³H]ethanol is transferred to pyruvate by the pyridine nucleotide NAD to form [³H]lactate. The ethanol substrate is evaporated and leaves a residue of [³H]lactate proportional to the NAD content of the sample.

lactate dehydrogenase (LDH), sp act 360 U/mg, were used throughout (Boehringer Mannheim Corp., New York). These enzymes were freed of contaminating pyridine nucleotides by placing each enzyme into a 2% activated charcoal suspension (Sigma Chemical Co., St. Louis, Mo.), agitating for 30 min, and then centrifuging at 15,000 g for 15 min. The enzymes were subsequently recrystallized with ammonium sulfate at 4°C. This purification procedure was repeated and the final enzyme preparations stored at 4°C in a volume of final crystallization liquid equal to the original volume of each enzyme. Aliquots of these enzyme preparations were dissolved in a 10-fold volume of 0.1 M pyrophosphate buffer, pH 9.5 (Sigma Chemical Co.) immediately before addition to the cycling assay cocktail. Absolute ethanol, USP reagent grade, was diluted to a concentration of 0.001 M with distilled water; pyruvate (Sigma Chemical Co.) was dissolved before use in 0.1 M pyrophosphate buffer, pH 9.5, to a final concentration of 0.018 M. NAD standards (Boehringer-Mannheim Corp.), ranging in concentration from 0.19 to 1.5 pmol/5 μl were prepared in advance in distilled water and frozen in aliquots to be thawed just before use.

The cycling assay for NAD was performed as follows. After lyophilization, samples or HBSS blanks were dissolved in 5 μl of distilled water. To each microtube was added 10 μl of cocktail containing LDH, ADH, 0.1 M pyrophosphate buffer, 0.001 M ethanol, tritiated ethanol, and 0.018 M pyruvate (5:5:5:1:1:1 by volume). In this system, the rate-limiting factor is the NAD content of the sample (10). The reaction is allowed to cycle for 15 h at 20°C; the reaction is terminated and the tritiated ethanol substrate removed by heating at 95°C for 25 min in an Eppendorf Thermostat no. 3401 micro-oven (Brinkmann Instruments, Inc., Westbury, N. Y.), which leaves a residue of tritiated lactate proportional to the NAD content of the sample, standard, or blank. 50 μl of water were added to each microtube to dissolve the tritium-labeled lactate residue. Each microtube was then transferred into a scintillation vial containing 15 ml of Aquasol (New England Nuclear) and counted by using a Packard Tri-Carb liquid scintillation spectrometer, model no. 4322 (Packard Instrument Co., Inc., Downers Grove, Ill.). The NAD content of each sample was calculated from a standard curve performed with each assay.
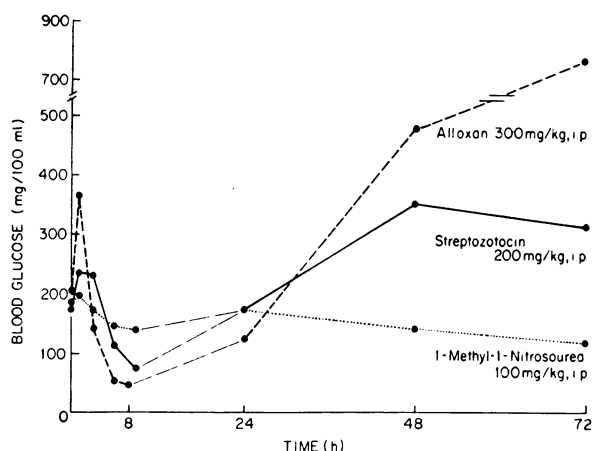
FIGURE 3 Temporal development of drug-induced diabetes. MNU, 100 mg/kg, streptozotocin, 200 mg/kg, or alloxan, 300 mg/kg, were administered intraperitoneally (i.p.) to groups of five mice. Each point represents the mean value for each group. When compared with pretreatment mean blood glucoses, alloxan and streptozotocin produced significant hypoglycemia at 6 and 9 h, respectively ($P < 0.01$), and significant hyperglycemia by 48 h ($P < 0.01$). At 72 h post-treatment, the degree of hyperglycemia produced by alloxan was significantly greater than that produced by streptozotocin ($P < 0.01$). MNU had no significant effect upon blood glucose.

To determine the comparability and time points of peak streptozotocin and MNU concentrations in plasma after intraperitoneal administration, 8 μCi of [methyl-^{14}C]streptozotocin, sp act 1 μCi/μmol (Monsanto Research Corp., Dayton, Ohio), and [methyl-^{14}C]MNU, sp act 1 μCi/μmol (New England Nuclear), were administered to groups of five mice. 10 μl aliquots of tail vein blood were serially collected at 2, 5, 10, 15, 30, and 60 min after injection and added to 90 μl of 5% perchloric acid. The precipitates were centrifuged down at 18,000 g for 2 min, and a 10-μl aliquot of the resulting supernate was placed in a counting vial containing 15 ml of Aquasol and counted in a Packard Tri-Carb liquid scintillation spectrometer (Packard Instrument Co.).

Plasma streptozotocin and MNU concentrations were determined by measurement of the intact N-nitroso group of the respective molecules by using the spectrophotometric method of Forist (6, 11).

The specificity of in vivo drug uptake into the islets of Langerhans, exocrine pancreas, liver, kidney, brain, muscle, adipose tissue, and bone marrow was determined by the following procedures: 15 min after intravenous administration of 16 μCi of [methyl-^{14}C]streptozotocin, sp act 1 μCi/μmol, or 16 μCi of [methyl-^{14}C]MNU, sp act 1 μCi/μmol, to groups of five mice, the animals were sacrificed. The islets of Langerhans were isolated as described above. The islets from each treatment group were pooled, solubilized in 5 μl of 10 N NaOH, and counted in 15 ml of Aquasol in the Packard Tri-Carb liquid scintillation spectrometer. The bone marrow was aspirated from the tibias by using a 25-gauge needle and a syringe of 0.0067 M phosphate-buffered 0.85% NaCl solution. All tissues were sonified in a known volume of this phosphate-buffered saline solution, and a 200-μl aliquot was counted in 15 ml of

Aquasol in the previously mentioned Packard spectrometer. DNA content of all tissues, except adipose tissue, was extracted by the method of Schneider (12) and was determined by the method of Burton (13). For the adipose tissue, triglycerides were measured by the method of Kessler and Lederer (14). The 15-min time period was selected because of the previously demonstrated plasma half-life of 5 min for biologically active streptozotocin and MNU (5, 6, 15). Internal quench techniques were utilized when calculating drug uptake.

Pancreatica were removed on day 2–3 after drug administration and immediately placed in 10% formalin. The tissues were embedded in paraffin, and sections 4-μm thick were stained for beta cell granules by using an aldehyde fuchsin stain (16).

RESULTS

Serial blood glucose determinations were performed to evaluate the temporal development of diabetes (Fig. 3). Streptozotocin, 200 mg/kg, produced a transient hyperglycemic response at 1 and 3 h after intraperitoneal administration. At 9 h post-treatment, the mean blood glucose, 76 mg/100 ml, was significantly reduced from the pretreatment level ($P < 0.01$) (17). All streptozotocin-treated mice were demonstrated to be hyperglycemic at 48 and 72 h, with mean blood glucose concentrations of 353 mg/100 ml and 317 mg/100 ml, respectively ($P < 0.01$). Alloxan, 300 mg/kg, also produced a hyperglycemic phase at 1 h post-treatment, with mean blood glucose of 364 mg/100 ml, but unlike streptozotocin, the mean blood glucose had decreased to below control levels by 3 and was significantly depressed to 53 mg/100 ml by 6 h ($P < 0.01$). At 8 h post-treat-

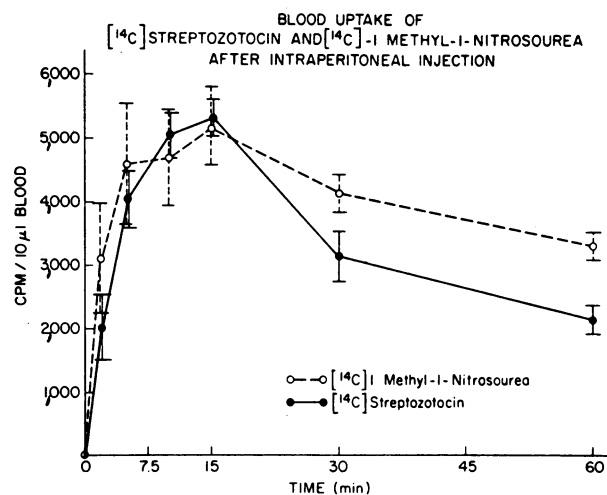

FIGURE 4 Uptake of [methyl-^{14}C]streptozotocin and [methyl-^{14}C]MNU into blood after intraperitoneal injection. 8 μCi, sp act 1 μCi/μmol, of each drug was administered in 0.1 ml of 0.005 M citrate buffer, pH 4.5. 10 μl aliquots of tail vein blood were taken from each animal serially at the designated time points. Values are expressed as mean counts per minute $\pm$SEM.

ment, these mice developed generalized seizures at a time when their mean blood glucose had decreased to 48 mg/100 ml. All surviving mice which had received alloxan were diabetic 48 h after drug administration, with mean blood glucose of 480 mg/100 ml; at 72 h, these animals had a mean blood glucose of 833 mg/100 ml, a degree of hyperglycemia significantly greater than that produced by streptozotocin, 200 mg/kg ($P < 0.01$). MNU, 100 mg/kg, did not produce any significant changes in blood glucose over the 72-h period studied.

To determine the relative uptake and half-life of streptozotocin and MNU in plasma after intraperitoneal injection, 8 μCi of each drug was administered, and the plasma was serially assayed for activity. The results were reproducible with this route of administration for both drugs, and there was no significant difference in uptake from the peritoneal cavity (Fig. 4). With this result, studies dealing with effects of the two drugs on NAD concentrations in islets and this relationship to subsequent diabetogenic activity were carried out by using the intraperitoneal route of administration.

Streptozotocin was administered intraperitoneally at doses of 100, 150, and 200 mg/kg, which produced maximum plasma drug concentrations of 0.136±0.0043, 0.161±0.0036, and 0.224±0.034 μmol/ml, respectively, based on the measurement of the intact N-nitroso group 15 min post-injection (Table I). This was associated with a dose-related depression of mean islet NAD content which could be correlated with the degree of diabetogenicity. A dose of 150 mg/kg produced a borderline diabetic state with a mean islet NAD content of 0.364±0.056 pmol. A dose of 200 mg/kg produced overt diabetes with a mean islet NAD content of 0.149± 0.023 pmol.

MNU was administered intraperitoneally at doses of 100 and 200 mg/kg, which produced maximum plasma drug concentrations of 0.162±0.014 and 0.344±0.040 μmol/ml and mean islet NAD contents of 0.573±0.042 and 0.380±0.080 pmol. No diabetogenic activity was

TABLE I
Relationship of Plasma Drug Concentration and Islet NAD Content for Diabetogenic Activity

Drug	Dose*	Mean peak plasma concn	Mean islet NAD content	Mean plasma glucose
	mg/kg	μmol/ml	pmol/islet	mg/100 ml
Control	—	—	0.777 ±0.085	128 ±7.5
Streptozotocin	100	0.136 ±0.0043	0.850 ±0.150	169 ±10.5
	150	0.161 ±0.0036	0.364 ±0.056	242 ±39.9
	200	0.224 ±0.034	0.149 ±0.023	317 ±58.4
MNU	100	0.162 ±0.014	0.537 ±0.042	121 ±5.6
	200	·.344 ±0.040	0.380 ±0.080	256 ±76.7
Alloxan	500	—	0.853 ±0.181	833 ±7.5

Peak plasma N-nitroso intact streptozotocin and MNU concentrations after intraperitoneal administration at graded doses are correlated with islet NAD content at 3 h post-injection and with plasma glucose concentrations at 2-3 days post-injection. The islet NAD content and plasma glucose concentration after intraperitoneal injection of alloxan, 500 mg/kg, are also compared. The plasma glucose and blood drug concentrations are reported as the means of five animals±SEM. NAD contents of isolated islets of Langerhans are reported as mean pmol±SEM.
* Given intraperitoneally.

found at the 100 mg/kg dose level; however, borderline diabetogenic activity was documented at the 200-mg/kg dose by plasma glucose determination and light microscopic examination of pancreatic islets which demonstrated degeneration and destruction of the beta cells. This degree of NAD depression in islets and the elevation of the plasma glucose produced by MNU at 200 mg/kg was comparable to that produced by streptozotocin at 150 mg/kg (a 3.5:1 M dose ratio). A 2.1-fold increase in plasma drug concentration of MNU was required to produce this effect. Alloxan at an LD of 500 mg/kg did not decrease mean islet NAD content, which was 0.850±0.181 pmol.

To explain the relative ineffectiveness of MNU as a diabetogenic agent, a comparative study of drug distribution of streptozotocin and MNU was performed. To avoid possible contamination of the pancreas and other

TABLE II
Mean Mouse Tissue Uptake of [¹⁴C]Streptozotocin and [¹⁴C]MNU 15 Min After Intravenous Administration

	Pancreatic islets	Exocrine pancreas	Liver	Kidney	Brain	Bone marrow	Muscle	Adipose tissue
	dpm/50 islets	dpm/μg DNA	dpm/μg DNA	dpm/μg DNA	dpm/μg DNA	dpm/μg DNA	dpm/μg DNA	dpm/mg triglycerides
Streptozotocin								
(16 μCi; sp act 1 μCi/μmol)	1,114	284	3,911	1,712	325	20	574	400
MNU								
(16 μCi; sp act 1 μCi/μmol)	290	670	1,598	468	1,394	23	1,160	428
P		<0.01	<0.01	<0.01	<0.01	>0.1	<0.01	>0.1

Groups of five mice were injected i.v. with 16 μCi of [methyl-¹⁴C]streptozotocin or [methyl-¹⁴C]MNU, sp act 1 μCi/μmole. 15 min post-injection the animals were sacrificed. The pancreatic islets, and the respective organs were assayed for uptake of radioactivity.

visceral organs by intraperitoneal isotope administration, these studies were carried out after intravenous administration.

To determine the relative tissue distribution of [methyl-^{14}C]streptozotocin and [methyl-^{14}C]MNU, equal molar doses were administered intravenously and uptake determined in respective organs at the time of maximum plasma concentration (Table II). Uptake of streptozotocin into islets was 3.8 times that of MNU, while uptake into whole pancreas favored MNU over streptozotocin 2.4:1. There was greater uptake of streptozotocin into liver and kidney compared to MNU, while MNU demonstrated greater uptake into brain and muscle. Uptake of the two compounds into bone marrow and adipose tissue was comparable.

DISCUSSION

The diabetogenic activity of streptozotocin has been closely correlated with a reduction in the concentration of pancreatic islet pyridine nucleotides (4). Streptozotocin has been shown to inhibit the incorporation of [^{14}C]nicotinamide into NAD by the mouse pancreatic islet (4). Nicotinamide administered in pharmacologic doses has been shown to overcome this partial block in NAD synthesis (2, 4) and is specific in its protective action against streptozotocin-induced diabetes (2, 18). It is proposed that this is accomplished by maintaining pyridine nucleotide concentrations at normal or greater than normal values (4, 5). Recently Lazarus and Shapiro have challenged this concept for the protective effect of nicotinamide (19). They base their conclusion on the finding that intraperitoneally administered NAD only partially prevented streptozotocin-induced diabetes, as determined by histologic examination. However, there has been no demonstration that intact NAD can be taken up into islets. In addition, the work of Kaplan, Goldin, Humphreys, Ciotti, and Stolzenbach has demonstrated that intraperitoneally injected NAD is a grossly ineffective means of increasing intracellular NAD when compared with intraperitoneally injected nicotinamide (20).

The diabetogenicity of alloxan is also mediated via pancreatic islet beta cell necrosis, although the specific mechanism of action remains controversial (7, 21, 22). Unlike streptozotocin, a wide range of chemically diverse agents have been shown to protect against alloxan-induced diabetes. Hypotheses regarding their mechanism(s) of protection include attachment to and subsequent inactivation of alloxan (the sulfhydryl-containing compounds glutathione and cysteine) (23), oxidation or reduction of alloxan to an inactive form (sodium bisulfite, thiouracil) (24, 25), and alteration of splanchnic bed perfusion with reduction in the delivery of drug to the beta cell (nicotinic acid) (26). Nicotinamide had

previously been reported to protect against alloxan-induced diabetes (26). However, recent work indicates that only when nicotinamide is administered before alloxan is the pancreatic islet protected, whereas nicotinamide administered up to 60 min after streptozotocin still has an antagonistic effect (27). In addition, the study of Lazarus and Shapiro has shown a failure of nicotinamide to prevent beta cell destruction by alloxan in the mouse (19).

The temporal development of diabetes is similar with both alloxan and streptozotocin. The hypoglycemic phases at 3–8 and 6–9 h, respectively for the two agents, have been shown to result from a release of stored insulin, temporally related to beta cell necrosis (7, 22, 28). For the comparative study of NAD content, the time period chosen for study, 3 h post-treatment, just precedes actual beta cell destruction. Based upon the time of onset of hypoglycemia, alloxan, at the doses employed in this study, acts at least as rapidly as streptozotocin in destroying the beta cell.

Alloxan, 500 mg/kg, did not decrease pancreatic islet NAD within 3 h after injection. This is consistent with previous work that has shown that, unlike streptozotocin, diabetogenic doses of alloxan do not depress hepatic NAD concentrations (5). The demonstration that the diabetogenicity of streptozotocin, but not alloxan, correlates with early interference of pancreatic islet pyridine nucleotide metabolism, and the failure of nicotinamide to prevent alloxan- but not streptozotocin-induced diabetes when given after the diabetogenic agents (19, 27). strongly suggests that the mechanism of diabetogenicity is different for the two drugs.

The addition of the glucose carrier to the MNU cytotoxic moiety results in important alterations in drug distribution for specific organs. Significant differences in the uptake of MNU and streptozotocin were observed for liver, kidney, brain, and most interestingly for the purpose of these studies, the pancreas. MNU does not produce diabetes when administered at an LD of 100 mg/kg, but will depress hepatic NAD concentrations to the same degree as streptozotocin, 200 mg/kg (5, 6). In contrast to streptozotocin, MNU is nonpolar and lipid soluble, properties which allow passive diffusion across cell membranes (29). Thus, measurable MNU uptake into islets, accompanied by a minor depression of pancreatic islet NAD concentration, was to be anticipated in the present study. At equimolar doses of MNU and streptozotocin, there was a 3.8 times increased uptake of [methyl-^{14}C]streptozotocin into pancreatic islets. When MNU was administered at a supralethal dose of 200 mg/kg, islet NAD was reduced to 0.380 pmol, a level equivalent to that produced by streptozotocin, 150 mg/kg. This represents a 3.5:1 increase in molar dosage of MNU over streptozotocin to produce a comparable dia-

betogenic activity as determined by plasma glucose concentrations and beta cell histology. It is of interest that the plasma N-nitroso intact streptozotocin concentration required for diabetogenic activity in mice, 0.161 μmol/ml, is equivalent to that observed with slow intravenous infusion of streptozotocin in man with therapeutic doses of 1.0–1.5 g/m² (30).

Although we have interpreted these biochemical-pharmacologic changes in specific reference to the beta cell, we recognize that the actual measurements were performed in the whole islet of Langerhans, which includes both alpha and delta cells as well. This can be justified in part by the recognition that the destructive changes within the islet associated with streptozotocin are confined to the beta cell (19). The findings of the present studies support the concept that the glucose portion of the streptozotocin molecule facilitates the active transport of the MNU cytotoxic moiety into the pancreatic beta cell; the end result is a decrease in beta cell NAD levels and subsequent necrosis.

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