Streptozotocin Diabetes: Time Course of Irreversible B-Cell Damage; Further Observations on Prevention by Nicotinamide[1] (34439)

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In susceptible species, the injection of diabetogenic doses of streptozotocin induces an initial hyperglycemic peak with no apparent change in serum insulin, followed by profound hypoglycemia caused by the liberation of large amounts of insulin from the pancreas. Twenty-four hours after the injection, the animals are permanently diabetic (1–4). Pancreatic insulin content remains within normal limits up to 6 hr after the injection but falls to about 1% of control values within 24 to 48 hr (2, 4).

As with alloxan (6), the effects of streptozotocin can be partially or completely prevented by the previous or simultaneous injection of nicotinamide. In contrast to alloxan, however, the injection of nicotinic acid affords no such protection (3, 4, 7, 8).

The studies reported here were prompted by the recent observation that, in contrast to earlier findings with alloxan (6), streptozotocin-treated rats, injected with nicotinamide up to 2 hr after streptozotocin, did not develop diabetes (8, 9). Having previously established that pancreatic content of immunoreactive insulin (IRI) was the most reliable expression of the diabetogenic effectiveness of the drug (3), we undertook to define the time course of onset of irreversible B-cell damage by observing the effects of nicotinamide injected at different time intervals after streptozotocin upon pancreatic IRI content.

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In addition, experiments were designed to test the hypothesis that the differential effectiveness of nicotinamide and nicotinic acid in affording protection against streptozotocin-induced diabetes might be the consequence of differences in the mechanisms governing their excretion after intraperifoneal injection (10).

While the exact mechanisms underlying the sequence of events induced by streptozotocin are not yet fully understood, the first hyperglycemic peak may be the consequence of adrenalin-independent hepatic glycogenolysis (4) and/or of the failure of pancreatic B-cells to respond to stimulation by glucose (5). The permanent diabetic state ensuing after the initial phases is due to selective, dose-dependent destruction of pancreatic B-cells (2, 3). On the basis of ultrastructural evidence, the hypoglycemic phase has been attributed to uncontrolled liberation of insulin from damaged B-cells (2). However, other mechanisms, such as streptozotocin-induced hyper-responsiveness of insulin secretory mechanisms to normal stimuli could also produce the massive release of insulin seen at that time. An attempt was made to suppress this insulin release with mannoheptulose which abolishes insulin secretion in response to physiological stimuli (11).

Materials and Methods. Animals. Male Wistar rats bred in the laboratory, weighing 180–200 g and fed rat chow *ad libitum* (Altromin-R, Kunath, S. A., Aarau, Switzerland) were used throughout. They were fasted for 16 hr prior to the injection of streptozotocin, for 24 hr prior to the injection of alloxan, and in all experiments for 16 hr before sacrifice.

Experimental samples and assay procedures: Blood for the measurement of

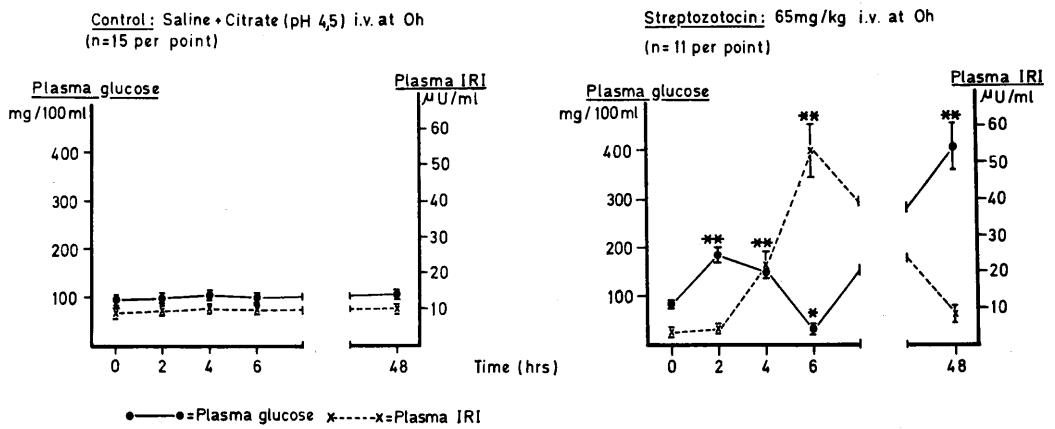

FIG. 1. Effect of streptozotocin (65 mg/kg, iv) on plasma glucose and IRI concentrations of fasted (16 hr) rats. Control animals left side panel, streptozotin-treated animals right side panel. (Mean values ± SEM), double asterisks indicate significance of difference vs controls ($p<.01$).

plasma glucose and plasma IRI was obtained from the tip of the tail at the time indicated and from the neck of the animals at decapitation. It was collected in heparinized microfuge tubes and centrifugated, plasma being kept frozen at —20° until the time of assay. At death pancreases were rapidly removed, dissected free of adipose tissue, and kept frozen at —20° until the time of extraction, which was performed according to Scott and Fisher (12). Plasma glucose was measured by an O-toluidine method (13) and IRI in plasma and pancreatic extracts according to Hales and Randle (14), free and antibody-bound insulin being separated by centrifugation at 4° (15). Rat insulin was used as a standard.

Reagents and solutions for injections. Streptozotocin (Upjohn, Kalamazoo, Michigan) was prepared fresh for each experiment. It was dissolved in 0.9% saline and the pH was immediately adjusted to 4.5 with 0.05 *M* citric acid. Final concentration was 26 mg/ml. Alloxan (Merck, Darmstadt, Germany) was dissolved in citric acid phosphate buffer, pH 4 (16), immediately before injection. Final concentration was 24 mg/ml. Nicotinamide (Hoffmann-La Roche, Basel, Switzerland) was dissolved in distilled water, final concentration being 50 mg/ml. In preliminary experiments it was established that nicotinamide did not affect pancreatic IRI content (data not shown). Nicotinic acid (Hoffmann-La Roche, Basel, Switzerland) was dissolved in 2 *N* NaOH, the concentration being brought to 50 mg/ml with distilled water (final pH 8–9). Mannoheptulose (Schering, Berlin, Germany) was freshly dissolved in distilled water before each experiment, final concentration being 200 mg/ml.

Experimental procedure. Streptozotocin (65 mg/kg) and alloxan (60 mg/kg) were injected into a tail vein under light ether anesthesia. Control animals received either the acidified saline or citric acid phosphate buffer. Nicotinamide (500 mg/kg) and mannoheptulose (400 mg/animal) were injected intraperitoneally, nicotinamide at the time intervals indicated (Fig. 2 and Table I) and mannoheptulose 30 min before and 90 min after the injection of streptozotocin. Nicotinic acid (1 g/kg) was given by gastric tube 4 and 2 hr before the injection of streptozotocin. When indicated, blood samples were obtained without anesthesia from the tip of the tail immediately before as well as 2, 4, and 6 hr after the injection of streptozotocin. Feeding was resumed after termination of blood sampling or immediately after injections when blood was taken only upon sacrifice. The animals were killed by decapitation under Nembutal anesthesia 48 hr after the injection of streptozotocin or alloxan.

Results. As indicated by Fig. 1, the injection of streptozotocin alone resulted in the

TABLE I. Plasma Glucose and Plasma IRI of Rats 48 hr after the Injection of Streptozotocin (65 mg/kg iv) Followed by That of Nicotinamide (500 mg/kg ip) at Varying Time Intervals.

Treatment	Interval between injection of streptozotocin and nicotinamide (min)	Plasma glucose[a] (mg/100 ml)	Plasma IRI[a] (μU/ml)
Controls		105 ± 2	10.4 ± 1.7
Streptozotocin		408 ± 47*	7.5 ± 1.9
Streptozotocin + nicotinamide	0	136 ± 9**	22.0 ± 3.4
Streptozotocin + nicotinamide	10	134 ± 6**	22.0 ± 3.7
Streptozotocin + nicotinamide	30	148 ± 5***	22.3 ± 3.5
Streptozotocin + nicotinamide	60	207 ± 10***	20.4 ± 3.5
Streptozotocin + nicotinamide	120	338 ± 17*	20.0 ± 4.6

[a] Means ± SEM; $n = 15$ in control animals, 11 in animals treated with streptozotocin and 6 in all other cases. Significance of differences: * $p < .01$ vs controls, ** $p < .01$ vs streptozotocin alone, *** $p < .01$ vs controls and streptozotocin alone.

expected biphasic response of plasma glucose and IRI concentrations. Forty-eight hours after the injection, all animals were severely diabetic. Animals injected with buffer alone showed no significant variations of plasma glucose or IRI concentrations. When nicotinamide (ip) was injected simultaneously with streptozotocin (iv) (Fig. 2, left side-panel), plasma glucose concentrations were generally higher than in control animals. However, neither the initial hyperglycemia nor the subsequent hypoglycemia characteristic of the injection of streptozotocin alone, occurred. At 48 hr, the animals were slightly hyperglycemic but free of polyuria, glycosuria, or ketonuria. Plasma IRI showed no significant changes apart from a small rise at 48 hr. When the interval between the injections was 10 min, the ensuing patterns of both plasma glucose and plasma IRI concentrations were identical with those obtained after the simultaneous injections. When nicotinamide was injected 2 hr after streptozotocin (Fig. 2, right side panel), the results were indistinguishable from those observed after the injection of streptozotocin alone. Intermediate patterns were obtained when nicotinamide was injected 30 min and 1 hr after streptozotocin as indicated by the results at 48 hr (Table I).

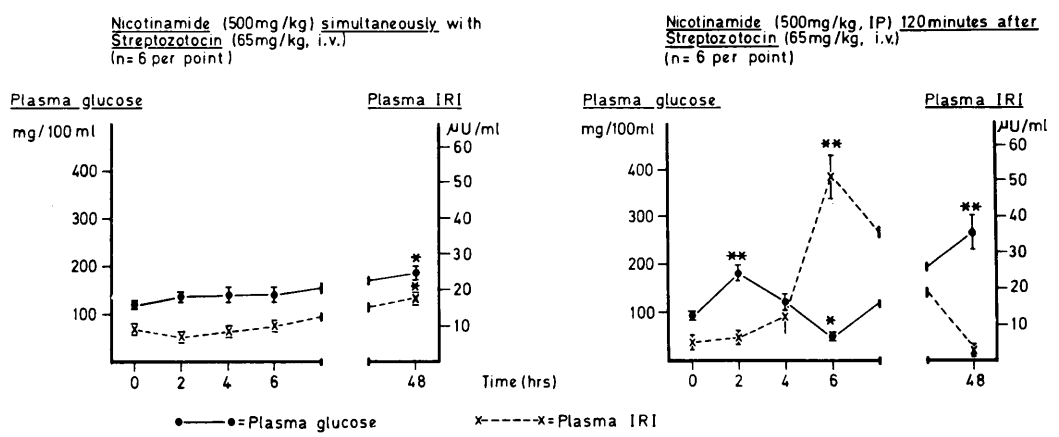

FIG. 2. Plasma glucose and IRI concentrations of rats injected with streptozotocin (65 mg/kg, iv) and nicotinamide (500 mg/kg, ip). Nicotinamide was injected simultaneously with (left side panel) or 2 hr after (right side panel) streptozotocin. (Mean values ± SEM). Significance of differences vs. zero time: *$p < .05$, **$p < .01$.

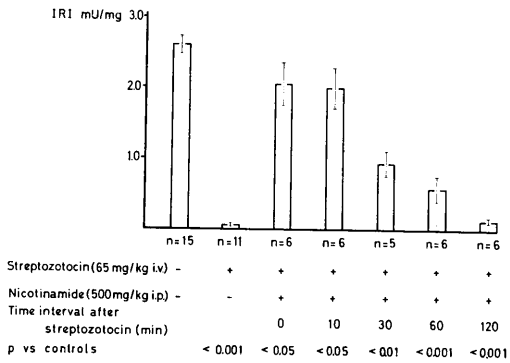

FIG. 3. Pancreatic IRI content of rats 48 hr after the injection of streptozotocin (65 mg/kg, iv) and nicotinamide (500 mg/kg, ip) at various time intervals. (Mean values ± SEM). Difference between streptozotocin alone and streptozotocin and nicotinamide at 120 min, $p<.05$.

As shown in Fig. 3, a small but significant fall in pancreatic IRI was observed 48 hr after the simultaneous injection of nicotinamide (ip) and streptozotocin (iv). No further decrease occurred when the injection of nicotinamide was delayed for up to 10 min but a progressive reduction in pancreatic IRI was observed with increasing intervals between the two injections from 10 to 120 min. The injection of nicotinamide 2 hr after streptozotocin still had a measurable protective effect on pancreatic IRI ($p<.05$). As shown in Table II, the intragastric administration of nicotinic acid did not modify the effectiveness of streptozotocin as measured by the reduction of pancreatic IRI content 48 hr after the injection.

Figure 4 summarizes an experiment in which mannoheptulose was injected prior to the administration of streptozotocin. While the hyperglycemia induced by this sugar prevented the severe hypoglycemia usually seen 6 hr after the injection of streptozotocin, a significant fall in plasma glucose occurred nonetheless and mannoheptulose evidently was without effect on the massive liberation of insulin from the pancreas occurring at that time. As suggested by a previous report (8), mannoheptulose did not modify the effect of streptozotocin on pancreatic IRI content (data not shown).

In contrast to the findings with streptozotocin, the diabetogenic effect of alloxan was not modified by the subsequent administration of nicotinamide (Table II).

Discussion. The intraperitoneal injection of nicotinamide together with, or 10 min after the intravenous injection of streptozotocin almost completely protected the pancreas of experimental animals from the destructive effects of streptozotocin, and plasma glucose concentrations of the these animals remained well within normal limits. The rats became diabetic only when the interval between the injection of streptozotocin and that of nicotinamide exceeded 30 min. Under these conditions pancreatic insulin content was grossly reduced. However, even then, nicotinamide

TABLE II. Plasma Glucose, Plasma IRI, and Pancreatic IRI Content of Rats 48 hr after the Injection of Streptozotocin Preceded by the Intragastric Administration of Nicotinic Acid and 48 hr after the Injection of Alloxan Followed Within 10 min by That of Nicotinamide.

Treatment	Plasma glucose[a] (mg/100 ml)	Plasma IRI[a] (μU/ml)	Pancreas IRI[a] (mU/mg)
Controls	88 ± 4	10.3 ± 3.0	2.8 ± 0.1
Streptozotocin (65 mg/kg)	416 ± 9	1.6 ± 0.3	0.028 ± 0.01
Streptozotocin (65 mg/kg) + nicotinic acid (2 × 1 g/kg)	386 ± 13**	0.8 ± 0.1**	0.023 ± 0.01**
Alloxan (60 mg/kg)	393 ± 35	5.8 ± 1.7	0.14 ± 0.06
Alloxan (60 mg/kg) + nicotinamide (500 mg/kg)	400 ± 21**	7.0 ± 1.8**	0.16 ± 0.05**

[a] Means ± SEM; $n = 15$ in control animals, 11 in animals treated with streptozotocin, and 6 in all other cases.

** Not statistically different from values obtained with streptozotocin or alloxan alone ($p > .1$).

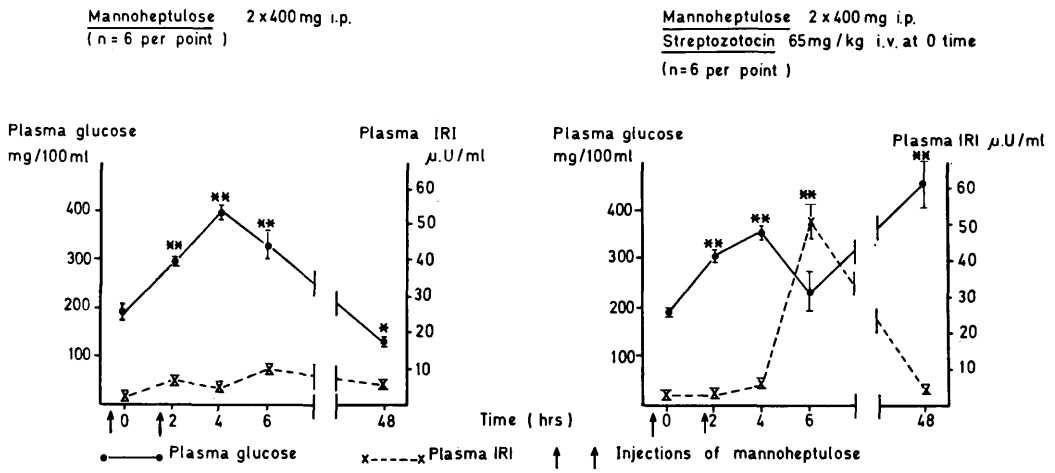

FIG. 4. Effect of mannoheptulose on plasma glucose and IRI concentrations of streptozotocin-treated rats. Mannoheptulose (400 mg/animal, ip) was injected 30 min before and 90 min after buffer alone (left side panel) and streptozotocin (65 mg/kg, iv, right side panel). (Mean values ± SEM). Significance of differences vs zero time: $*p < .05$, $**p < .01$.

exerted a significant protective effect which was progressively reduced as the time interval between the two injections increased. Nicotinamide reduced the initial hyperglycemic peak and the subsequent hypoglycemia and hyperinsulinemia in proportion to its protective effect on pancreatic IRI content. This parallelism between degree of pancreatic protection and modification of the initial blood changes, supports the view that the intermediate hypoglycemia and hyperinsulinemia induced by streptozotocin are the direct consequence of its cytotoxic effect on B-cells. The morphological evidence in favor of this interpretation (2) is supported by the observation that mannoheptulose did not suppress this insulin release, suggesting that the release was independent of glucose metabolism.

Available data do not rule out the possibility that the early hyperglycemic peak might be caused by nicotinamide-sensitive but extrapancreatic effects of streptozotocin (4). However, the observation that, 1 hr after the injection of streptozotocin, islets of Langerhans fail to secrete insulin in response to stimulation by glucose *in vitro* (5), supports the hypothesis that the primary lesion does occur at the level of the B-cell.

The protective effect of nicotinamide injection as long as 2 hr after streptozotocin suggests either that the irreversible β-cytotoxic effect of streptozotocin does not occur immediately after the injection or that it remains partly reversible for prolonged periods of time. In conjunction with these results, the report that pyrazinamide and 2-deoxyglucose prevent the diabetogenic effect of streptozotocin only when injected prior to or simultaneously with it (8), may suggest that nicotinamide exerts its effect at a relatively late step in the chain of events induced by streptozotocin. Alternatively, different B-cell populations might respond to streptozotocin at different times and/or with varying intensity. In the latter event, nicotinamide, when injected after the longer time intervals could prevent the β-cytotoxic effects on late responding cells only. While no proof in favor of this hypothesis can be advanced at the present time, it may be recalled that the degree of granulation of the B-cells of a given pancreas is not uniform and that on the basis of ultrastructural evidence obtained in diabetic spiny mice, it has been suggested that degranulated B-cells may be less sensitive to streptozotocin than well-granulated ones (17).

Dulin *et al.* (8, 9) have reported that animals which had received nicotinamide as late as 2 hr after streptozotocin were normoglycemic 1 week after injection. This finding contrasts with the hyperglycemia and the re-

duction of pancreatic IRI content to less than 5% of control values observed in the present study 48 hr after the injection of streptozotocin and nicotinamide with the same time interval. Since these authors did not report on early changes in blood glucose concentrations or on pancreatic IRI content, the two sets of experiments are difficult to compare.

The mechanism by which nicotinamide prevents the effect of streptozotocin is unknown. Streptozotocin reduced the nicotine-adenine-dinucleotide (NAD) content of liver and of certain tumors and nicotinamide abolished this effect (7). Accordingly, it has been suggested that the diabetogenic effect of streptozotocin might be due to a reduction in synthesis, an increase in destruction, or an increase of metabolic requirements for NAD and that the antagonistic effect of nicotinamide could reside in its role as a precursor to NAD-synthesis (4, 7–9). As previously reported (3, 6, 7) and confirmed by the present study, nicotinic acid, generally considered to be metabolically interchangeable with nicotinamide, has no protective effect against the diabetogenic activity of streptozotocin. In order to test the possibility that the ineffectiveness of nicotinic acid in preventing streptozotocin-induced diabetes might be due to its rapid renal excretion after parenteral administration (10), large amounts of nicotinic acid were administered by gastric tube before the injection of streptozotocine. As shown in Table II, nicotinic acid was ineffective in these experiments.

Early changes in plasma glucose and insulin concentrations after the inejction of alloxan are almost identical to those observed after the administration of streptozotocin (2, 4, 18). This, and the fact that nicotinamide abolished the diabetogenic activity of both when injected before or together with the diabetogenic agent, has been interpreted as evidence that they exert their β-cytotoxic effects by the same or similar mechanisms. However, in contrast to streptozotocin, the diabetogenic effect of alloxan is abolished by the previous or simultaneous administration of nicotinic acid (6, 19, 20), but not by the subsequent administration of nicotinamide (Table II). These observations suggest that the mechanisms of action of these two substances are not necessarily identical (4, 8, 9).

Summary. Further studies on the mode of action of streptozotocin and the prevention of its effects by nicotinamide have yielded the following results: 1. The injection of nicotinamide at varying time intervals after streptozotocin afforded partial protection against its β-cytotoxic effects, decreasing with the length of the intervals but still significant when the interval was 2 hr. 2. Mannoheptulose failed to suppress the insulin release occurring between 6 and 10 hr after the injection of streptozotocin. 3. Nicotinic acid administered by gastric tube failed to protect against the diabetogenic effect of streptozotocin. 4. Nicotinamide injected 10 min after alloxan afforded no protection against the diabetogenic effect of the latter. It is concluded (1) that the β-cytotoxic effect of streptozotocin is not immediately irreversible or that it does not affect all B-cells simultaneously; (2) that the insulin release observed between 6 and 10 hr after the injection of streptozotocin occurs by way of leakage from damaged B-cells; (3) that the ineffectiveness of nicotinic acid in preventing the β-cytotoxic effect of streptozotocin is probably not the consequence of its insufficient conversion to nicotinamide after parenteral administration; and (4) that streptozotocin and alloxan differ in their mechanism of action on the pancreatic B-cell.

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