# Modification of Chemically Induced Diabetes in Rats by Vitamin E

## SUPPLEMENTATION MINIMIZES AND DEPLETION ENHANCES DEVELOPMENT OF DIABETES

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ABSTRACT Administration of the antioxidant vitamin E to rats, prior to administration of either streptozotocin or alloxan, provided protection against the diabetogenic effect of both these agents. This was demonstrated by their response to a glucose load, their pancreatic insulin content and light microscopy findings. In addition, rats whose antioxidant state was depleted, by being maintained on a vitamin E and selenium-deficient diet, demonstrated increased diabetogenic susceptibility to normally nondiabetogenic doses of streptozotocin. These findings provide indirect support for the suggestion that the chemical agents streptozotocin and alloxan may exert their diabetogenic effect by acting as oxidants or free radical producers.

## INTRODUCTION

The mechanism by which the chemical agents streptozotocin and alloxan cause diabetes has stimulated considerable investigation and controversy, and has yet to be completely resolved. Many similarities have been noted between the mode of action of these two drugs, yet a number of distinct differences have been documented (1). Both agents cause selective destruction of pancreatic islet cells with resultant diabetes.

Heikkila et al. (2) have proposed that the diabetogenic action of alloxan is mediated via its action as an oxidant and its ability to form hydroxyl free radicals. Alloxan's action as an oxidant is thought to be the cause of the change in the cellular redox state observed when alloxan has been administered to rats, as exemplified by a fall in blood (3) and islet (4) reduced glutathione (GSH). Indirect support for the oxidant action of alloxan comes from the ability of a number of free radical scavengers to protect against the diabetogenic effect of alloxan (5).

The mechanism of action of streptozotocin seems to be less clearly defined. Schein and co-workers (6) have suggested that alkylation of DNA is the principal mode of streptozotocin's antileukemic action, while depression of NAD is responsible for its diabetogenic effect. We have recently demonstrated that streptozotocin, as well as alloxan, induces a change in the cellular redox state, causing a fall in blood GSH in rats (7), and a fall in GSH and rise in oxidized glutathione (GSSG) in rat islets and erythrocytes in vitro (8). These findings suggest that streptozotocin may also act as an oxidant, and this action may be associated with its diabetogenic effect. Recent support for this supposition was the finding of Sandler and Andersson (9), that partial protection against streptozotocin-induced diabetes in the mouse was achieved by prior administration of the hydroxyl radical scavenger dimethyl-urea.

The biological antioxidants vitamin E (10) and selenium-dependent glutathione peroxidase (11), are among the most effective protective systems in animals that function to limit oxidant damage. Administration of supplementary vitamin E to animals has ameliorated the cytotoxic effect of such free radical-producing agents as carbon tetrachloride (12), adriamycin (13), ozone (14), and irradiation (15), whereas animals fed vitamin E and selenium-deficient diets have demonstrated increased tendency for membrane lipid peroxidation (16). In examining the possibility that the diabetogenic action of streptozotocin, in addition to

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alloxan, may be mediated via an oxidant action, the following two questions have been posed in the present study. Firstly, does supplementation of rats with the antioxidant vitamin E protect against the diabetogenic effect of these agents? Secondly, do rats, depleted of their antioxidant protective mechanisms by being maintained on a vitamin E and selenium-deficient diet, become more susceptible to the diabetogenic action of streptozotocin?

## METHODS

Male Wistar rats weighing between 200 and 300 g were used for the two sets of experiments carried out in this study. Experiment A was designed to examine whether rats, supplemented with vitamin E, were able to modify the diabetogenic action of streptozotocin or alloxan. Experiment B was designed to examine whether rats, maintained on a vitamin E and selenium-deficient diet for 10 wk, were more susceptible to the diabetogenic action of streptozotocin.

### *Experiment A: vitamin E supplementation*

The rats were divided into groups according to their treatment protocol. Four separate studies were carried out with each group, and each group consisted of at least 12 rats. The rats were fed a regular diet of Wayne Lab-blox chow (Allied Mills, Chicago, IL) with free access to water. Streptozotocin, which was administered in a dose of 45 mg/kg, was dissolved in citrate buffer pH 4.6, and alloxan administered in a dose of 90 mg/kg was dissolved in 0.9% NaCl, just before intravenous injection for their respective studies. The following groups were studied:

*Vitamin E + streptozotocin (group I).* Three different protocols were used for supplementing groups of rats with vitamin E prior to their receiving streptozotocin. Each rat received either: (*a*) 200 U at 72 h, 100 U at 48 h, and 100 U at 2 h. (= vitamin E 72 + 48 + 2 h before streptozotocin); (*b*) 200 U at 72 h, 100 U at 48 h, but none at 2 h. (= vitamin E 72 + 48 h before streptozotocin) or; (*c*) 100 U at 2 h, but none at 72 h or 48 h. (= vitamin E 2 h before streptozotocin).

*Streptozotocin alone (group II).* Vehicle was administered intraperitoneally in an equal volume and at the same times as vitamin E was administered in (*a*), before streptozotocin.

*Control for streptozotocin studies (group III).* Vitamin E was administered in an equal concentration and at the same times as was administered in (*a*), before intravenous administration of citrate buffer pH 4.5, instead of streptozotocin.

*Vitamin E + alloxan (group IV).* Vitamin E was administered in an equal concentration and at the same times as in (*a*), before intravenous administration of alloxan.

*Alloxan alone (group V).* Vehicle was administered intraperitoneally prior to alloxan.

*Control for alloxan studies (group VI).* Vitamin E was administered as in (*a*), prior to intravenous administration of 0.9% NaCl, instead of alloxan.

The supplemental dose of vitamin E used in these experiments was commensurate with the dose used by previous investigators in which the ameliorating effects of vitamin E on the toxicity of adriamycin (13) and carbon tetrachloride (12) were examined. No adverse toxic effects were noted with this concentration of vitamin E used in these experiments.

Following an overnight (16 h) fast but with unlimited water, the rats were lightly anesthetized with ether and then either streptozotocin, citrate buffer, alloxan, or NaCl was injected via a tail vein. Food was returned to the rats 1–2 h after injection.

8 d later the rats underwent glucose tolerance tests (GTT). The weights in grams (mean±SE) of the different groups of rats at this stage were; controls 311±22, vitamin E supplemented 282±25, control rats made diabetic by streptozotocin (219±24) and by alloxan (235±31). After light ether anesthesia, glucose solution (1 g/kg) was administered via a tail vein. Blood was drawn from a tail vein before (time 0), and at 15, 30, and 60 min after glucose administration. Blood was collected into microfuge tubes and immediately centrifuged for 2 min in a Beckman Spinco microfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA). Plasma was frozen for later insulin and glucose assay. After 3 h, the animals were killed and the pancreas dissected free from surrounding fat. A third of the pancreas was fixed in Bouin's solution for histological examination, and total insulin content was measured after acid-ethanol extraction of the remaining tissue.

Pancreatic histological sections for light microscopy were stained with hematoxylin and eosin. Slides were graded 0, +, ++, +++ according to the degree of islet tissue damage. Between 100 and 150 islets of each group of rats were histologically examined. 0 (zero) indicates normal islet; +, mild damage; islets less compact, interstitial edema present, and evidence of recovery with active nuclei apparent; ++, moderate damage; islets demonstrated architectural disarray, increased intercellular space, and irregular staining with variable beta cell degranulation; +++, severe damage; nuclei pyknotic and inflammatory cells consisting mainly of lymphocytes were observed, usually at the periphery and sometimes within the interior of the islets. This finding of insulitis was consistently found in some areas of the pancreas.

The slides were numbered randomly so that the pathologist (Dr. Page), although aware of the compounds utilized in the study, did not know what treatment, if any, a given animal had received.

Total pancreatic liver and vitamin E content was measured in a group of vitamin E-supplemented rats [group I (*a*)], and a group that received vehicle (groups II and V), but neither group received streptozotocin or alloxan.

### *Experiment B: vitamin E and selenium depletion*

The following groups of rats were involved in the studies: I. Rats fed a vitamin E and selenium-deficient diet for a period of 10 wk. II. Rats fed a control Lab-blox diet.

Four separate studies were carried out in each experiment. Each group consisted of at least 12 rats. All the rats were allowed water ad lib. Prior to streptozotocin administration or the performance of a GTT, the rats were deprived of food for a period of 16 h.

*Experiment A.* The two groups of rats were administered streptozotocin in a dose of 65 mg/kg, and were monitored daily for weight, urinary glucose, ketones, and mortality.

*Experiment B.* A reduced dose of streptozotocin (45 mg/kg i.v.) was administered to two further groups of rats that had received the two types of diets. To assess whether the vitamin E + Se-deficient rats were dying as a result of di-

abetes, or some other toxic effect of streptozotocin, a further group of vitamin E + Se-deficient rats made diabetic with streptozotocin (45 mg/kg i.v.) were treated with daily subcutaneous insulin injections (NPH 2 U/kg/d s.c.) starting 24 h after streptozotocin administration. In addition, citrate buffer was administered to a group of vitamin E + Se-deficient rats. These groups of rats were monitored daily for weight, urinary glucose and ketones, and mortality.

*Experiment C.* To evaluate the effect of a normally nondiabetogenic dose of streptozotocin on the deficient animals, a lower dose of streptozotocin (25 mg/kg i.v.) was administered to a group of rats fed a vitamin E + Se-deficient diet and to a group of rats fed a control diet. In addition, citrate buffer was administered to a group of rats fed a vitamin E + Se-deficient diet and to a group of rats fed a control diet. 14 d later these four groups of rats underwent GTT (1 g/kg i.p.).

To assess the effect of maintaining rats on a vitamin E and selenium-deficient diet, pancreatic and liver vitamin E, and islet glutathione peroxidase concentrations were measured in a group of vitamin E and selenium-deficient rats and in a group of control rats that had not been injected with streptozotocin.

### *Statistical analyses*

Multiple mean comparisons of unpaired data were made using one-way analysis of variance, then Duncan's new multiple range test for means of equal sample size, or Student-Neuman-Keul's test for means of unequal sample size. Analysis of discrete data utilized Fisher's exact test. Significance was chosen as $P < 0.01$.

### *Materials*

Vitamin E ($\alpha$-tocopherol acetate suspended in soybean oil, 1,000 U/g) was obtained from Sigma Chemical Co., St. Louis, MO. Concentration of alphatocopherol injected was 200 U/0.2 $cm^3$.

Vehicle used was soybean oil, in which concentration of alpha tocopherol was 2 U/0.2 $cm^3$.

Streptozotocin, lot 6014 CU-9889, was kindly supplied by Dr. W. Dulin of Upjohn Co., Kalamazoo, MI.

Vitamin E and selenium-deficient ICN diet 10300 was purchased from ICN Pharmaceuticals (Plainview, NY). The pelleted vitamin E + Se-deficient diet is based on the formula of Schwartz and Fredga (17), and was constituted from the following components: Torula yeast 30%, sucrose 55.7%, stripped lard 5%, cod liver oil 3%. The vitamin E + Se-deficient diet contained 8% fat, 57% carbohydrate, and 24.5% protein and had a caloric content of 4.15 kcal/g, while the Lab-blox diet contained 4.2% fat, 24.5% protein, and 55% carbohydrate, and a caloric content of 4.06 kcal/g.

### *Assays*

Urinary glucose and ketones were measured by keto-diastix (Ames Co., Div. Miles Laboratories, Elkhart, IN). Serum glucose was measured by the glucose oxidase method, using a Beckman glucose analyzer. Serum insulin and pancreatic insulin content were measured by means of radioimmunoassay, free hormone being separated from antibody bound hormone by dextran-charcoal (18); porcine insulin was used as standard. Vitamin E content of pancreas and liver was measured by the thin-layer chromatographic method of Bieri (19). Glutathione peroxidase of pancreatic islets was measured by the method of Lawrence and Burk (20). Pancreatic islets were isolated as previously described (21).

## RESULTS

*Experiment A.* The plasma glucose and insulin responses to GTT (mean±SE) in rats treated with vitamin E given 72, 48, and 2 h before streptozotocin (group I [*a*]), streptozotocin alone (group II), and control rats treated with vitamin E prior to citrate injection (group III), are shown in Fig. 1. The glucose response of the streptozotocin-treated rats was significantly different from control rats at all points in time. Glucose levels of rats treated with vitamin E plus streptozotocin were not significantly different from control rats prior to, and 15 min after, glucose administration but were significantly different ($P < 0.01$) 30 and 60 min after intravenous glucose. However, at all points measured, vitamin E- and streptozotocin-treated rats were different ($P < 0.01$) from the glucose levels of rats treated with streptozotocin alone. The basal insulin levels of rats treated with vitamin E plus streptozotocin were not significantly different from those

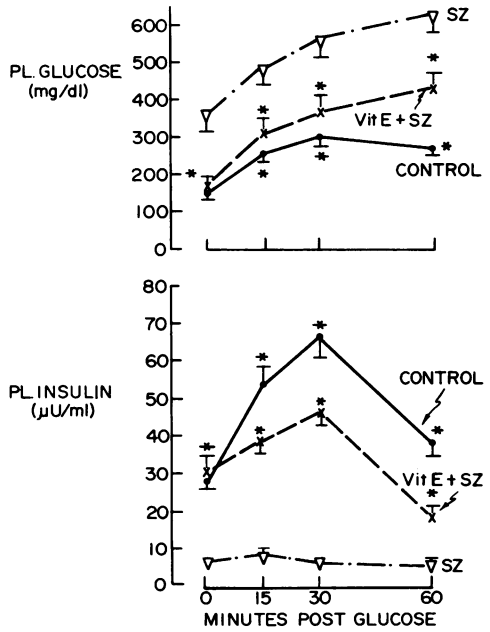


FIGURE 1 Plasma (PL.) glucose and insulin response (mean±SE) to GTT (1 g/kg i.v.) in three groups of rats; a group in which streptozotocin (SZ) (45 mg/kg i.v.) was administered, a group in which vitamin E was administered intraperitoneally 72, 48, and 2 h before streptozotocin (Vit. E + SZ), and a group in which vehicle was administered intraperitoneally 72, 48, and 2 h prior to citrate buffer (control). ($n$ = 12 rats for each group). $^*P < 0.01$ vs. SZ value.

of control rats, while the insulin levels at other points in time were significantly less ($P < 0.01$) than those of control rats. Insulin levels of rats treated with streptozotocin alone were significantly lower ($P < 0.01$) than the two other groups of rats at all points.

Fig. 2 compares the glucose responses (mean±SE) to GTT of groups of rats that underwent differing protocols of vitamin E administration (groups I [*a*], I [*b*], and I [*c*]) prior to streptozotocin administration. These groups of rats were also compared to rats treated with streptozotocin alone (group II) and control rats (group III). Rats that received differing amounts of vitamin E before streptozotocin administration did not differ significantly from each other, or from control rats at times 0, 15, and 30 min, while at 60 min all three groups I (*a*), (*b*), and (*c*) differed significantly ($P < 0.05$) from control rats (group III). All four groups of rats were significantly different ($P < 0.01$) from rats treated with streptozotocin alone (group II) at all points in time.

The plasma glucose and insulin responses (mean±SE) of rats injected with vitamin E plus alloxan (group IV), alloxan alone (group V), and a group of control rats (group VI) are shown in Fig. 3. The response of these groups of alloxan-treated rats was similar to that observed following streptozotocin (Fig. 1). The glucose levels of alloxan-treated rats were significantly higher ($P < 0.01$) than both the vitamin E plus alloxan, and control rats, at all points in time. The glucose levels of vitamin E plus alloxan rats did not differ significantly from those of control rats. The insulin levels of rats treated with vitamin E plus alloxan did not differ significantly from those of control rats at all points. Insulin levels of rats treated with alloxan were very low and differed significantly ($P < 0.01$) from both other groups of rats at all points.

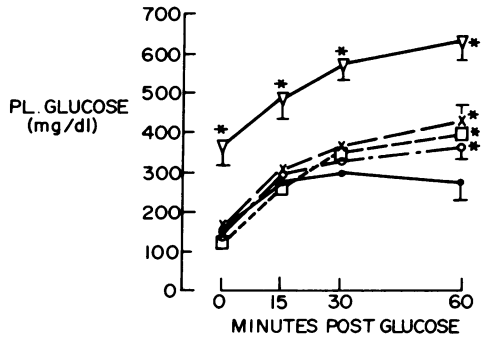


FIGURE 2 Plasma (PL.) glucose levels (mean±SE) following GTT (1 g/kg i.v.) in rats injected with different amounts of vitamin E at different times prior to streptozotocin (Sz, 45 mg/kg i.v.). × – – – ×, vitamin E at 72 h (200 U), 48 h (100 U), and 2 h (100 U), before Sz; □ ----- □, vitamin E at 72 h (200 U) and 48 h (100 U) before Sz; ○ – · – · – ○, vitamin E (100 U) at 2 h before Sz; ▽ —— ▽, Sz alone; ● —— ●, controls (no Sz administered) ($n$ = 12 rats for each group) *$P < 0.05$ vs. control value.

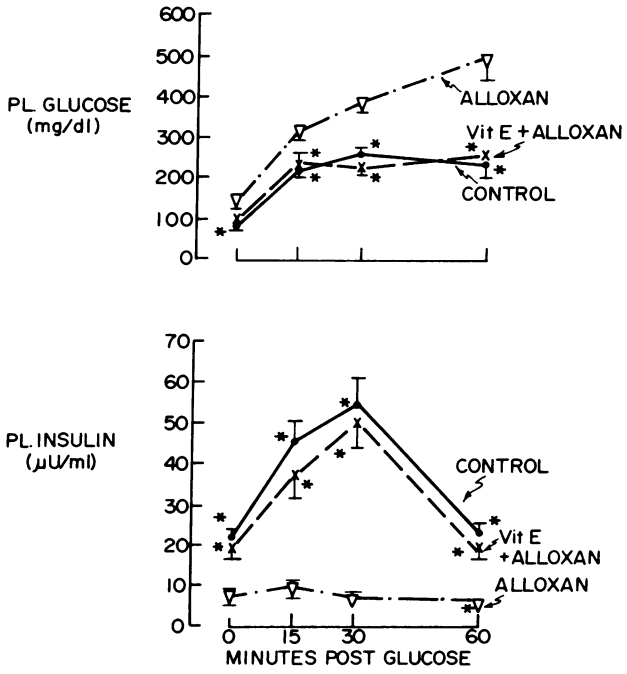


FIGURE 3 Plasma (PL.) glucose and insulin response (mean±SE) to (GTT 1 g/kg i.v.) in three groups of rats; a group in which alloxan (95 mg/kg i.v.) was administered (alloxan), a group in which vitamin E (Vit) was administered intraperitoneally 72, 48, and 2 h before alloxan (Vit E + alloxan), and a group in which vehicle was administered intraperitoneally 72, 48, and 2 h prior to citrate buffer (control). ($n$ = 12 rats for each group) *$P < 0.01$ vs. alloxan value.

Table I documents the pancreatic insulin content of the different groups of rats. Pancreatic insulin contents of rats treated with vitamin E before either streptozotocin or alloxan were significantly greater than those

TABLE I

*Pancreatic Insulin Content (IRI) in Rats Injected with Either Streptozotocin or Alloxan, and in Control Rats (Mean±SE)*

| Type of animal preparation | Group | Number of animals | Pancreatic IRI |
|---|---|---|---|
| | | | *ng/g wet wt* |
| Vitamin E + streptozotocin | I*a* | 12 | 23,370±1,250* |
| Streptozotocin | II | 14 | 8,115±380 |
| Vitamin E + alloxan | IV | 12 | 47,458±2,260* |
| Alloxan | V | 12 | 10,675±485 |
| Control | III and VI | 21 | 53,294±1,250* |

*, Significance ($P < 0.01$) between either vitamin E + streptozotocin and streptozotocin, and vitamin E + alloxan, and alloxan.

TABLE II

*Assessment of Islet Damage in Rats following Streptozotocin or Alloxan Administration, with or without Pretreatment with Vitamin E*

| Type of animal | Group | Islet damage 0 | + | ++ | +++ |
|---|---|---|---|---|---|
| Control | III | 12 | — | — | — |
| Vitamin E + streptozotocin | Ia | — | 7 | 4 | 1 |
| Streptozotocin | II | — | 1* | —* | 11* |
| Control | VI | 11 | 1 | | |
| Vitamin E + alloxan | IV | 2 | 8 | 2 | — |
| Alloxan | V | — | 1* | 2 | 9* |

*, Significance ($P < 0.01$) at each assessment between either vitamin E + streptozotocin and streptozotocin, and vitamin E + alloxan and alloxan.

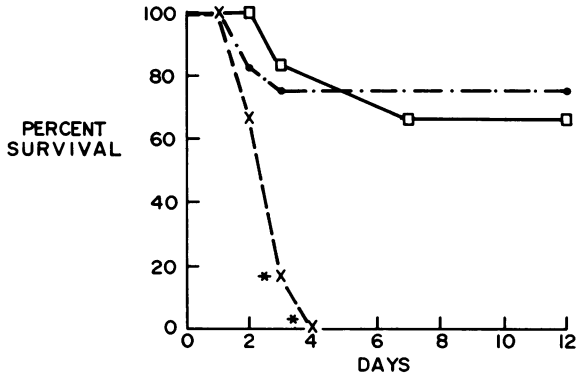


FIGURE 4 Mortality rate of different groups of rats following streptozotocin (Sz) administration (45 mg/kg i.v.). $n = 12$ rats for each group. *$P < 0.01$ vs. normal diet + Sz. Mortality rate of rats maintained on a vitamin E + Se-deficient diet (X - - - X) was significantly different from rats on a normal diet (□ —— □) on days 3 and 4, after Sz. Mortality rate of rats maintained on a vitamin E + Se-deficient diet but treated with insulin (2 U/kg per d s.c.) 24 h after Sz (● -·-·- ●) did not differ from rats on a normal diet at any time during the 12 d of the experiment.

of rats treated with streptozotocin alone or alloxan alone ($P < 0.01$).

Table II summarizes the light microscopy findings of the pancreatic islets of the different groups of rats. The streptozotocin-treated rats and alloxan-treated rats manifested severe islet cell damage, the vitamin E plus streptozotocin-treated rats showed mild to moderate damage, while the majority of the vitamin E plus alloxan-treated rats showed only mild damage.

The vitamin E content of the whole pancreas of vitamin E-supplemented rats was 89±11 μg/g wet wt, which was significantly greater than that of rats injected with vehicle (6.4±0.8, $P < 0.01$). Similarly, vitamin E content of liver from vitamin E-supplemented rats was 143±21 μg/g wet wt, which was greater than that of rats injected with vehicle (8.2±1.2, $P < 0.01$).

*Experiment B.* Prior to streptozotocin administration, the body weight in grams (mean±SE) of the vitamin E + Se-deficient rats (389±31) did not differ significantly from control rats (364±25), although the majority of the deficient animals had lost a considerable amount of body hair. After streptozotocin administration (65 mg/kg), the vitamin E + Se-deficient animals demonstrated moderate to large amounts of glucosuria and ketonuria within 24 h, and all died within 72 h. The Lab-blox-fed rats demonstrated glucosuria within 48 h, but all were still alive 5 d after streptozotocin administration. The mean fasting plasma glucose of the surviving vitamin E + Se-deficient rats 48 h after streptozotocin administration was 642 mg/dl, compared to Lab-blox-fed rats injected with streptozotocin (320 mg/dl), and vitamin E + Se-deficient control rats injected with citrate buffer (163 mg/dl).

The mortality rate of rats injected with a lower dose of streptozotocin (45 mg/kg) is shown in Fig. 4. Within 96 h all the vitamin E + Se-deficient rats injected with streptozotocin had succumbed, while 75% of the Lab-blox-fed rats injected with streptozotocin, and 83% of vitamin E + Se-deficient rats injected with streptozotocin and then treated with insulin were still alive after 12 d, when the study was discontinued. None of the vitamin E + Se-deficient rats that had been injected with citrate buffer had died.

The glucose responses to GTT in rats given an even lower dose of streptozotocin (25 mg/kg) are shown in Fig. 5. The plasma glucose levels of the vitamin E + Se-deficient rats injected with streptozotocin were significantly higher ($P < 0.01$) than those of the other three groups of rats at all points. The normal response of the Lab-blox fed rats injected with streptozotocin indicates that the dose of streptozotocin administered (25 mg/kg) was normally not a diabetogenic dose.

The vitamin E content of whole pancreas of rats maintained on a vitamin E + Se-deficient diet was 2.1±0.2 μg/g wet wt, which was significantly less than that of rats maintained on Lab-blox diet (6.4±0.8, $P < 0.05$). The glutathione peroxidase activity of pancreatic islets of rats maintained on a vitamin E + Se-deficient diet was 4.6±0.55 mM NADPH oxidized/min per mg protein, which was significantly less than that of rats maintained on Lab-blox diet (8.6±0.79, $P < 0.05$).

## DISCUSSION

From the work of Heikkila et al. (2, 5, 22) it appears that the diabetogenic action of alloxan is mediated by its function as an oxidant, and its ability to form hy-

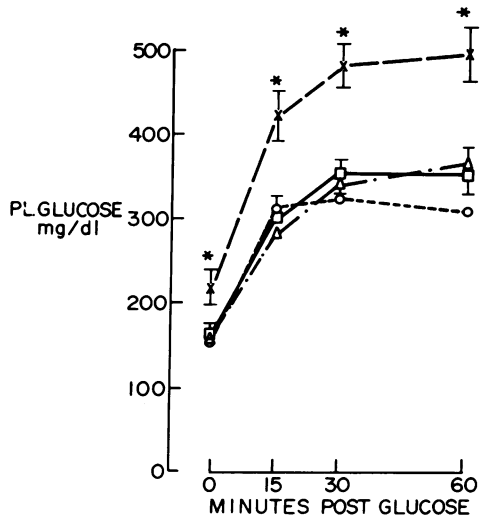


FIGURE 5 Plasma glucose response (mean±SE) to GTT (1 g/kg i.v.) in four groups of rats; a group maintained on a vitamin E + Se-deficient diet in which streptozotocin (25 mg/kg i.v.) was administered (× - - - ×), a group maintained on a vitamin E + Se-deficient diet in which citrate buffer was administered (△ -·-·- △), a group maintained on normal Lab-blox diet in which streptozotocin was administered (□ —— □), and a group maintained on a normal diet in which citrate buffer was administered (○ ----- ○). ($n$ = 12 rats for each group). *$P < 0.01$ vs. normal diet.

droxyl radicals. In animal experiments and in vitro studies, vitamin E has been shown to act as an antioxidant and free radical scavenger (23). The ability for vitamin E to protect against the diabetogenic action of alloxan in the present study is further evidence that alloxan probably acts as an oxidant or free radical producer.

Both streptozotocin and alloxan produce similar diabetogenic effects, in that they both produce irreversible damage to the beta cells in a variety of species, and both produce a triphasic change in blood sugar (1). Of significance to their possible action as oxidants is the finding that both drugs have been shown to cause an acute change in the cellular redox state, as exemplified by a fall in the reduced to oxidized glutathione (GSH/GSSG) ratio. However, these two drugs differ from each other in a number of respects. Glutathione, cysteine, nicotinic acid, nicotinamide, glucose, 3-*O*-methyl glucose (1) and superoxide dismutase (24) administered prior to alloxan are known to protect against the diabetogenic action of alloxan, whereas only nicotinamide (25), superoxide dismutase (26), 3-*O*-methyl glucose (27), and glucose in high concentrations (28) are known to protect against the beta cell toxicity of streptozotocin. Hepatic and pancreatic oxidized and reduced nicotinamide adenine dinucleotide (NAD, NADH) levels were shown to be acutely depressed in mice after streptozotocin administration, whereas alloxan had no effect on NAD levels (29). From experiment A of this study it is apparent that the diabetogenic effect of streptozotocin, as well as that of alloxan, is ameliorated by the prior administration of vitamin E, suggesting that streptozotocin may act, at least in part, as an oxidant. To produce an antioxidant-depleted state, rats were made selenium deficient as well as vitamin E deficient. The selenium-dependent enzyme, glutathione peroxidase, plays a significant role in the defence of the cell against oxidative challenge by its ability to reduce a large variety of harmful hydroperoxides to their corresponding alcohols (30). The usual streptozotocin dose of 65 mg/kg was highly toxic to these animals, all becoming severely diabetic within 24 h, and all dying within 72 h. This heightened morbidity and mortality was apparent in vitamin E and selenium depleted rats even when a lower dose of streptozotocin (45 mg/kg) was administered. This enhanced mortality was shown to be due to the severity of the diabetes rather than due to some other toxic effect of streptozotocin, since the mortality rate was greatly reduced when the vitamin E and selenium-deficient rats were treated with daily insulin injections, soon after the onset of diabetes. When even a lower dose of streptozotocin was administered (25 mg/kg) control rats did not become diabetic, whereas the vitamin E and selenium-deficient rats demonstrated significant glucose intolerance. These studies demonstrate that animals with impaired antioxidant abilities have an enhanced susceptibility to the diabetogenic action of streptozotocin.

The results of experiments A and B reinforce the concept that streptozotocin, in addition to alloxan, may cause diabetes through the generation of free radicals.

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