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ALLOXAN-INDUCED DIABETES—EVIDENCE FOR HYDROXYL RADICAL AS A CYTOTOXIC INTERMEDIATE

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Abstract—Four aliphatic alcohols, namely methanol, ethanol, *n*-propanol and *n*-butanol, prevented the diabetic state induced by alloxan in Swiss–Webster mice, as judged at 72 hr by two criteria: blood glucose levels and granulation of the β-cells of the pancreas. *n*-Propanol and *n*-butanol were better protective agents than methanol or ethanol. Thiourea also proved to be protective, while hydroxyurea and urea did not. In experiments *in vitro*, the order of reactivity of the above mentioned compounds with the hydroxyl radical was found to correlate reasonably well with their capacity to prevent alloxan diabetes. None of the compounds reacted with superoxide radicals to any significant extent, while only thiourea was shown to react directly with hydrogen peroxide. There was no correlation between the capacity of the compounds to act as substrates for the peroxidatic activity of catalase and their ability to prevent alloxan diabetes. It had been previously postulated that hydrogen peroxide or the hydroxyl radical might be involved in the diabetogenic action of alloxan. The data of the present study are consistent with a role for the hydroxyl radical and appear to rule out a direct causative role for hydrogen peroxide. Other data rule out any protective role for transiently elevated levels of blood glucose resulting from the administered compounds.

Dialuric acid is the reduced form of alloxan (a quinone). The reaction of dialuric acid with oxygen (autoxidation) leads to the formation of three highly reactive species: hydrogen peroxide (H_2O_2), the superoxide radical (O_2^-) and the hydroxyl radical ($^{\cdot}OH$) [1]. Both dialuric acid and alloxan can induce a diabetic state in experimental animals [2] and erythrocyte hemolysis in vitamin E-deficient animals [3]. Alloxan is reduced by ascorbic acid *in vitro* to dialuric acid which, in turn, spontaneously autoxidizes to form H_2O_2 [4] as well as O_2^- and $^{\cdot}OH$. The detection of H_2O_2 in erythrocytes after injection of alloxan [5] implies that tissue-reducing agents similarly carry out the conversion of alloxan to dialuric acid *in vivo*.

H_2O_2, O_2^- and $^{\cdot}OH$ have been shown or postulated to be cytotoxic in a variety of biological systems. For example, O_2^- and $^{\cdot}OH$ have been implicated as causative agents in lipid peroxidation [6, 7], and there is evidence that O_2^- is toxic to micro-organisms which metabolize oxygen [8]. It appears that O_2^- [9] and H_2O_2 [10, 11] are both required for the destruction of micro-organisms by phagocytes. In addition, $^{\cdot}OH$ reacts rapidly with nucleic acids and may be responsible for the damage caused by X-irradiation [12, 13]. In light of these observations, it appeared likely [1] that H_2O_2, O_2^- or $^{\cdot}OH$ could be responsible for the destruction of the β-cells of the pancreas by dialuric acid and alloxan.

In a previous study [5], it was shown that ethanol prevented the diabetogenic action of alloxan. This result was consistent with two possible modes of protection: removal of H_2O_2 by the peroxidatic activity of catalase or removal of $^{\cdot}OH$ by direct reaction with ethanol. We have now extended the study with ethanol to include three other aliphatic short chain alcohols, namely, methanol, *n*-propanol and *n*-butanol. The use of a homologous series of alcohols allowed a differentiation to be made between the two previously mentioned modes of protection. Thiourea was also tested because this compound, like the alcohols, reacts rapidly with $^{\cdot}OH$ [14]. The data obtained in the present study point to a contributory role of $^{\cdot}OH$ in the development of alloxan diabetes.

MATERIALS AND METHODS

Experiments were carried out with male Swiss–Webster mice in the weight range of 23–33 g. Animals were routinely fasted 3–4 hr before alloxan injection and given free access to food 1 hr later. Alloxan monohydrate (50 mg/kg) was injected into a tail vein in 0·2 ml saline. All pretreatment compounds (methanol, ethanol, *n*-propanol, *n*-butanol, urea, thiourea and hydroxyurea) were given as i.p. injections in saline [0·9% (w/v) NaCl in water]. Animals received alloxan alone or were pretreated prior to alloxan injection. Control animals were either untreated, or pretreated with one of the above compounds or saline, but not alloxan.

Blood glucose was measured 72 hr after alloxan administration. Animals were decapitated and blood was collected from the neck. Blood glucose was measured in $Ba(OH)_2$-$ZnSO_4$ filtrates by a glucose oxidase method (Worthington Biochemicals).

The pancreases from 43 mice including controls, alloxan-treated and alloxan plus various alcohols were excised and fixed overnight in Zenker formol [15]. Six-μm thick paraffin sections were treated with aldehyde fuchsin reagent [16] to stain the β-cells of the pancreatic islets.

The reaction of the various pretreatment compounds with hydroxyl radicals, superoxide radicals and H_2O_2 were evaluated as follows. An $^{\cdot}OH$-generating system consisted of autoxidizing 6-aminodopamine in 0·2 M acetate buffer at pH 6·4 containing 10^{-4} M EDTA [1]. Hydroxyl radicals are known to react with 3-thiomethylpropanal [1, 17] or with 2-keto-4-thiomethylbutyric acid (used in this investigation) to form the hydrocarbon gas, ethylene, which can be measured by gas chromatography [1]. The reactivity of compounds with $^{\cdot}OH$ is given by the suppression of ethylene formation from 10^{-2} M 2-keto-4-thiomethylbutyric acid.

Reactivity with O_2^- was studied in a system consisting of autoxidizing 10^{-4} M 6-hydroxydopamine in 0·05 M phosphate buffer, pH 7·4, containing 10^{-4} M EDTA. The effect of the various compounds on the rate of formation of quinoidal products was followed at 490 nm [18]. The rate is catalyzed by O_2^- generated during the autoxidation and suppressed by agents that react with and remove the O_2^- catalyst [18, 19]. Reactivity with O_2^- is given by suppression of color development.

The reaction with H_2O_2 was studied by incubating the various compounds with H_2O_2 and then measuring residual H_2O_2 with the peroxidase chromogen procedure employed in the glucose oxidase method (glucose omitted). Additionally, the effect of the various compounds was studied by incorporating them into the glucose oxidase reaction mixture during the oxidation of 10^{-4} M glucose.

Chemicals

Alloxan monohydrate, urea, thiourea and hydroxyurea were purchased from Sigma Chemical Co. (St. Louis). Methanol, *n*-propanol and *n*-butanol were purchased from Fisher Scientific (Fairlawn, N.J.). Ethanol was obtained from the Mount Sinai Hospital (distributed by Publicker Industries Co., Linfield, Pa.). Glucose oxidase kits were purchased from Worthington Biochemicals, Freehold, N.J. 6-Hydroxydopamine hydrobromide was purchased from Regis Chemicals (Morton Grove, Ill.). 6-Aminodopamine dihydrochloride was a gift of Dr. E. Englehardt, Merck, Sharp & Dohme, West Point, Pa.). 2-Keto-4-thiomethylbutyric acid was a gift from Dr. Morris Lieberman (U.S. Dept. of Agriculture, Beltsville, Md.); we thank Dr. Lieberman for recommending the use of this odorless compound.

RESULTS

Effects of short chain alcohols and urea analogues on the induction of experimental diabetes by alloxan

Alloxan, administered intravenously in a 50 mg/kg dose, produced the expected increase in blood glucose levels 72 hr later ($P < 0·001$ compared to untreated controls, Fig. 1). Pretreatment with methanol (4 g/kg), ethanol (4 g/kg), *n*-propanol (1·6 g/kg), or *n*-butanol

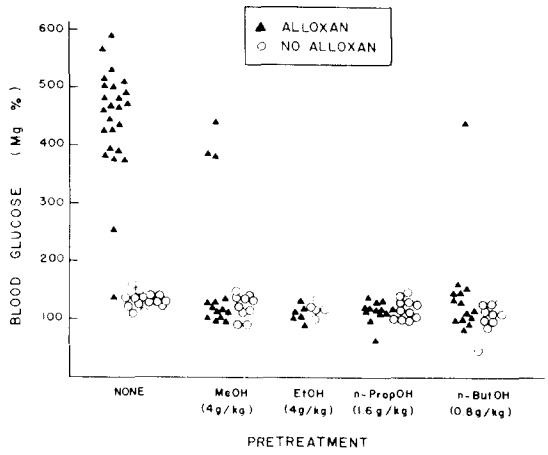

Fig. 1. Effect of pretreatment with various alcohols on blood glucose levels 72 hr after alloxan. Alloxan (50 mg/kg, i.v.) was administered alone or 30 min after an intraperitoneal injection (0·02 ml/g body weight) of 25% methanol (v/v in saline), 25% ethanol, 10% propanol or 5% *n*-butanol. Control animals were treated with one of the above alcohols or were untreated. Blood glucose levels are shown for individual animals. The following abbreviations were used: methanol (MeOH), ethanol (EtOH), *n*-propanol (n-PropOH) and *n*-butanol (n-ButOH).

(0·8 g/kg) prevented this hyperglycemic effect. Average blood glucose levels (mg/100 ml ± S.E.M.) for each group were as follows: alloxan (445 ± 19), methanol + alloxan (175 ± 33), ethanol + alloxan (107 ± 6), propanol + alloxan (111 ± 5), *n*-butanol + alloxan (141 ± 24), and combined controls (120 ± 3). The protective action for each alcohol was statistically significant ($P < 0·001$) compared to the group receiving alloxan alone. The ethanol dose corresponded to that used in a previous study [5]. Much smaller doses of *n*-propanol or *n*-butanol were used because these alcohols proved lethal at higher doses.

The pancreases from some animals in each group were removed and stained with Gomori's aldehyde fuchsin reagent. The β-cells from untreated animals were fully granulated (Fig. 2A), whereas those from animals treated with alloxan alone were totally degranulated (Fig. 2B, Table 1). In the alcohol-protected groups, certain animals displayed some degranulation of β-cells (Fig. 2, panels C and D), although blood glucose was in a normal range (Table 1). However, the β-cells from most of the alcohol-protected animals were fully granulated (Fig. 2, panels F, G and H), as were those from the alcohol-treated controls (Fig. 2E).

In order to compare directly the relative protective actions of the alcohols, mice were injected i.p. with equimolar doses of the alcohols (10^{-2} moles/kg, Fig. 3). Thiourea was also tested. For comparison with the doses listed in Fig. 1, 10^{-2} moles/kg is equivalent per kg to 0·32 g methanol, 0·46 g ethanol, 0·60 g *n*-propanol, 0·74 g *n*-butanol or 0·76 g thiourea. The pretreatment compounds appeared to fall into two categories: thiourea, methanol and ethanol at 10^{-2} moles/kg afforded weaker protection, based on mean blood glucose levels, than *n*-propanol and *n*-butanol

(Fig. 3). It should be noted that the mean blood glucose level for each group was obtained by averaging low (protected, 50 250 mg/100 ml), intermediate (250–400 mg/100 ml) and high (unprotected, 401 mg/100 ml and higher) blood glucose values. The distribution of animals in low, medium and high blood glucose ranges were as follows: saline + alloxan (0%, 18%, 82%), thiourea + alloxan (39%, 11%, 50%), methanol + alloxan (33%, 17%, 50%), ethanol + alloxan (17%, 33%, 50%) propanol + alloxan (72%, 17%, 11%), butanol + alloxan (90%, 5%, 5%), and combined controls (100%, 0%, 0%). In the experiments with higher doses of methanol and ethanol (Fig. 1), on the other hand, very few animals were found in the intermediate or high blood glucose ranges.

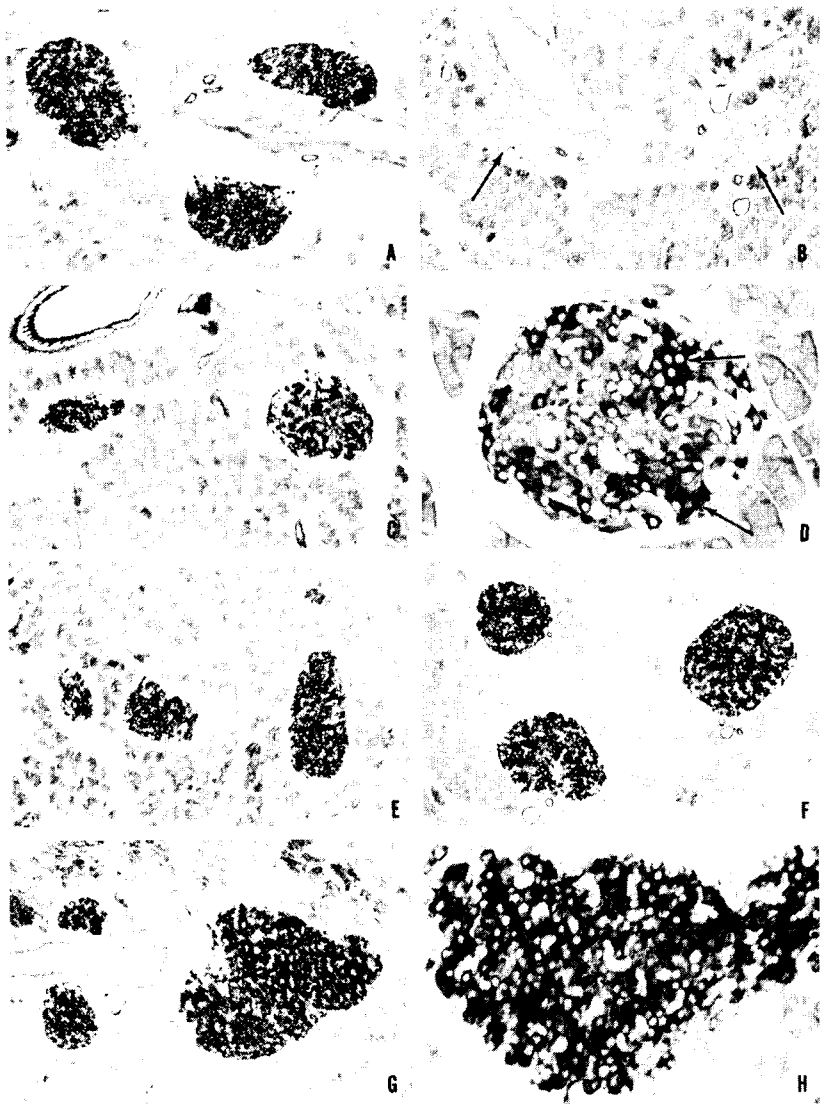

Fig. 2. (A–H) Mouse pancreas stained with Gomori's aldehyde fuchsin reagent. (A) Section from an untreated control mouse demonstrating three islets which consist mostly of well-stained, fully granulated β-cells (X 88). (B) Section from an alloxan-treated mouse demonstrating two completely degranulated islets (arrows) (X 88). (C) Section from a mouse treated with methanol plus alloxan. The smaller islet on the left contains fully granulated β-cells and the larger one on the right contains numerous, partially degranulated β-cells (X 88). (D) Another islet from a mouse treated with methanol plus alloxan, viewed under higher power than those shown in C. The islet exhibits β-cells in various stages of degranulation. Only two small groups (arrows) and several scattered β-cells are fully granulated (X 313). (E) Section from a control mouse treated with *n*-propanol alone. Three islets with fully granulated β-cells can be seen (X 88). (F) Section from a mouse treated with *n*-propanol plus alloxan, demonstrating three islets with fully granulated β-cells (X 88). (G) Section from a mouse treated with *n*-butanol plus alloxan, demonstrating three islets with fully granulated β-cells (X 88). (H) Portion of another islet from a mouse treated with *n*-butanol plus alloxan, viewed under a higher power than the islets shown in G. Nearly all of the β-cells are fully granulated (X 313).

Table 1. Correlation between morphologic characteristics of the β-cells of the pancreas and blood glucose levels 72 hr after alloxan.*

Experimental group	Appearance of β-cells	Blood glucose (mg/100 ml)
Combined controls†	Granulated (normal)	87, 88, 96, 99, 111, 111, 114, 115, 117, 125, 129, 130, 131, 136, 138
Alloxan	Totally degranulated	376, 444, 483, 501, 567, 587
Methanol (4 g/kg) + alloxan	Granulated	99, 101, 128
	Partially degranulated	97, 117
	Mostly degranulated	112
Ethanol (4 g/kg) + alloxan	Granulated	85, 100, 103, 112, 114
	Partially degranulated	130
n-Propanol (1·6 g/kg) + alloxan	Granulated	59, 107, 117
n-Butanol (0·8 g/kg) + alloxan	Granulated	103, 125, 129
	Partially degranulated	151

* Animals were treated as described in the legend to Fig. 1. The tissues were stained with Gomori's aldehyde fuchsin reagent. Blood glucose levels are given for individual animals.

† Controls were either non-treated or given only i.p. methanol, ethanol, propanol or butanol at the doses indicated for the other experimental groups.

In order to compare structure–activity relationships for some structural analogues of thiourea, we pretreated mice with 3·0 g/kg of urea, hydroxyurea or thiourea (Table 2). Urea and hydroxyurea did not give significant protection when administered either 0·5 hr or 2 hr prior to alloxan. Thiourea, on the other hand, exerted a strong protective action with an apparently greater effect in the 2-hr pretreatment schedule (see Table legend). It should be noted that the protective action observed for 3 g/kg of thiourea in experiment B was as powerful as that seen with any of the alcohols (compare Fig. 1).

Reaction of short chain alcohols and urea derivatives with hydroxyl radicals, superoxide radicals and H_2O_2

The hydroxyl radical can be detected in aqueous solution by its ability to generate ethylene from certain aldehydes or keto acids (see Methods). In a system containing a known ·OH-generating agent (viz. 6-aminodopamine), the alcohols and urea derivatives were compared directly with regard to their relative abilities to scavenge ·OH (inhibit ethylene formation).

In preliminary experiments, it was noted that suppression of ethylene generation by the alcohols was dose dependent. The results summarized in Fig. 4 show that, when the alcohols were compared at 10^{-2} M (which was the effective dose used in the experiments *in vivo* in Fig. 3), the reactivity with ·OH increased with increasing chain length. Similar results had been noted by other investigators who employed other kinds of detection systems [14]. The urea derivatives were also compared in the same system. Urea itself was inactive, while thiourea was a powerful inhibitor of ethylene formation (Fig. 4); hydroxyurea was intermediate in activity.

As shown below, ·OH can arise from a reaction between H_2O_2 and O_2^- [17, 20]. Since both H_2O_2 and O_2^- are generated during the autoxidation of

$$H_2O_2 + O_2^- \rightarrow {}^{\cdot}OH + OH^- + O_2$$

6-aminodopamine or dialuric acid [1], it follows that inhibition of ethylene formation could have resulted from removal of either H_2O_2 or O_2^-, rather than a direct reaction with ·OH. In separate experiments we tested these possibilities, as follows.

The reactivity of the alcohols and urea compounds with O_2^- was tested in a system in which O_2^- was generated during the autoxidation of 6-hydroxydopamine. The rate of quinone formation from 6-hydroxydopamine is catalyzed by O_2^- and is markedly suppressed by scavengers of O_2^- such as superoxide dismutase [18] or catecholamines [19]. In this system, none of the compounds at 10^{-2} M inhibited the rate of quinone formation from 6-hydroxydopamine. Therefore, these compounds did not react directly with O_2^-.

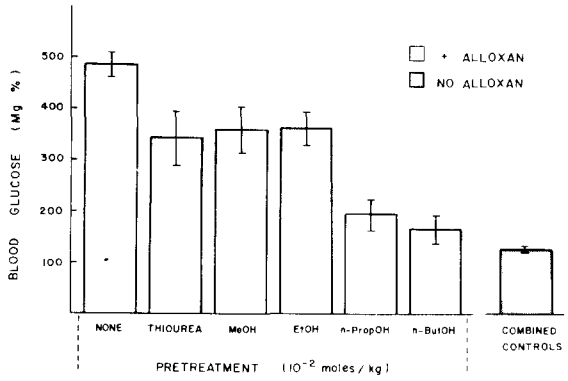

Fig. 3. Comparison of the effect of various alcohols or thiourea (10^{-2} moles/kg) on blood glucose levels 72 hr after alloxan. Data are the mean blood glucose values in mg/100 ml ± S.E.M. Except for the dose of pretreatment compound, animals were treated as described in the legend to Fig. 1. Some alloxan-treated animals and some controls were pretreated with i.p. saline. There were 17–19 animals in each experimental group and 87 animals in the combined control group which consisted of mice receiving saline alone, or one of the alcohols or thiourea. These controls exhibited blood glucose levels ranging from 73 to 211 mg/100 ml.

Table 2. Comparison of the protective effects of urea, hydroxyurea and thiourea on the induction of hyperglycemia by alloxan*

Treatment	Blood glucose (mg/100 ml ± S.E.M.) Experiment A†	Experiment B†
Saline + alloxan	433 ± 43 (9)	449 ± 9 (24)
Urea + alloxan	443 ± 44 (7)‡	478 ± 17 (21)‡
Hydroxyurea + alloxan	364 ± 86 (6)‡	390 ± 29 (13)‡
Thiourea + alloxan	250 ± 43 (11)§	147 ± 11 (20)‖
Combined controls¶	118 ± 5 (25)	124 ± 2 (61)

* Animals were pretreated with test compounds (i.p., 3 g/kg) either 0·5 hr (Expt. A) or 2 hr (Expt. B) before alloxan. Other details are the same as those described in the legend to Fig. 1. The number of animals in each group is shown in parentheses.

† The distribution of animals in three blood glucose ranges (50–250, 251–400, and 401 mg/100 ml and higher) was as follows (expressed as the per cent of total in each group): Experiment A: saline + alloxan (11%, 22%, 67%), urea + alloxan (14%, 0%, 86%), hydroxyurea + alloxan (33%, 17%, 50%), thiourea + alloxan (64%, 18%, 18%) and combined controls (100%, 0%, 0%); and Experiment B: saline + alloxan (0%, 4%, 96%), urea + alloxan (0%, 14%, 86%), hydroxyurea + alloxan (15%, 23%, 62%), thiourea + alloxan (95%, 5%, 0%) and combined controls (100%, 0%, 0%).

‡ Not significant when compared to saline + alloxan group.

§ $P < 0.01$ when compared to saline + alloxan group.

‖ $P < 0.001$ when compared to saline + alloxan group.

¶ Control animals received only i.p. saline, urea, hydroxyurea or thiourea (3 g/kg).

Reaction directly with H_2O_2 was studied in modifications of the glucostat system used in this investigation for the measurement of glucose in blood. The measurement of glucose is dependent upon the generation of H_2O_2 by glucose oxidase and subsequent reaction of H_2O_2 with 3,3-dimethoxybenzidine (catalyzed by peroxidase) to yield a chromophore which is measured spectrophotometrically. We tested the effect of adding 10^{-2} M concentrations of alcohols or urea compounds on the rate of color formation in the presence of 10^{-4} M glucose. Hydroxyurea (10^{-2} M) could not be tested because it interfered with color development from H_2O_2 in this system. None of the compounds showed an effect except for thiourea, which inhibited color development by less than 10 per cent.

Additionally, we incubated 10^{-2} M compounds with 10^{-4} M H_2O_2 in separate tubes at room temperature for 10 min. Aliquots were removed for determination in the glucose oxidase–peroxidase system (no glucose added). Only thiourea reacted with H_2O_2 under these conditions; approximately half of the H_2O_2 was decomposed in 10 min. The lack of reactivity of the alcohols excluded a direct reaction of the alcohols with H_2O_2 as a contributory factor in the suppression of ethylene.

With regard to thiourea, it had been shown by others [14] to be a good 'OH trap. In our system, suppression of ethylene by 10^{-2} M thiourea was undoubtedly due both to removal of H_2O_2 as well as a direct reaction with 'OH. However, 10^{-3} M thiourea suppressed ethylene formation by 43·7 ± 1·4 per cent (mean ± S.D., N = 4 experiments run in quadruplicate) in 10 min but decomposed only 12·1 ± 1·3 per cent (N = 3) of 10^{-4} M H_2O_2. These data indicated that thiourea reacted more rapidly with 'OH than with H_2O_2 in the ethylene system. In comparison, butanol at 10^{-3} M suppressed ethylene formation by only 4·9 ± 3·2 per cent (N = 4) in 10 min. The combined data indicated that thiourea is a better 'OH scavenger than *n*-butanol. As noted earlier, hydroxyurea could not be evaluated because it interfered with color development in the peroxidase system.

Effect of short chain alcohols and urea derivatives on blood glucose levels

Transiently elevated blood glucose levels can protect against the diabetogenic action of alloxan [21]. Previously, we observed that pretreatment with ethanol (4 g/kg) significantly increased blood sugar levels at 30 min, normally the time of alloxan administration [5]. Experiment A of Table 3 shows that methanol, ethanol, *n*-propanol and *n*-butanol administered in the doses used in Fig. 1 significantly increased blood glucose at 30 min ($P < 0.001$ for each alcohol

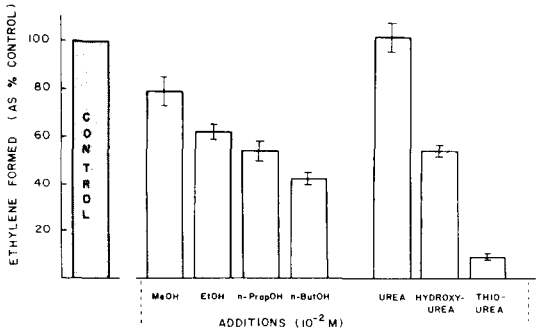

Fig. 4. Effect of several alcohols and urea derivatives at 10^{-2} M on ethylene generation. The system consisted of 10^{-2} M 2-keto-4-methylthiobutyric acid in 0·2 M acetate buffer at pH 6·4, containing 6-aminodopamine (10^{-4} M) as a hydroxyl radical-generating agent. Results are the mean ± S.E.M. for three to five experiments with each compound, run in quadruplicate and expressed as a per cent of the control. Control generation of ethylene was 0·79 nmole at 40 min

Table 3. Blood glucose levels 30 min after i.p. injection of alcohols and thiourea.*

Treatment	Experiment A Dose (g/kg)	Experiment A Blood glucose (mg/100 ml ± S.E.M.)	Experiment B Dose (g/kg)†	Experiment B Blood glucose (mg/100 ml ± S.E.M.)
Saline		95 ± 5 (14)		100 ± 4 (18)
Methanol	4	163 ± 9 (14)‡	0·32	106 ± 3 (18)§
Ethanol	4	159 ± 14 (15)‡	0·46	88 ± 5 (18)
n-Propanol	1·6	211 ± 27 (15)‡	0·60	115 ± 5 (18)‖
n-Butanol	0·8	153 ± 14 (15)‡	0·74	145 ± 7 (18)‡
Thiourea			0·76	114 ± 5 (18)‖

* The doses in Expt. A correspond to those in Fig. 1, while those in Expt. B correspond to Fig. 3. In both experiments, food was withheld 3–4 hr prior to injection. The number of animals in each group is shown in parentheses.

† Doses in Expt. B were all 10^{-2} moles/kg.

‡ $P < 0{\cdot}001$ when compared to saline control.

§ Not significant when compared to saline control.

‖ $P < 0{\cdot}05$ when compared to saline control.

group compared to saline control). However, in essentially lower, equimolar doses (10^{-2} moles/kg, Experiment B of Table 3), identical to those used in Fig. 3, only butanol caused a clear-cut and strong elevation in blood glucose. (Note, however, that the dose of butanol was essentially identical in Experiments A and B.) Thiourea and *n*-propanol gave small increases of borderline significance ($P < 0{\cdot}05$), while methanol produced no change and ethanol appeared to produce a slight decrease in blood glucose.

Blood glucose was also determined 2 hr after i.p. injection of 3 g/kg of urea, hydroxyurea or thiourea (cf. Experiment B of Table 2). No elevation in blood glucose was observed at 2 hr after injection of these compounds (Table 4).

Earlier experiments with starved animals [5] demonstrated that protection by ethanol could not be attributed to an elevated blood glucose level *per se*; however, it was not clear whether or not an upward change in blood glucose occurring prior to the injection of alloxan could play a significant protective role. In order to test this possibility we performed experiments with 10^{-2} moles (0·6 g)/kg of *n*-propanol as a protective agent (cf. Fig. 3), or 4 g/kg of urea as a negative control (cf. Table 2). Blood was sampled from the tail of each animal immediately prior to i.p. injection of propanol or urea and again 30 min later (immediately prior to alloxan). The data of Table 5 show that out of a total of seventeen animals receiving *n*-propanol, five animals became diabetic; however, the blood glucose increases in these five animals during the 30-min period after *n*-propanol injection was not different from that observed in the twelve protected animals. All urea-treated animals became diabetic; however, some of these mice displayed relatively large increases in blood glucose levels during the period from 0 to 30 min, comparable to the increases seen in the propanol-protected animals. Therefore, it can be concluded that an upward change in blood glucose cannot be the overriding factor in protection against alloxan.

Table 4. Blood glucose levels 2 hr after i.p. injection of urea, hydroxyurea and thiourea.*

Treatment	Blood glucose (mg/100 ml ± S.E.M.)
Saline	116 ± 8 (10)
Urea	113 ± 12 (9)†
Hydroxyurea	116 ± 12 (9)†
Thiourea	99 ± 10 (13)†

* Food was withheld 3–4 hr before injection. Each compound was administered at 3 g/kg, the same dose used for the experiments described in Table 2. The number of animals per group is shown in parentheses.

† Not significant when compared to saline control.

DISCUSSION

It had been shown previously that ethanol protected mice against the diabetogenic action of alloxan [5]. Two possible modes of protection were suggested: removal of H_2O_2 through the peroxidatic activity of catalase with ethanol as substrate, or removal of $^{\cdot}OH$ by direct reaction with ethanol.

In the present study, methanol, *n*-propanol and *n*-butanol were shown also to protect against alloxan. These alcohols prevented degranulation of the β-cells of the pancreas (Table 1, Fig. 2), as well as the rise in blood glucose 72 hr after alloxan (Fig. 1). The ability of the alcohols to scavenge $^{\cdot}OH$ radicals *in vitro* was studied in a system where $^{\cdot}OH$ gave rise to ethylene. Reactivity with $^{\cdot}OH$ (suppression of ethylene formation) for the homologous series of alcohols increased with increasing chain length (Fig. 4). The protective actions of the alcohols were compared *in vivo* at a fixed dose (10^{-2} moles/kg, equivalent to the concentration of 10^{-2} M used in the ethylene-generating system *in vitro*). Although fine distinctions between the various alcohols could not be made, the results of Fig. 3 showed that *n*-butanol and *n*-propanol were more effective protective agents than either ethanol or methanol. Furthermore, a breakdown of the distribution of animals with regard to final blood glucose levels indicated very few diabetic animals in the butanol and propanol groups, but considerably larger numbers of diabetic animals in the ethanol and methanol groups.

These data permit a distinction to be made between the two suggested protective mechanisms. The rank

Table 5. Change in blood glucose after urea or *n*-propanol and subsequent hyperglycemia 72 hr after alloxan.*

Group	Change in blood glucose 30 min after propanol or urea (mg/100 ml)	Blood glucose 72 hr after alloxan (mg/100 ml)
Propanol + alloxan (protected)	6, 16, 25, 38, 39, 44, 48, 49, 52, 54, 55, 55	226, 178, 121, 136, 137, 160, 107, 113, 134, 148, 130, 147
Propanol + alloxan (not protected)	19, 19, 30, 33, 56	421, 381, 422, 365, 396
Urea + alloxan (protected)	None	
Urea + alloxan (not protected)	−24, −21, −12, −11, −9, −7, −7, −2, 5, 23, 26, 29, 34, 37, 41, 41, 66, 147	474, 478, 618, 457, 454, 661, 507, 393, 457, 440, 468, 444, 561, 388, 452, 440, 441, 390

* Tail blood was sampled from individual mice immediately before and 30 min after i.p. urea (3 g/kg) or *n*-propanol (10^{-2} moles/kg = 0·60 g/kg). Alloxan (50 mg/kg) was injected i.v. immediately after the second blood sample. Data are listed so that the numbers listed in the "Change in blood glucose" column correspond to the same animals in the "Blood glucose 72 hr" column (i.e. the animal that showed a change of 6 mg/100 ml on injection of *n*-propanol showed a final blood glucose after alloxan of 226 mg/100 ml and so on).

order of the homologous series of alcohols for reaction with ·OH *in vitro* (*n*-butanol > *n*-propanol > ethanol > methanol) [14] (Fig. 3) is exactly opposite to their rank order as substrates for the peroxidatic activity of catalase (methanol > ethanol > *n*-propanol > *n*-butanol) [22, 23]. The results with regard to protection against alloxan (*n*-butanol or *n*-propanol > ethanol or methanol) are inconsistent with the peroxidatic activity of catalase but are in keeping with a reaction with ·OH radicals.

Urea and its analogues, hydroxyurea and thiourea, exhibited marked differences in their reactivity in the ·OH-detecting system (Fig. 4). Urea did not inhibit ethylene production and, therefore, cannot be considered an ·OH trapping agent. In addition, urea did not protect mice against alloxan (Table 2). Thiourea, on the other hand, has been shown by others [14] to be a good ·OH scavenger, and in our experiments it was an effective protective agent against alloxan-induced diabetes (Table 2). Thus, these results were qualitatively supportive of the point of view that the trapping of ·OH radicals provided protection against alloxan.

Hydroxyurea was an exception in that it appeared to exhibit moderate ability to trap ·OH (Fig. 4), but did not give statistically significant protection against alloxan (Table 2). However, although thiourea was decidedly superior to *n*-butanol as an ·OH scavenger, it was a less potent protective agent *in vivo* when the two compounds were compared at the same dosage (10^{-2} moles/kg). It may be that thiourea and other urea compounds are not as rapidly absorbed after i.p. injection or not taken up into the pancreatic β-cells as readily as the alcohols. From the relative potencies of hydroxyurea and thiourea *in vitro* (Fig. 4), it might be anticipated that a very much higher dose of hydroxyurea would be required *in vivo* to achieve a level of protection similar to that given by thiourea (Table 2).

One source of concern in the interpretation of these experiments was that an elevation in blood glucose levels might protect against the diabetogenic action of alloxan. However, thiourea failed to give any detectable elevation in blood glucose in a regimen which provided significant protection (Table 4 compared to Experiment B of Table 2). Additionally, in experiments with *n*-propanol (a protective agent) and urea (non-protective), there was no correlation between the rise in blood glucose and whether or not an animal was protected (Table 5). Therefore, blood glucose did not appear to be an important factor in these experiments.

Although direct evidence is lacking for the presence of hydroxyl radicals in the β-cell, the results of the current study tend to support the view that hydroxyl radicals play a primary role in the cytotoxic action of alloxan. None of the protective agents reacted with O_2^- and, with the exception of thiourea, none reacted directly with H_2O_2. Thiourea was, however, more reactive with ·OH than with H_2O_2. These data tend to exclude a direct reaction with either O_2^- or H_2O_2 as a basis for the protective actions of the compounds studied. Moreover, as noted earlier, the peroxidatic action of catalase can also be ruled out. On the other hand, the correlation between protective action *in vivo* and reaction with ·OH *in vitro* makes scavenging of ·OH a likely mechanism for protection by the aliphatic alcohols and thiourea.

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