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INHIBITION OF ALLOXAN-INDUCED HYPERGLYCAEMIA BY COMPOUNDS OF SIMILAR MOLECULAR STRUCTURE

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Abstract—In this study we have shown that a range of compounds that are structurally similar to alloxan are able to protect mice against the diabetogenic effect of alloxan. The compounds include a group of five barbiturates, a group of five hydantoins, the methylxanthines caffeine and theophylline, the related compound uric acid, and ethosuximide. They were injected intraperitoneally prior to intravenous injection of alloxan, and blood glucose concentration was used as an index of alloxan toxicity. The salient structural feature possessed by all of these protective compounds is a pair of carbonyl oxygen atoms separated by a distance of 4.5 Å and projecting from an approximately planar heterocyclic five- or six-membered ring; in all cases the carbonyl groups are separated by a ring nitrogen. We suggest that this feature is required for the protective effect of these compounds. In order to test further the requirement for two ring carbonyl groups, we also examined the effects of two compounds containing hydroxyl groups projecting from a six-membered ring, inositol and glucuronic acid. In agreement with previous studies on hexoses, we found that the effects of compounds such as these are unpredictable, with inositol protecting against alloxan toxicity but glucuronic acid not. We are unable to identify the critical difference in structure between these two compounds.

The diabetogenic action of alloxan was first discovered 50 years ago by Dunn *et al.* [1]. In normal, non-fasted animals, a characteristic triphasic pattern of blood glucose concentration is seen after a single injection of alloxan [2]. An initial hyperglycaemia is thought to be a consequence of a rapid block of insulin secretion from the β-cells, possibly combined with a direct glycogenolytic effect on the liver [3]. Hypoglycaemia follows, as the β-cells are destroyed and release their insulin content. Finally, a permanent hyperglycaemia is produced as a result of long-term insulin deficiency.

Early studies have shown that certain compounds are able to protect against the toxic effects of alloxan. For example, Battacharya [4] showed that glucose and, to a lesser extent, fructose and mannose inhibited the diabetogenic effect of alloxan. The non-metabolizable glucose analogue 3-*O*-methylglucose (3-OMG) was found to have a similar protective effect [5], whilst another analogue, mannoheptulose, removed the protective property of both glucose and 3-OMG without providing any protection itself [6]. Tomita *et al.* [7] used isolated perifused islets to demonstrate the protective effect of various hexoses against alloxan toxicity; glucose provided greater protection than 3-OMG, followed by mannose, 2-deoxyglucose, galactose and fructose. In an *in vivo* study in rats, Rossini *et al.* [8] found that 3-OMG provided even greater protection than glucose, and that mannoheptulose actually sensitized the β-cell to the toxic effect of alloxan in the fasting state, probably by inhibiting the protection provided by endogenous glucose. They also suggested that fructose and mannose give protection only as a result of their *in vivo* conversion to glucose.

As indicated above, most of the compounds that have been shown to protect against alloxan toxicity are hexoses, and it has been suggested that, in order to produce its effect, alloxan must at some stage bind to a site that normally recognizes glucose, such as the glucose transporter in the membrane of the β-cell [9]. There have, however, been reports of protection by other compounds. These compounds fall into two categories: those which are structurally similar to alloxan and those which are not. The first group includes 5,5′-diphenylhydantoin [10] and the methylxanthines caffeine and theophylline [11], while the latter group includes NADH and NADPH [11], the monoamine oxidase inhibitors nialamide and tranylcypromine [12, 13], and a series of alcohols [14].

The purpose of this study was to test the ability of a range of compounds that are structurally similar to alloxan to protect mice against alloxan toxicity, with a view to obtaining information about the molecular characteristics required for this effect. Most of the compounds studied are either of pharmacological interest (three of the barbiturates used have hypnotic properties, 5,5′-diphenylhydantoin and ethosuximide are anti-convulsants, and caffeine and theophylline are central nervous system stimulants and diuretics), or are physiologically significant (allantoin, uric acid, inositol and glucuronic acid).

MATERIALS AND METHODS

Drugs. All compounds used in these experiments were obtained from the Aldrich Chemical Co. (Gillingham, U.K.), except for alloxan monohydrate,

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ethosuximide, 5,5'-diethylbarbiturate and phenobarbitone, which were from the Sigma Chemical Co. (Poole, U.K.).

Test for protection against alloxan toxicity. Male albino mice (30–35 g) were used in all experiments. Alloxan monohydrate was dissolved in 0.9% saline at pH 5.0 and used within 20 min. Alloxan was injected via a tail vein, at the maximally effective dose of 60 mg/kg in 0.1 mL of solution. All other compounds were injected intraperitoneally, again in 0.9% saline. Where possible, the volume used was 0.1 mL, although solubility problems in some cases necessitated the use of volumes up to 1 mL. Blood samples (0.1 mL) were collected by cardiac puncture and immediately deproteinized by addition of 1.0 mL uranyl acetate solution followed by centrifugation at 4000 rpm for 5 min in a bench centrifuge. A sample (0.1 mL) of the supernatant was taken for determination of glucose concentration, using glucose oxidase and peroxidase and the ammonium salt of 2,2'-azino-di-(3-ethyl-benzothiazoline-6-sulphonic acid) as a redox indicator, as described by Werner *et al.* [15].

In early experiments, the compound under test was administered 1 hr before the alloxan injection, and blood glucose was assayed after a further 48 hr (i.e. during the second hyperglycaemic phase [2]). The results for three compounds (5,5'-diphenylhydantoin, caffeine and uric acid) were obtained at this time-point. In preliminary experiments with the hypnotic barbiturates, however, the survival of the animals over this period was poor. Consequently, it was decided to use the first hyperglycaemic phase of the alloxan response and assay blood glucose 45 min after the alloxan injection [2]. Since this procedure proved convenient, it was used for the remaining compounds, with a pre-injection 15 min prior to the alloxan injection. Alloxan is known to be unstable, with a half-time *in vivo* of the order of minutes [3]. Consequently, the triphasic blood glucose response following a single alloxan injection must be a result of a short-lived effect of this agent. It is reasonable to expect, therefore, that although the mechanisms underlying the two hyperglycaemic phases are different, both should be inhibited by a compound that is able to block the initial effect of the alloxan.

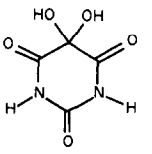
Results are expressed as means ± standard deviations (SD). Differences between groups were assessed for statistical significance by Student's unpaired *t*-test.

RESULTS

The effects of alloxan on blood glucose concentration at the two time-points used in these experiments are shown in Table 1. It can be seen that glucose concentration is significantly elevated at both 45 min and 48 hr post-injection. Tables 2–5 show the effects on alloxan-induced elevation of blood glucose concentration of pre-treatment of the mice with the various compounds under study. Two groups of five compounds, the barbiturates (Table 2) and the hydantoins (Table 3), provided significant protection against alloxan toxicity. Only relatively low doses of the hypnotic barbiturates (5,5'-diethylbarbiturate, phenobarbitone and 1'-methylphenobarbitone) were tolerated, higher doses proving lethal. Consequently, the significance of the results obtained with these drugs is lower than that of the results obtained with barbituric acid and thiobarbituric acid, which are non-hypnotic and could therefore be given at a higher dose. Doses of the majority of the other compounds used in this study were in the same range as those of the barbiturates. In addition to the above two groups, five other compounds were shown to protect against the diabetogenic effect of alloxan. The results obtained with four of these compounds (the methylxanthines caffeine and theophylline, the related compound uric acid, and ethosuximide) are shown in Table 4. In addition, we examined the effects of two hexose analogues, inositol and glucuronic acid. It can be seen (Table 5) that the former compound provided protection but that the latter did not.

None of the compounds tested had any significant effect on blood glucose concentration when injected alone (data not shown). The compounds were also screened for any effect on the glucose assay reaction, at a concentration equal to the amount injected intraperitoneally in 2.4 mL (i.e. the blood volume of a mouse). This concentration represents the maximum possible concentration of the compound in the blood after injection. At this concentration, only uric acid had any effect on the reaction, causing a significant inhibition. The concentration of uric acid in the blood 48 hr after injection will, of course, be much lower than the concentration tested. Furthermore, the blood glucose concentration determined 48 hr after administration of uric acid alone was not significantly different from the control, which suggests that the concentration of uric acid in the blood at this time was not sufficient to inhibit the assay reaction.

Table 1. Effect of alloxan on blood glucose concentration



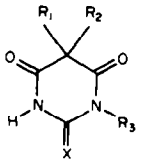
Condition	Time	Blood glucose concentration (mg/100 mL)
Control	—	106 ± 29 (5)
Alloxan	45 min	355 ± 85 (22)*
Alloxan	48 hr	432 ± 121 (9)*

Alloxan (60 mg/kg) was injected via a tail vein, and blood glucose concentration was determined at the time indicated.

Values are means ± SD, for the numbers of animals shown in parentheses.

Asterisks indicate that blood glucose concentration is significantly higher in the treated group than in the control group (*$P < 0.001$).

Table 2. Protection against alloxan-induced hyperglycaemia by barbiturates



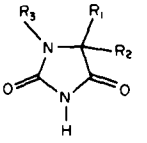
Compound	R_1	R_2	R_3	X	Dose (mg/kg)	Blood glucose concentration (mg/100 mL)
Barbituric acid	H	H	H	O	320	158 ± 39 (6)‡
Thiobarbituric acid	H	H	H	S	300	179 ± 39 (12)‡
5,5'-Diethylbarbiturate	C_2H_5	C_2H_5	H	O	160	256 ± 71 (6)*
Phenobarbitone	C_2H_5	C_6H_5	H	O	120	228 ± 25 (6)†
1-Methyl-phenobarbitone	C_2H_5	C_6H_5	CH_3	O	160	219 ± 93 (5)†

Compounds were injected intraperitoneally and alloxan (60 mg/kg) was injected via a tail vein 15 min later. Blood glucose concentration was determined after a further 45 min.

Values are means ± SD, for the numbers of animals shown in parentheses.

Symbols indicate that blood glucose concentration is significantly lower than in animals treated with alloxan alone (*$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$).

Table 3. Protection against alloxan-induced hyperglycaemia by hydantoins



Compound	R_1	R_2	R_3	Dose (mg/kg)	Blood glucose concentration (mg/100 mL)
5,5'-Diphenylhydantoin*	C_6H_5	C_6H_5	H	80	172 ± 46 (9)§
5-Methyl-5'-phenylhydantoin	CH_3	C_6H_5	H	160	198 ± 55 (6)§
5,5'-Dimethylhydantoin	CH_3	CH_3	H	160	247 ± 64 (6)‡
1-Methylhydantoin	H	H	CH_3	160	231 ± 46 (6)‡
Allantoin	NH_2	H	H	300	276 ± 58 (6)†
	|				
	CO				
	|				
	NH—				

Compounds were injected intraperitoneally and alloxan (60 mg/kg) was injected via a tail vein, usually 15 min later. Blood glucose concentration was usually determined after a further 45 min.

* 5,5'-Diphenylhydantoin was administered 1 hr prior to alloxan, and blood glucose concentration was determined 48 hr later.

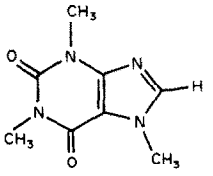
Values are means ± SD, for the numbers of animals shown in parentheses.

Symbols indicate that blood glucose concentration is significantly lower than in animals treated with alloxan alone (†$P < 0.05$, ‡$P < 0.01$, §$P < 0.001$).

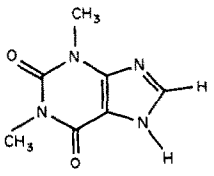
DISCUSSION

In this study the observation made by Mennear and Gossel [10] that 5,5'-diphenylhydantoin protects against alloxan toxicity in the mouse has been confirmed, and protection by caffeine and theophylline has been shown to occur *in vivo*, as well as in isolated islets [11]. In addition, 12 other compounds that share structural features with alloxan have been shown to inhibit its diabetogenic effect. The *in vivo* system used here inevitably introduces complications with regard to the absorption, distribution and metabolism of the compounds used. It would undoubtedly have been preferable, for example, to administer both the test compound and alloxan by the intravenous route. We decided to give the test compound intraperitoneally because we could not be confident of reliably performing two intravenous injections into each mouse. In fact, some attempts at intravenous injection of alloxan were unsuccessful, so that some mice had to be excluded from the study. No meaningful data on relative potencies

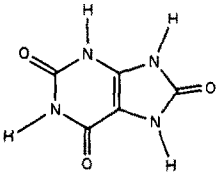
Table 4. Protection against alloxan-induced hyperglycaemia by other compounds of similar structure



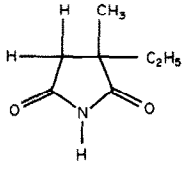
Caffeine



Theophylline



Uric acid



Ethosuximide

Compound	Dose (mg/kg)	Blood glucose concentration (mg/100 mL)
Caffeine*	80	218 ± 129 (12)‡
Theophylline	160	224 ± 64 (5)†
Uric acid*	160	150 ± 44 (6)‡
Ethosuximide	300	216 ± 52 (6)‡

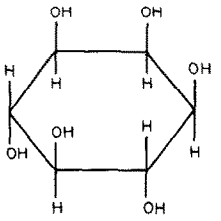
Theophylline and ethosuximide were injected intraperitoneally 15 min before intravenous injection of alloxan (60 mg/kg), and blood glucose concentration was determined after a further 45 min.

*Caffeine and uric acid were administered 1 hr prior to alloxan, and blood glucose concentration was determined 48 hr later.

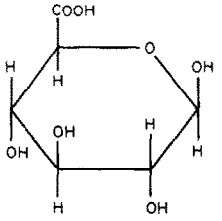
Values are means ± SD, for the numbers of animals shown in parentheses.

Symbols indicate that blood glucose concentration is significantly lower than in animals treated with alloxan alone (†$P < 0.01$, ‡$P < 0.001$).

Table 5. Effects of other compounds on alloxan-induced hyperglycaemia



Inositol



β-D-Glucuronic acid

Compound	Dose (g/kg)	Blood glucose concentration (mg/100 mL)
Inositol	4	160 ± 33 (9)*
Glucuronic acid	4	308 ± 62 (11)

Compounds were injected intraperitoneally and alloxan (60 mg/kg) was injected via a tail vein 15 min later. Blood glucose concentration was determined after a further 45 min.

Values are means ± SD, for the numbers of animals shown in parentheses.

Asterisk indicates that blood glucose concentration is significantly lower than in animals treated with alloxan alone (*$P < 0.001$).

could have been obtained in this study, therefore, and a strict structure–activity study was impossible. Nevertheless, the finding that this group of compounds, that have diverse actions of their own, share the ability to block the effect of alloxan is of considerable interest.

Almost all of the compounds used in this study are similar in structure to each other and to alloxan. The molecules are approximately planar five- or six-membered heterocyclic rings bearing carbonyl oxygen atoms. Analysis of the crystal structure of alloxan [16] revealed that the three carbonyl oxygen atoms in its six-membered ring are separated by distances of between 4.50 and 4.75 Å. The barbiturates also have a six-membered ring bearing three carbonyl oxygens (or, in the case of thiobarbituric acid, two oxygens and one sulphur), which have similar spacings to those in alloxan. The hydantoins have a five-membered ring with two carbonyl oxygens, again separated by about the same distance. The methylxanthines caffeine and theophylline have two carbonyl oxygens with similar spacing, while the related compound uric acid has three carbonyl oxygens, but only two with the typical spacing. Finally, ethosuximide has a five-membered ring, again containing two appropriately spaced carbonyl oxygens; it bears a resemblance to the hydantoins. The feature common to all of these molecules is the presence of two negatively charged carbonyl oxygen atoms separated by about 4.5 Å and projecting from an approximately planar ring; further, the carbonyl groups are in all cases separated by a ring nitrogen. It is reasonable to propose, therefore, that this feature is required for the protective effect of these compounds.

The cytotoxicity of alloxan appears to involve the generation of hydrogen peroxide, superoxide anion radicals and hydroxyl radicals [11, 14, 17, 18]. Two additional features coincide to render the β-cell uniquely sensitive to the toxic actions of alloxan: a rapid cellular uptake of the drug, which is also seen in the liver, and a low activity of glutathione peroxidase, an enzyme that catalyses the reduction of peroxides [19]. The ability of glucose to protect β-cells from damage by another generator of peroxide radicals, *tert*-butyl-hydroperoxide, which enters cells non-specifically [19], suggests that at least part of its protective effect against alloxan is a consequence of intracellular effects, such as an increase in the rate of generation of reducing equivalents [20]. It has also been pointed out that the protective effect of methylxanthines, NADH and NADPH, and *n*-alkanols may be a consequence of their ability to scavenge hydroxyl radicals [11, 14]. On the other hand, there are also clear correlations between the uptake of alloxan and glucose, which indicate a possible interaction at the plasma membrane. For example, alloxan is taken up only slowly by muscle cells, in which glucose transport is a rate-limiting step in metabolism [19]. Further evidence for an effect of alloxan at the plasma membrane was provided by Dean and Matthews [21], who showed

that alloxan caused a rapid depolarization of the β-cell membrane. Recently, Kozlowski and Ashford [22] showed that alloxan and a group of barbiturates were all able to inhibit ATP-sensitive potassium channel activity in patch-clamped membranes from CRI-G1 insulin-secreting cells, and speculated that this channel may be at least one target of alloxan *in vivo*.

The mechanism by which the compounds used in this study protect against alloxan-induced diabetes, therefore, is still not clear. A more extensive survey of the structural features required for protection may provide further information. The rationale behind the use of inositol and glucuronic acid in this study was that they both contained hydroxyl groups, rather than carbonyl groups, projecting from ring structures. It was found that only inositol protected against the diabetogenic effect of alloxan. The unpredictability of the effect of hexoses has been discussed above, and we are not convinced that our results with these two compounds provide much information about the structural features required for protection, or even that inositol is acting by the same mechanism as the main group of compounds. In addition to having hydroxyl groups in place of carbonyl groups, of course, inositol does not have a planar ring, which is a further feature of the other protective agents. It might have been more revealing to have tested compounds having a flat ring but, say, only a single carbonyl group. It would also be helpful to know whether the "two-carbonyl" motif must be present in a ring structure, and whether it is essential for the carbonyl groups to be separated by a nitrogen. Finally, it would be interesting to test whether any of the compounds other than the barbiturates have any effect on the ATP-sensitive potassium channel in CRI-G1 cells. These approaches might shed more light on the mechanism underlying the protective effect.

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