

Mechanism of Barbituric-acid Protection Against Inhibition by Alloxan of Glucose-induced Insulin Release

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SUMMARY

Isolated rat islets were maintained in a simple static incubation system and were exposed to alloxan for a period of five minutes. Alloxan inhibited subsequent glucose-induced insulin release in a dose-dependent manner at 37° C., with 650 μ M alloxan producing 94 per cent inhibition of insulin release. Barbituric acid, a compound structurally related to alloxan, provided complete protection (at 37° C.) against this inhibition of insulin release when present during the alloxan exposure. At 23° C., barbituric acid was shown to be absent from the intracellular space of the islet yet still protected completely against alloxan inhibition of insulin release. Thus, barbituric acid apparently provided protection against alloxan in the extracellular medium. By fluorometric and chromatographic analyses, it was determined that barbituric acid reacted rapidly with alloxan to produce a new compound. These findings indicate that barbituric acid protected against alloxan by a chemical reaction in the medium. *DIABETES* 27:71-77, February, 1978.

Previous in-vitro studies have shown that a five-minute exposure of isolated rat islets to alloxan inhibits subsequent glucose-induced insulin release.¹ The concomitant presence of high concentrations of either D-glucose, D-mannose, or 3-O-methyl-D-glucose during the alloxan exposure protects the islets against the inhibitory effect of alloxan on glucose-induced insulin release. This hexose protection shows stereochemical preference for the alpha anomer of D-glucose, which is a better protective agent than the beta anomer at low concentrations.² These same hexoses have been observed to protect against the diabetogenic effects of alloxan when administered be-

fore alloxan in vivo.³⁻⁶ The specificity of the hexose protection supports the hypothesis that alloxan may interact with a receptor for D-glucose and that alloxan may serve as a direct probe for establishing the presence of glucose receptors on the beta-cell membrane.

Barbituric acid, a compound that shares certain structural similarities with alloxan (figure 1), has been shown in vivo to provide protection against the diabetogenic effects of alloxan.⁷ Because of this structural relationship, both Martinez⁷ and Webb⁸ suggested that barbituric acid may compete with alloxan for a specific structural site.

In preliminary studies with isolated rat islets, we found that barbituric acid protected against alloxan inhibition of glucose-induced insulin release.⁹ The purpose of the present study was to determine the dose dependency of this protection, the direct effect of barbituric acid on insulin release, the uptake of barbituric acid by isolated rat islets, and the mechanism of barbituric acid protection against alloxan.

MATERIALS AND METHODS

Medium and chemicals. All incubations were accomplished with a modified Krebs-Ringer bicarbonate medium containing 115 mM NaCl, 24 mM NaHCO₃, 5.0 mM KCl, 2.5 mM CaCl₂, albumin (0.5 per cent wt./vol., bovine plasma albumin, Armour Pharmaceutical, Chicago) and, as required, alloxan monohydrate (Sigma Chemical, St. Louis), barbituric acid (Eastman Chemical, Rochester, N.Y.), D-glucose (dextrose, National Bureau of Standards, Washington, D.C.), (³H) sucrose (New England Nuclear, Boston), and (2-¹⁴C) alloxan and (2-¹⁴C) bar-

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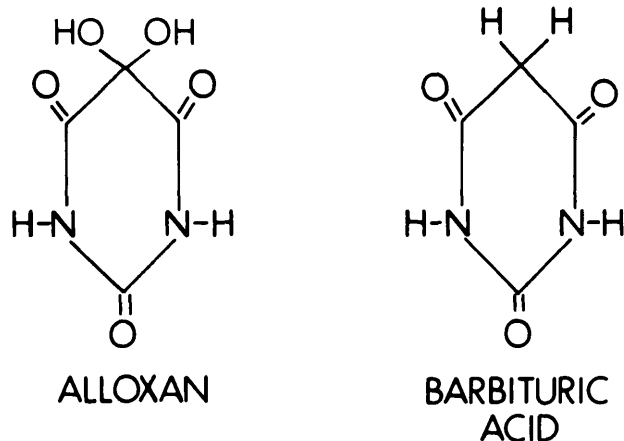


FIG. 1. Chemical structures of alloxan (hydrated form) and barbituric acid.

bituric acid (California Bionuclear, Sun Valley, Calif.). Barbituric acid was recrystallized from water and neutralized with NaOH. The medium was equilibrated to pH 7.4 with a mixture of oxygen/carbon dioxide (95 per cent/5 per cent).

In-vitro determination of insulin release. Pancreatic islets were isolated from the pancreas of Wistar rats (200-300 gm.) by the collagenase technique.^{10,11} Islets were placed in a glass vial (11 mm. inner diameter \times 20 mm. high) that contained 200 μ l. of medium. Each vial contained 20-25 islets, and 8-10 vials were used for each experiment. This medium was removed and glucose was added with the aid of a micropipet and a dissection microscope. The glass vial was inserted in a scintillation vial equipped with rubber stoppers, gassed with oxygen/carbon dioxide (95 per cent/5 per cent), and shaken in a Dubnoff metabolic shaker (100 strokes per minute). In general, the islets were preincubated for 20 minutes in 200 μ l. of glucose-free medium and stimulated with 200 μ l. of D-glucose (27.5 mM) medium for 30 minutes at 37° C. Islets were exposed to alloxan in the following manner: the preincubation medium was removed and replaced with 200 μ l. of freshly mixed medium containing alloxan. After five minutes, the alloxan medium was removed and replaced with 200 μ l. of D-glucose (27.5 mM) medium for 30 minutes. Insulin release from alloxan-treated islets was compared with that from parallel groups of untreated islets. At the end of the stimulation period, the media were removed and frozen for subsequent insulin assay by the method of Wright et al.¹² Results are expressed as microunits of insulin per islet per minute.

Barbituric acid uptake. The uptake of (2-¹⁴C) barbituric acid into pancreatic islets was determined by a dual-isotope procedure with (³H) sucrose as the ex-

tracellular marker. This procedure is based on the work of Hellman et al.¹³ and has been described previously.¹⁴ Approximately 25 islets were placed in glass vials (see above) and preincubated for 20 minutes in 200 μ l. of glucose-free medium at a temperature identical to the subsequent incubation. The medium was then removed and replaced with 100 μ l. of glucose-free medium containing (2-¹⁴C) barbituric acid (20 mM, 0.56 mCi./mmol.) and (³H) sucrose (20 mM, 1.5 mCi./mmol.). At the end of the incubation period, the medium was removed and the vial containing the islets was frozen in liquid nitrogen. The frozen vial was placed in a precooled lyophilization tube¹⁵ and lyophilized (-40° C., $p < 0.01$ mm. Hg) for 18 to 36 hours. The lyophilized islets were transferred to a scintillation vial, digested with 100 μ l. of Hyamine-10X (Packard), and then counted with a liquid scintillation spectrometer. The number of counts per minute were equated to the compound amount by comparison with external standards consisting of 5 μ l. of medium dissolved in 100 μ l. Hyamine-10X. The cellular uptake of barbituric acid was calculated by the method of Bloom et al.,¹⁶ and sample calculations have been shown previously.¹⁷

Alloxan assay. A stock solution of alloxan was prepared in 1 mM HCl and measured in a Zeiss spectrophotometer at 270 nm ($\epsilon = 980 \text{ M}^{-1} \text{ cm.}^{-1}$) in 50 mM phosphate buffer (pH 7.4) by the method of Patterson et al.¹⁸ Since alloxan decomposed rapidly at pH 7.4 and 37° C., a portion of the stock solution (54 mM) was added to previously warmed and gassed medium within 10 seconds of the medium's use.

The interaction between alloxan and barbituric acid was examined by two methods. The decomposition of alloxan (650 μ M) in the presence and absence of barbituric acid was measured fluorometrically by the method of Bilic and Felber.¹⁹ Alloxan was added to 10 ml. of Krebs-Ringer medium containing barbituric acid (1, 5, and 20 mM) at 37° C. and pH 7.4. Portions (4-20 μ l.) of the reaction mixture were removed at 0.5-minute intervals and added to 1 ml. of 1,2-phenylenediamine (276 μ M) in citrate buffer, pH 4.0. After one hour's incubation, the amount of product formed between alloxan and 1,2-phenylenediamine was measured fluorometrically. Alloxan concentration in the medium was calculated by comparison with the amount of fluorescence produced from known standards of alloxan (final concentrations 0.3-3.0 μ M) incubated in the 1,2-phenylenediamine buffer. The presence of barbituric acid in the 1,2-phenylene-diamine buffer did not alter the amount of fluorescence produced in the assay.

Thin-layer chromatography (TLC) was used to monitor the reaction between alloxan and barbituric acid in the following manner: ($2\text{-}^{14}\text{C}$) alloxan ($650\ \mu\text{M}$) and barbituric acid ($20\ \text{mM}$) were incubated in Krebs-Ringer medium for five minutes at $37^\circ\ \text{C}$. A $5\text{-}\mu\text{l}$. sample of the reaction mixture was examined by thin-layer chromatography (TLC) on 0.25-mm . silica gel F plates (New England Nuclear) in a solvent system of *n*-butanol, water, and glacial acetic acid (volume ratios of 4, 2, and 1). The plates were scanned for radioactivity by a Packard Radiochromatography Scanner (model 7200). Parallel standards of unlabeled alloxan and decomposed alloxan were placed on separate sections of each TLC plate. Sections of the silica gel were suspended in Instagel (Packard) and counted in a liquid scintillation counter for final quantitation.

Statistics. The results from the alloxan dose-response (figure 2) study were fitted to the sigmoidal equation $y = A/[1 - B \exp(-kt)]$ by logarithmic transformation and least-squares linear analysis.²⁰ The uptake of barbituric acid (figure 4) was fitted to the inverted exponential equations $y = A[1 - \exp(-kt)]$ by an iterative nonlinear least-squares method.²¹ The decomposition of alloxan (figure 5) was fitted by an iterative nonlinear least squares method to the equation $y = A \exp(-kt) + C$, where C was the alloxan concentration remaining in the medium after 10 minutes.²¹ Statistical analysis was performed with the un-

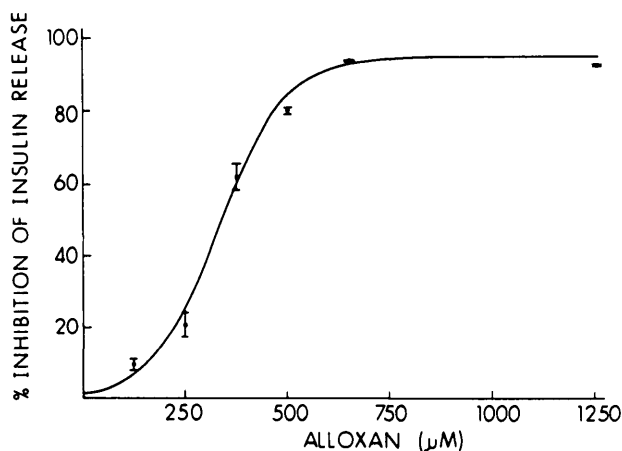


FIG. 2. The effect of alloxan concentration on glucose-induced insulin release. Islets in the experimental group were exposed to alloxan for five minutes at $37^\circ\ \text{C}$., the medium was removed, and the islets were then incubated in glucose ($27.5\ \text{mM}$) medium for 30 minutes at $37^\circ\ \text{C}$. The insulin release from the alloxan-treated islets was compared with that from parallel, untreated groups of islets (mean rate, $3.99\ \mu\text{U}$. per islet per minute). Mean \pm S.E.M. $N=8-12$. The curve was machine-generated from least-squares analysis ($r = 0.9903$). Per cent inhibition = [(untreated rate minus alloxan-treated rate) divided by (untreated rate)] times 100 per cent.

paired Student's *t*-test, and results were expressed as Mean \pm S.E.M., with the number of observations indicated in parentheses.

RESULTS

Alloxan inhibition of insulin release. The effect of different alloxan concentrations on subsequent glucose-induced insulin release from statically incubated islets is shown in figure 2. A five-minute exposure to alloxan ($125\text{-}1,250\ \mu\text{M}$) in the absence of glucose produced a 9 to 93 per cent inhibition of insulin release compared with that from parallel groups of untreated islets. A sigmoidal fit of the results was used to estimate the inhibitor K_i of alloxan (the concentration at one-half maximal inhibition) as $330\ \mu\text{M}$. Since $650\ \mu\text{M}$ was the minimal concentration of alloxan required to produce maximal inhibition (94 per cent), this concentration was chosen for all subsequent experiments.

Alloxan decomposes rapidly at pH 7.4 and $37^\circ\ \text{C}$. to a nondiabetogenic product.⁸ To determine the effects of the decomposition product on insulin release, islets in the experimental group were exposed to $1.25\ \text{mM}$ alloxan that had been allowed to decompose in the medium for 10 minutes at $37^\circ\ \text{C}$. After a five-minute exposure to the decomposition product, the islets were incubated in fresh medium containing glucose ($27.5\ \text{mM}$), as in figure 2. The insulin release as expressed in microunits per islet per minute was 3.78 ± 0.23 ($n=12$) from the untreated islets, as against 3.94 ± 0.09 ($n=11$) from islets exposed to the alloxan-decomposition product ($p>0.4$).

Protection by barbituric acid at $37^\circ\ \text{C}$. The ability of barbituric acid to affect alloxan inhibition of glucose-induced insulin release was examined by exposing islets to alloxan ($650\ \mu\text{M}$) in the presence of barbituric acid ($1, 5, 10,$ and $20\ \text{mM}$). Barbituric acid protected the islets against alloxan inhibition of subsequent glucose-induced insulin release in a dose-dependent manner, with 99 per cent protection of insulin release provided by $20\ \text{mM}$ barbituric acid (figure 3). Table 1 shows the effect of barbituric acid alone on glucose-induced insulin release. Islets that were pulsed for five minutes with barbituric acid ($10, 20\ \text{mM}$) showed no alteration of subsequent glucose-induced insulin release. In addition, barbituric acid ($5, 20\ \text{mM}$), when present with glucose ($16.5\ \text{mM}$) during the incubation period, produced no significant alteration in insulin release.

Barbituric acid uptake at $23^\circ\ \text{C}$. and $37^\circ\ \text{C}$. To determine the site of protection of barbituric acid, the uptake into islets of ($2\text{-}^{14}\text{C}$) barbituric acid ($20\ \text{mM}$)

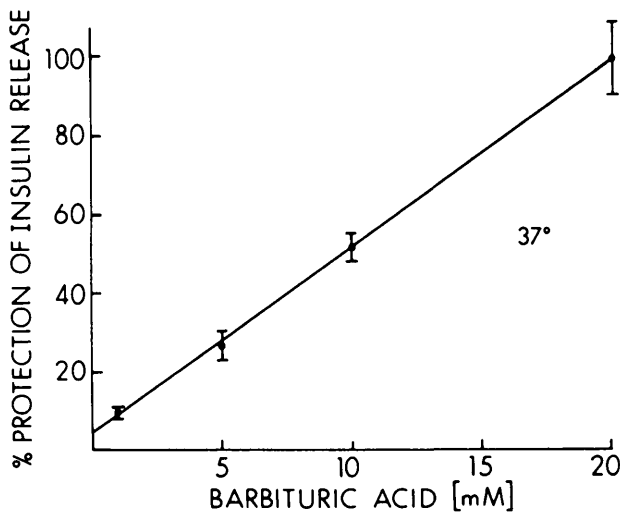


FIG. 3. Barbituric acid protection against alloxan inhibition of glucose-induced insulin release. Islets were exposed to alloxan (650 μ M) for five minutes at 37° C. in the presence of barbituric acid and then stimulated with glucose medium as in figure 2. Insulin release from alloxan-treated islets was compared with that from parallel groups of untreated islets (mean rate, 2.84 μ U. per islet per minute). Mean \pm S.E.M. The line was drawn from linear least-squares analysis ($r = 0.9997$). $N=9-15$. Per cent protection = [(alloxan-treated rate minus basal rate) divided by (untreated rate minus basal rate)] times 100 per cent.

was measured in the static incubation system (figure 4). At 37° C. there was a slow uptake of barbituric acid into the intracellular space of the islet, reaching 16.2 ± 1.3 picomoles per islet (0.81 nanoliters per islet) after 50 minutes of incubation. Based on an intracellular islet volume of approximately 3.5 nanoliters,¹⁷ this final value at 37° C. was equal to the presence of barbituric acid in about 23 per cent of the intracellular space. At 23° C., however, there was no detectable uptake of barbituric acid into the intracellular space of the islet during the 50-minute period of incubation.

Protection by barbituric acid at 23° C. The ability of barbituric acid to protect against alloxan was examined under the conditions when it was absent from the intracellular space. In these studies, islets were exposed to alloxan at 23° C. for five minutes in the presence or absence of barbituric acid (20 mM), washed at 23° C. to remove any residual alloxan, and then raised to 37° C. and stimulated with glucose (27.5 mM) medium. Table 2 shows that a five-minute exposure to alloxan (650 μ M) at 23° C. inhibited subsequent glucose-induced insulin release by 94 per cent ($p < 0.001$). Barbituric acid (20 mM), when present during the alloxan exposure at 23° C., provided 98 per cent protection against the inhibition of

TABLE 1

Effect of barbituric acid on glucose-induced insulin release

Experimental conditions	Insulin release (μ U./islet/min.)
Control*	2.08 \pm 0.17 (10)
10 mM B.A. pulse	1.92 \pm 0.18 (10)
Control*	2.43 \pm 0.33 (8)
20 mM B.A. pulse	2.23 \pm 0.28 (9)
Control†	1.93 \pm 0.18 (12)
5 mM B.A. incubation	2.01 \pm 0.23 (11)
20 mM B.A. incubation	2.47 \pm 0.20 (12)

All values N.S.

*Islets were pulsed for five minutes in the presence or absence of barbituric acid (B.A.). The medium was then removed and the islets were incubated in glucose (27.5 mM) medium for 30 minutes at 37° C.

†Islets were incubated in glucose (16.5 mM) medium for 30 minutes at 37° C. in the presence or absence of barbituric acid. Mean \pm S.E.M. Number of individual observations in parentheses.

glucose-induced insulin release. Thus, barbituric acid could protect against alloxan when absent from the intracellular space of the islet.

Alloxan assay. Martinez⁷ suggested originally that the protection by barbituric acid may result from a chemical reaction between alloxan and barbituric acid. In our initial spectrophotometric studies, we could not detect a change in the rate of the decomposition of alloxan in the presence of barbituric acid.⁹ These studies, however, were limited to low concentrations (100 μ M each) of alloxan and barbituric acid by the intense absorption spectrum of barbituric acid. The interaction between alloxan and barbituric acid was reexamined with higher concentrations of each agent by fluorometric and chromatographic methods.

Alloxan (650 μ M) decomposition in Krebs-Ringer medium at pH 7.4 and 37° C. in the presence and absence of barbituric acid was monitored by the 1,2-phenylenediamine assay of Bilic and Felber.¹⁹ This assay allowed for the determination of alloxan con-

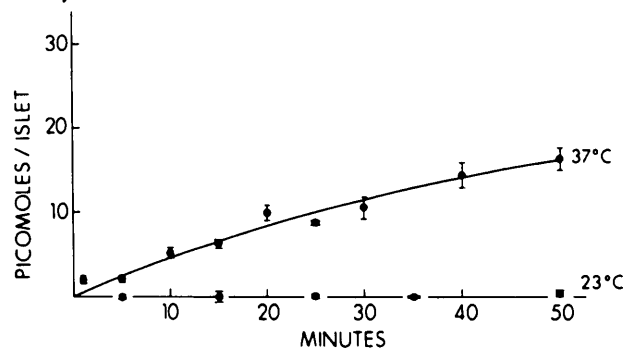


FIG. 4. Time study of barbituric acid uptake. Islets were incubated for the indicated intervals in 100 μ l. of medium containing ($^{2-14}$ C) barbituric acid (20 mM, 0.56 mCi./mmol) and (3 H) sucrose (20 mM, 1.5 mCi./mmol) at 37° C. or 23° C. Curve was machine-generated from least-squares analysis. $N=4-6$.

TABLE 2

Barbituric acid protection at 23° C. against alloxan inhibition of insulin release

Exposure	Conditions (23° C.)		Glucose-induced insulin release (37° C.) (μ U./islet/min.)
	Alloxan	B.A.	
Control	-	-	2.72 \pm 0.21 (11)
Experimental	+	-	0.18 \pm 0.03 (12)*
Control	-	-	2.54 \pm 0.19 (11)
Experimental	+	+	2.52 \pm 0.40 (12)†

* $p < 0.001$.

†N.S.

The experimental group of islets was exposed for five minutes to alloxan (650 μ M) in the presence or absence of barbituric acid (20 mM) at 23° C. The islets were washed with glucose-free medium for five minutes at 23° C., preincubated in the same medium for 20 minutes at 37° C., and stimulated with glucose (27.5 mM) medium for 30 minutes at 37° C. Mean \pm S.E.M. Number of individual observations in parentheses.

centration in the medium by the fluorometric measurement of the product formed between alloxan and 1,2-phenylenediamine. In the absence of barbituric acid, alloxan (650 μ M) decomposed rapidly ($t_{1/2}$ 1.38 minutes); in the presence of barbituric acid, however, the decomposition of alloxan was altered markedly in a dose-dependent manner (figure 5). After 10 minutes in the presence of 1, 5, and 20 mM barbituric acid, the amount of fluorescence produced in the subsequent assay was equivalent to 120 \pm 12, 464 \pm 20, and 652 \pm 16 μ M alloxan ($n=4$ to 7), respectively. It appeared, therefore, that barbituric acid reacted rapidly with alloxan in such a way as to inhibit the apparent decomposition of alloxan.

The mechanism of this apparent inhibition of alloxan decomposition was examined by TLC using (14 C) alloxan. Most (86 per cent) of the total activity

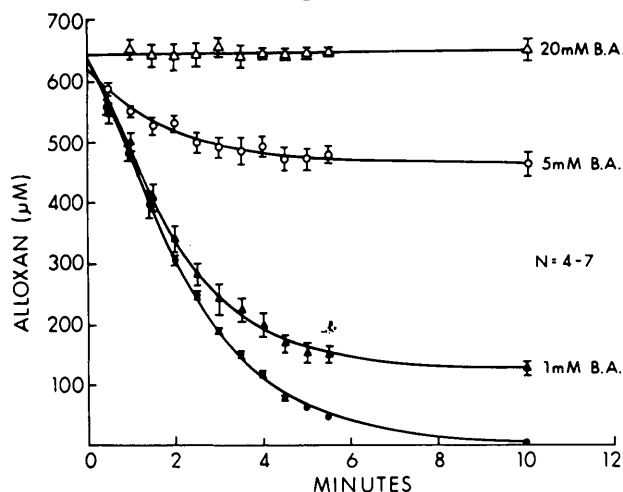


FIG. 5. The decomposition of alloxan (650 μ M) in the presence (Δ , \circ , \square) and absence (\bullet) of barbituric acid (B.A.). Alloxan was added to 10 ml. of Krebs-Ringer medium with and without barbituric acid (1, 5, and 20 mM) at pH 7.4 and 37° C. Portions (4-20 μ l.) of the reaction mixture were removed at 0.5-minute intervals and assayed fluorometrically for alloxan (see MATERIALS AND METHODS). Mean \pm S.E.M. The curves were machine-generated from least-squares analysis.

associated with (14 C) alloxan migrated rapidly ($R_f=0.7$, figure 6a). When (14 C) alloxan (650 μ M) was incubated in the medium for five minutes at 37° C., 93 per cent of the total activity co-migrated with the parallel standard of decomposed alloxan ($R_f=0.14$, figure 6b). However, when barbituric acid (20 mM) was present with (14 C) alloxan in the medium, a new peak was observed, which accounted for 86 per cent of the total activity ($R_f=0.53$, figure 6c). Under these conditions, less than 2 per cent of the

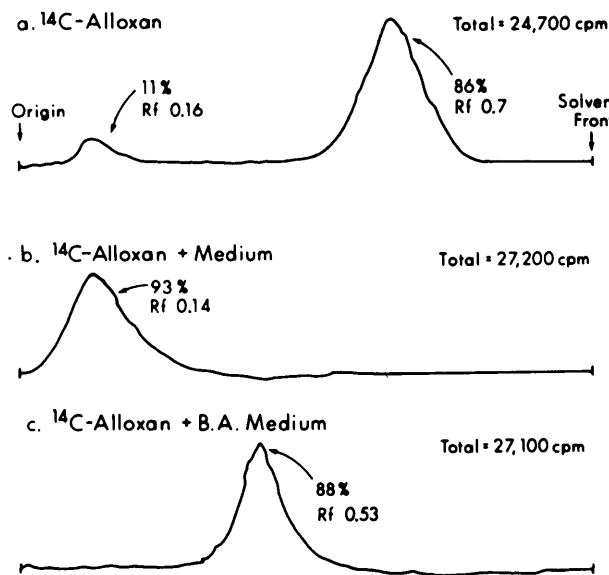


FIG. 6. The reaction between (14 C) alloxan (650 μ M, 3.0 mCi/mmol) and 20 mM barbituric acid (B.A.). Shown are tracings of radiochromatograms from thin-layer chromatographic (TLC) plates that contained the following: (a) 5 μ l. of (14 C) alloxan (650 μ M) in 1 mM HCl, (b) 5 μ l. of (14 C) alloxan incubated for five minutes at 37° C. and pH 7.4 in 100 μ l. of Krebs-Ringer medium, (c) 5 μ l. of (14 C) alloxan incubated for five minutes at 37° C. in 100 μ l. of medium supplemented with 20 mM barbituric acid. Final quantitation of the radioactivity associated with each plate was obtained by liquid scintillation counting of sections of the TLC plate.

total activity co-migrated with the parallel standards of alloxan or decomposed alloxan. The addition of barbituric acid (20 mM) to previously decomposed (2-¹⁴C) alloxan medium did not affect the migration of the decomposition product (Rf=0.14). It appeared, therefore, that barbituric acid reacted with (2-¹⁴C) alloxan in the medium to produce a new migratory product.

DISCUSSION

The rapid decomposition of alloxan in the medium must be considered in the calculation of the effective concentration of alloxan required to inhibit glucose-induced insulin release. In previous perfusion studies, we found that 1,250 μ M alloxan was required to produce more than 90 per cent inhibition of insulin release;¹ since the perfusion system had a 1-1.5-minute delay between the addition of alloxan to the medium and the medium's appearance around the islets, the effective concentration of alloxan was not known. In the static incubation system used here, the effective concentration of alloxan was determined precisely by the addition of alloxan medium directly to the islets. Under these conditions, a five-minute exposure of alloxan inhibited insulin release in a dose-dependent manner, and 650 μ M alloxan was the minimal concentration to produce maximal (94 per cent) inhibition of insulin release.

Barbituric acid, a compound that shares some structural similarity with alloxan, had no detectable effect on glucose-induced insulin release from isolated rat islets. The presence of barbituric acid during the alloxan exposure at 37° C., however, protected against alloxan inhibition of insulin release in a dose-dependent manner, with 20 mM barbituric acid providing 99 per cent protection. At 23° C., barbituric acid (20 mM) was absent from the intracellular space of the islet yet provided 98 per cent protection against alloxan inhibition of insulin release. These results are in agreement with in-vivo experiments that showed that barbituric acid protected against the diabetogenic effect of alloxan.⁷ To explain these in-vivo findings, Webb⁸ suggested that barbituric acid may have competed with alloxan for a complementary site. Present studies on the chemical interaction between alloxan and barbituric acid in the incubation medium do not support a competitive mechanism.

Evidence of a chemical interaction between alloxan and barbituric acid was obtained by two methods of analysis. The decomposition of alloxan in the incubation medium was measured fluorometrically and

shown to be decreased in the presence of barbituric acid. The decrease in alloxan decomposition was dependent on the concentration of barbituric acid, with 20 mM barbituric acid completely preventing the apparent decomposition of alloxan during the 10-minute incubation. In addition, complete conversion of (2-¹⁴C) alloxan to a new migratory product on TLC plates occurred in the presence of 20 mM barbituric acid. These analyses indicated that barbituric acid reacted rapidly with alloxan to produce a new migratory product.

The reaction between alloxan and barbituric acid occurred in the same concentration range of barbituric acid (1-20 mM) as that which protected against alloxan. Since 20 mM barbituric acid provided complete protection against alloxan and completely converted alloxan to a new migratory product, it is likely that this product was incapable of inhibiting glucose-induced insulin release. These findings indicate that barbituric acid protects against inhibition by alloxan of glucose-induced insulin release by means of a chemical reaction with alloxan.

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