Glucose tolerance in *Bufo gutturalis* (Power) after destruction of the islets of Langerhans

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An investigation was undertaken to determine the glucose tolerance response in *Bufo gutturalis* after alloxan induced diabetes. The pancreatic β-cells were completely destroyed 24 h after the administration of 240 mg alloxan/kg body mass. However, animals treated in this manner showed a normal response curve. When the alloxan dosage and treatment period were increased, typically diabetic tolerance was apparent.

S. Afr. J. Zool. 1982, 17: 35 – 40

Hierdie ondersoek is onderneem om die glukosetoleransierespons by *Bufo gutturalis* na alloksaan-geïnduseerde diabetes te bepaal. Die β-selle van die pankreas is totaal vernietig 24 h nadat 240 mg alloksaan/kg liggaamsmassa toegedien is. Diere wat op hierdie wyse behandel is, het nog 'n normale responskurwe getoon. Toe die alloksaandosis vergroot is en die toedieningsperiode verleng is, is 'n tipiese diabetiese kurwe verkry.

S.-Afr. Tydskr. Dierk. 1982, 17: 35 – 40

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Received 22 May 1981; accepted 13 August 1981

Although many attempts have been made to induce diabetes mellitus in experimental animals with the use of alloxan (Rerup 1970), there is still a great deal of controversy on the action of this diabetogenic agent. It has been reported that the sensitivity of animals to alloxan varies according to the species, the nutritional state of the animal and the route of administration (Gater & Balls 1977). Miller (1960) reported that alloxan had little effect on amphibian islet cells, but Nace, Blair & Dass (1965) found that an intracardiac injection of 200 mg alloxan/kg body mass raised the blood glucose level in *Amphiuma* and reduced the number of pancreatic β-cells. In addition, doses of up to 800 mg alloxan/kg body mass, administered intraperitoneally, failed to raise the blood glucose level significantly in bullfrogs (Wright 1959), whilst 280 mg alloxan/kg body mass, administered intraperitoneally, was sufficient to induce diabetes mellitus in *B. arenarum* within 48 h (Prieto-Diaz, Iturriza & Rodriquez 1967).

Due to these contradictory reports it was of interest to determine whether alloxan induced diabetes mellitus in *B. gutturalis*. It should however be noted that this study did not investigate the dose response of alloxan. The study was then extended to include an investigation on glucose tolerance in alloxanized toads.

Materials and Methods

Collection and care of animals

Toads were collected in and around Durban (29°49′S/30°56′E) and acclimatized to laboratory conditions for one month prior to experimentation (Vawda, Burger & Smit 1981). The animals were fasted for 48 h before the performance of glucose tolerance tests.

Glucose analysis

Blood was drawn from the truncus arteriosus and analysed for glucose by means of the glucose hexokinase method (Epple, Jorgensen & Rosenkilde 1966; Gater & Balls 1977). Urine samples were collected by pithing the toads over a beaker, and the urine was immediately tested for glycosuria with Ames Labstix.

Microtomy and staining of the pancreas

The pancreas was removed immediately after pithing the animal and fixed in 10% formalin. The tissue was then processed for histological study according to the pro-

cedure described by Gray (1954) and sectioned serially at 10 μm.

The Gomori staining technique described by Disbrey & Rack (1970) was modified according to Halmi's (1952) specification. This modified staining technique successfully distinguished α (yellowish-brown) from β (purple) cells. Staining with haematoxylin and eosin (Pantin 1964) and phloxine and tartazine (Humason 1967) only served to distinguish the islets of Langerhans from the exocrine tissue. The stained sections were studied under a light microscope.

Statistical analysis

The experimental results were subjected to an analysis of variance to establish whether the blood glucose levels changed significantly with time. The possible influence of haematocrit measurements on blood glucose levels was also considered by introducing a haematocrit variable into the statistical equation. The fitted statistical model and the level of significance are indicated in the accompanying text.

Results

The effect of alloxan on pancreatic histology

In order to determine the effectiveness of alloxan as a diabetogenic agent, eight toads were treated with varying doses of alloxan and sampled for histological study after 24, 48 and 72 h (Table 1). Freshly dissolved alloxan prepared as a 5% solution (Seiden 1945) was injected into the triceps femoris muscle of the hindlimb. It should be noted that a detailed histological study was not undertaken. Serial sections were made merely to determine the extent of tissue destruction after the administration of alloxan and to verify the destruction of all the β-cells.

Table 1 Alloxan dosages used to induce diabetes mellitus in *B. gutturalis*

Toad No.	Alloxan dosage (mg/kg body mass)	Treatment period (h)
1	0 – control	0
2	0 – control	0
3	240	24
4	240	48
5	240	72
6	500	24
7	500	48
8	500	72
9	750	24
10	750	46

All the toads except the one numbered 10 (Table 1) which died after 46 h, survived the alloxan treatment for the required experimental duration. Microscopic examination of serial sections revealed that all the alloxan dosages used, damaged all the islets of Langerhans. In every case most of the pancreatic acinar tissue and all the α- and β-cells of the islets were destroyed. Furthermore, there was no apparent increase in the degree of tissue destruction after a corresponding increase in alloxan dosage and/or experimental duration. From these results it was concluded that 240 mg alloxan/kg body mass administered intramuscularly as a 5% solution would effectively destroy all the pancreatic β-cells in *B. gutturalis* within 24 h, although this dose may be in excess of that needed for complete destruction of β-cells. For all practical purposes toads treated in this manner were regarded as diabetic.

Glucose tolerance tests on alloxanized toads

In order to test the sugar tolerance response in toads with damaged pancreatic islets and to determine whether an increase in alloxan dosage and or experimental duration influenced the assimilation of glucose, glucose tolerance tests were performed on the following groups of toads:

(a) toads treated with 240 mg alloxan/kg body mass, at 24, 48 and 72 h after treatment with alloxan.
(b) toads treated with 500 mg alloxan/kg body mass at 24, 48 and 72 h after treatment with alloxan.

It should be noted that a glucose dosage of 0,3 g glucose/kg body mass as a 20% solution in 0,9% saline (intramuscular injection) was used in all the tolerance tests because it had been shown in a previous study (Vawda *et al.* 1981) that this dosage would readily demonstrate any abnormality in glucose metabolism in *B. gutturalis*.

Forty nine toads were selected at random, fasted for 24 h and treated with 240 mg alloxan/kg body mass. The toads were then left in the laboratory terrarium for 24 h, during which period they were allowed water but no food. This gave a total fasting period of 48 h.

After the alloxan treatment period, seven toads were killed and blood and urine samples were collected for glucose analysis. These toads served as the controls. The remaining toads were given, by intramuscular injection, 0,3 g glucose/kg body mass as a 20% solution in 0,9% saline and were sampled, in groups of seven toads each, after 0,5; 1; 1,5; 2; 3 and 4 h. The results of this experiment are graphically depicted in Figure 1. The fitted statistical model with a peak in blood glucose concentration at 1 h is accepted at the 1% level of significance.

Although the toads used in this experiment were regarded as diabetic toads, the tolerance curve in Figure 1 partially contradicts this fact. In addition no glucose was present in the urine. It appears as though Figure 1 depicts a tolerance curve of non-diabetic toads. It can now be argued that since the pancreatic β-cells had already been destroyed, insulin was being secreted by an alternative source, thereby producing a normal response curve, with an initial increase in blood glucose concentration over the first hour and a subsequent decrease over the next 2 hours. It should however be noted that in a previous study (Vawda *et al.* 1981) toads with intact β-cells did not produce a tolerance response when they received 0,3 g glucose/kg body mass; yet alloxanized toads in the present test, receiving the same glucose dosage produced a normal response curve. This is a clear indication that normal toads can assimilate this glucose dosage more successfully than alloxanized toads.

In an attempt to determine whether the duration of the alloxan treatment period had any effect on the sugar tolerance response, the treatment period was increased to 48 h. Forty nine toads were treated as described previously. The results of this test are summarized in Figure 2. The fitted statistical model with a glucose concentration

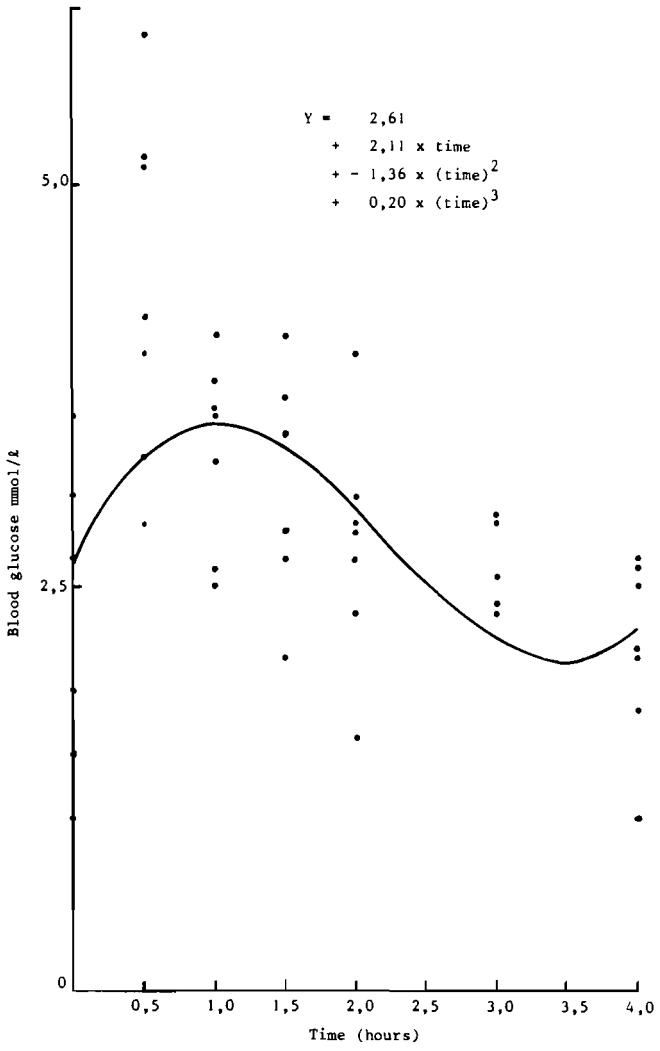

Figure 1 Glucose tolerance in toads treated with 240 mg alloxan/kg body mass for 24 h.

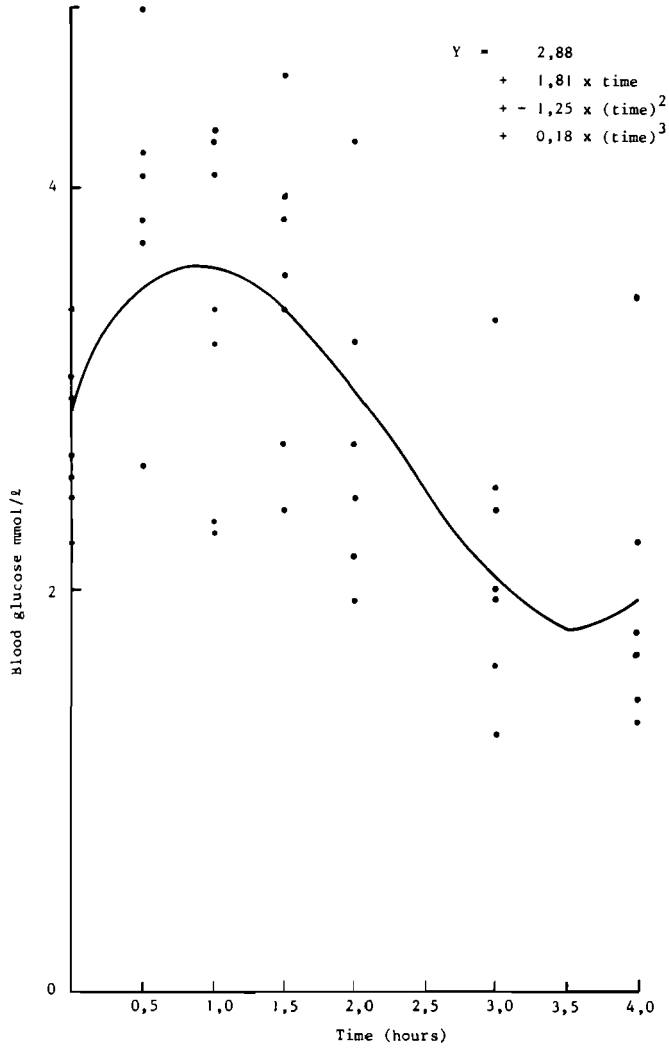

Figure 2 Glucose tolerance in toads treated with 240 mg alloxan/kg body mass for 48 h.

peak at 54 min is significant at the 1% level.

Here again the tolerance curve appears to be that of non-diabetic toads with only two toads (4%) exhibiting glycosuria. It should be noted that the control glucose levels and the peaks in glucose concentration are approximately the same for this and the previous test.

Since the results of the preceeding two tests indicated that the toads were to some extent non-diabetic, the alloxan treatment period was further increased to 72 h to determine the effects thereof.

Forty nine toads were selected at random, injected with 240 mg alloxan/kg body mass and left in the terrarium for 72 h. The toads were fasted for the last 48 h of the 72-h treatment period. Only 45 toads survived the treatment period. Of these, three control toads were sampled immediately and glucose tolerance tests were performed on the remaining toads. They were sampled, in groups of seven toads each, after 0,5; 1; 1,5; 2; 3 and 4 h.

The results of this experiment are shown in Figure 3. The fitted statistical model is a straight line. Considerable random variation is inherent in the experimental data in this test and statistical analysis was difficult. Although there was no evidence of glycosuria, the slow decrease in blood glucose concentration as seen in Figure 3 is typical of the diabetic state.

Since some of the toads did not survive the 72-h treatment period in the previous test, it was decided to increase the alloxan dosage rather than increasing the treatment period. As a result, the alloxan dosage was increased to 500 mg/kg body mass and its effect on the sugar tolerance response determined on 49 toads after 24 h of alloxan treatment.

The results of this experiment are summarized in Figure 4. The fitted statistical model is based on 'a quadratic dependence in time, with a linear dependence on the haematocrit measurement'. The peak in glucose concentration is at 1 h 16 min. These results are accepted at the 1% level of significance.

It should be noted that the statistical curve could not be included with a scatter plot of the experimental data because haematocrit measurements had entered into the mathematical equation thereby making it impractical to plot the statistical curve with two independent variables against a third dependent variable on a two-dimensional graph.

From a physiological point of view the following have to be considered. First, glycosuria was exhibited by approximately 37% of the animals, indicating that some of the toads may have been diabetic. Secondly, according to the statistical model there is an increase in glucose con-

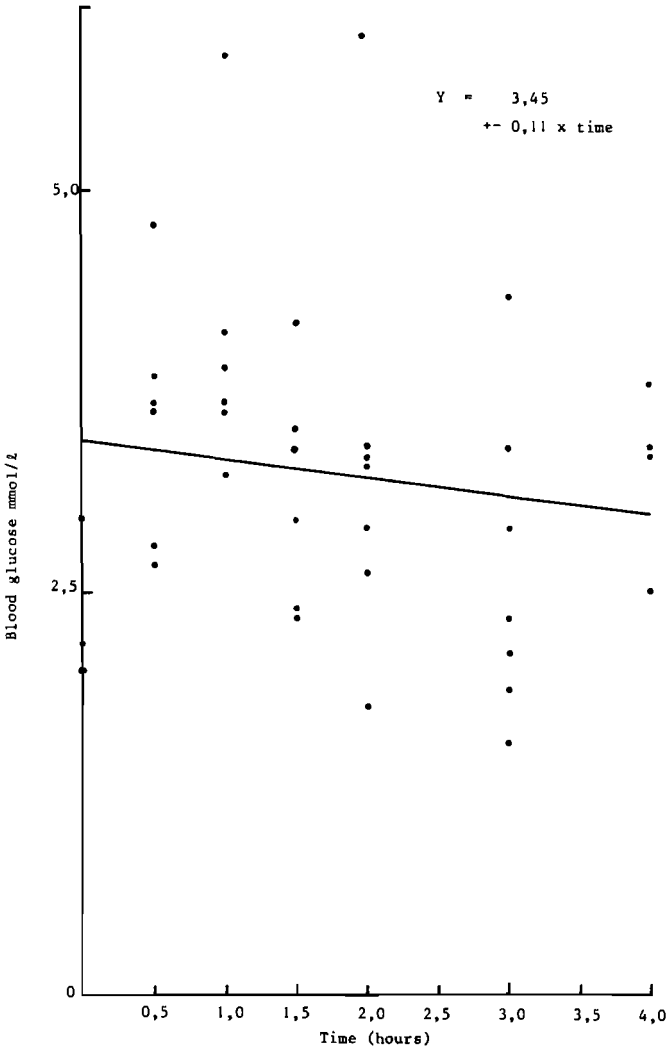

Figure 3 Glucose tolerance in toads treated with 240 mg alloxan/kg body mass for 72 h.

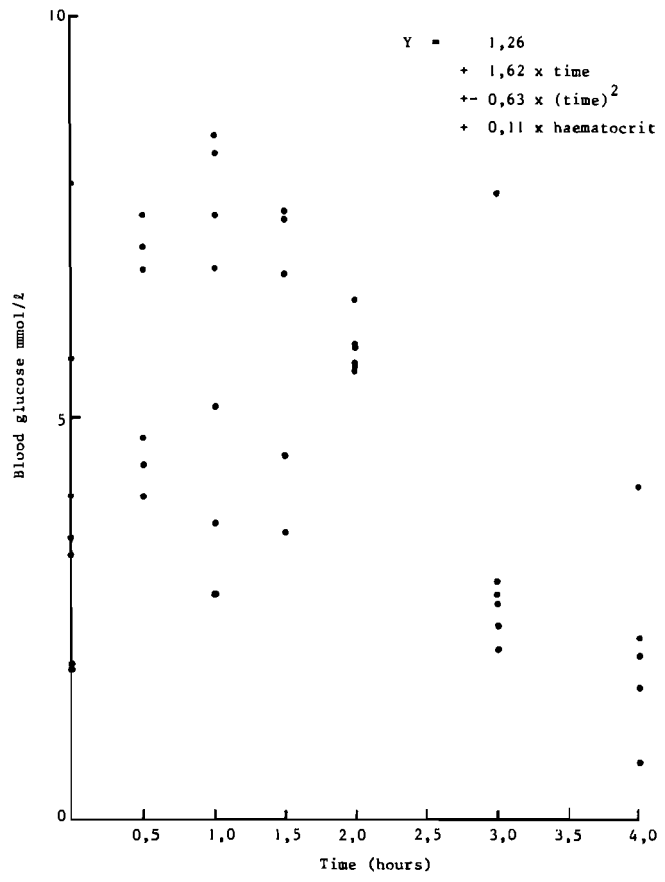

Figure 4 Glucose tolerance in toads treated with 500 mg alloxan/kg body mass for 24 h.

centration with a peak and a subsequent decline. This indicates that some emergency insulin is present in the blood.

Since these results still indicated some non-diabetic tolerance, the alloxan treatment period was now increased to 48 h.

When 28 toads were treated with 500 mg alloxan, seven toads died before the end of the 48-h treatment period, thereby giving a mortality rate of 25%. As a result only 21 toads were used for this tolerance test. The fitted statistical model showed no apparent peak in glucose concentration (Figure 5).

The statistical plot of blood glucose against time indicates a linear decrease in glucose concentration with time. This plot (Figure 5) is very similar to the one in Figure 3 except that the control glucose level is now higher (4,16 mmol/ℓ compared to 3,44 mmol/ℓ in Figure 3). Furthermore, the decrease in glucose concentration is more pronounced in Figure 5 (from 4,16 to 1,78 mmol/ℓ). Here again the slow decrease in blood glucose concentration is typical of the diabetic state.

Since it appeared as though some insulin was still present in the blood after a 48-h alloxan treatment period, the treatment period was now increased to 72 h.

When 28 toads were alloxanized for 72 h only 15 toads survived the treatment, thereby giving a mortality rate of 46% for this experiment. Two toads died approximately 24 h after the alloxan injection, nine died about 50 h after the injection and a further two toads died after 60 h of treatment. Of the remaining 15 toads, two toads were sampled as controls and the balance were given 0,3 g glucose/kg body mass as a 20% solution in 0,9% saline and sampled in groups of two toads each (except for the half-hour group which had three toads), after 0,5; 1; 1,5; 2; 3 and 4 h.

The results of this experiment are summarized in Figure 6. The fitted statistical model gives a peak in glucose concentration at 53 min. Both model and peak are significant at the 1% level.

Physiologically this tolerance curve appears similar to that of non-diabetic toads. However, it must be stressed that only 15 toads were used in this test because of the high mortality rate (46%) and the toads that survived the treatment period could have been the exception rather than the rule. For this reason the tolerance curve in Figure 6 was not analysed physiologically. The experiment was included in this report merely to provide information for future studies.

From the foregoing tolerance tests it was concluded that although the pancreatic β-cells are completely destroyed within 24 h by the intramuscular administration of 240 mg alloxan/kg body mass, emergency insulin is secreted by some secondary source and this insulin regulates the blood sugar to a limited extent.

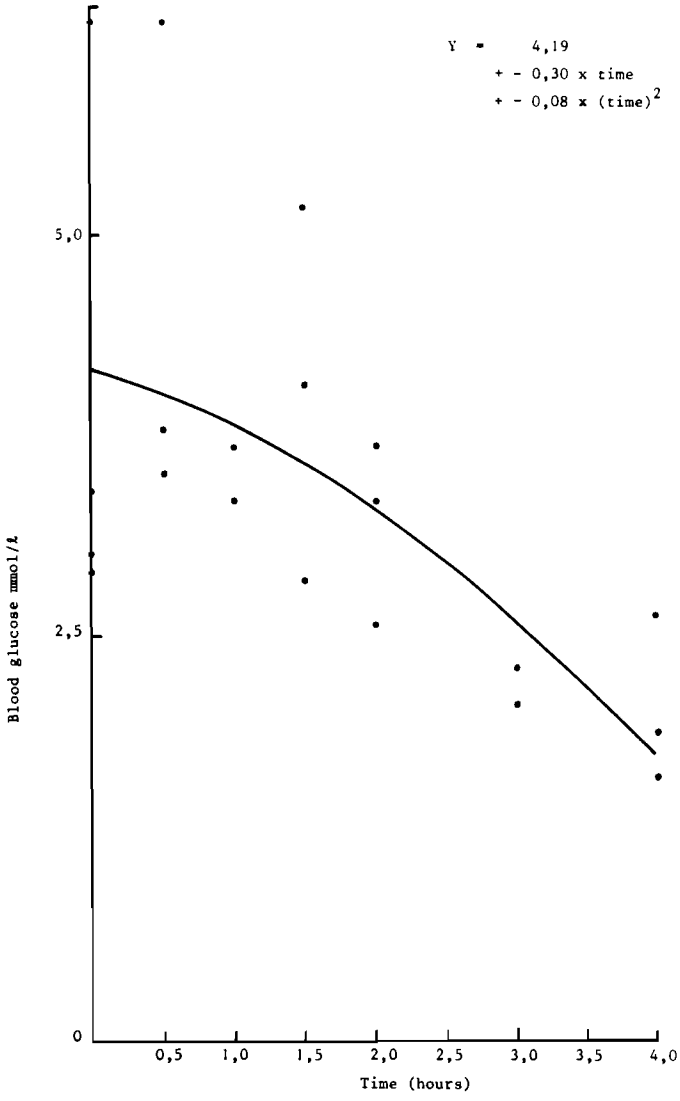

Figure 5 Glucose tolerance in toads treated with 500 mg alloxan/kg body mass for 24 h.

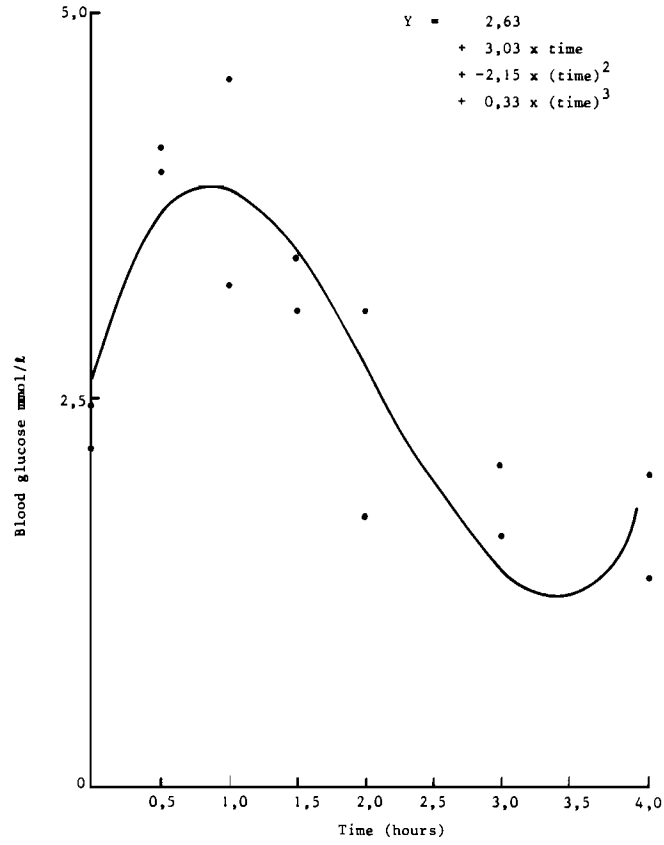

Figure 6 Glucose tolerance in toads treated with 500 mg alloxan/kg body mass for 72 h.

Discussion

Results on the effects of alloxan on the amphibian pancreas have been variable (Miller 1960; Nace *et al.* 1965). In the present study it was found that alloxan completely destroyed the pancreatic β-cells. This finding is based on histological observations of serial sections of the pancreas. Although alloxan is thought to normally affect only the β-cells, we have found that it destroys both the islets of Langerhans and also parts of the exocrine pancreas. Similar findings have been reported for *Rana temporaria* (Yaglov 1977). The destruction of α-cells and acinar tissue in *B. gutturalis* is therefore not unique.

Probably the major factor involved in alloxan experimentation is that the dosage of alloxan required to destroy the β-cells of the islets is only slightly less than the dosage that causes death. Therefore it is difficult to adjust the dosage precisely so that all the β-cells will be destroyed. Consequently the number of functional β-cells that may remain in an alloxan diabetic animal is unknown unless the pancreas is examined in serial sections. This was done in the present study. It must be stressed that this study did not deal with the dose response of alloxan, nor did it involve a detailed histological study of the pancreas. The lowest dosage of alloxan used (240 mg/kg body mass) may not necessarily be the minimum dosage required to destroy the β-cells.

The alloxan dosage problem is clearly evident in the literature. Dosages have ranged from 150 mg/kg body mass to 1000 mg/kg body mass (Seiden 1945; Wright 1959; Yaglov 1977). Closely associated with the problem of dosage is the fact that the routes of administration of alloxan have differed from laboratory to laboratory. In addition different species of amphibians have been investigated by different workers.

Experimental diabetes can also be induced by pancreatectomy (Penhos & Lavintman 1964). Although pancreatectomy appears to be a more reliable diabetogenic agent than alloxan, it was not used in the present study for the following reasons:

(a) the mortality rate is generally higher after pancreatectomy than after alloxan administration;
(b) preliminary experiments showed that successful pancreatectomy required skilled surgery, without which blood vessels and pancreatic ducts were irreparably damaged;
(c) the survival time after pancreatectomy in toads is limited (Dosne 1943);
(d) it has also been reported that acinar and islet tissue can regenerate from the biliary ducts after pancreatectomy (Dosne 1943).

From a physiological point of view, Figures 1 and 2 appear to be non-diabetic tolerance curves. The almost normal tolerance response in these toads immediately suggests the presence of insulin. However the pancreatic β-cells have all been destroyed in this group of experimental toads. Therefore the insulin is probably being secreted

into the blood from a source other than the β-cells. The idea of bond insulin in the human liver and kidney (Ganong 1969) lends support to these speculations. However additional work is required before any conclusions can be reached.

Analysis of the tolerance curve in Figure 3 indicates slight hyperglycemia at 0 h (3,44 mmol/ℓ compared to a normal of 2,94 mmol/ℓ). This is followed by a gradual decrease in glucose concentration over the next 4 h, thereby indicating a typically diabetic state. There is an increase in the degree of hyperglycemia at 0 h in Figure 5, and the subsequent decrease in glucose concentration, although more pronounced, is still typical of the diabetic state.

From the results of the tolerance tests on alloxanized toads it was interesting to note that although the pancreatic β-cells were completely destroyed, some toads were still capable of almost normal glucose tolerance. This is probably due to an alternative insulin source. On the other hand, when alloxanization is more severe, typically diabetic tolerance is apparent, probably in response to an insufficiency of 'secondary insulin' in relation to the needs of the animal. As pointed out earlier, however, additional work in this respect is necessary.

Acknowledgements

The authors wish to thank Mr K.S. Ganga for assistance with the field work and the microtomy, Professor Leon Troskei and Mr R.J. Baston for statistical computations and Mr Wyman for permission to collect toads at the Durban Botanic Gardens.

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