Glucose tolerance in the toad *Bufo gutturalis* (Power)

A. Vawda, F.J. Burger and A.L. Smit
University of Durban-Westville, Durban

Some aspects of glucose homeostasis were investigated in the toad *Bufo gutturalis*. Normal blood glucose and liver glycogen levels were determined and glucose tolerance tests were performed on laboratory acclimatized toads. The mean blood glucose concentration in such animals fasted for 48 h was 2,94 ± 0,65 mmol/l and 8,8 ± 2,62% of the wet mass of the liver consisted of glycogen. The rate of removal of glucose from the blood was directly dependent on the dose of glucose administered; the greater the dose the faster the removal rate. The renal threshold for glucose is between 3,22 and 5,64 mmol/l blood glucose.

S. Afr. J. Zool. 1981, 16: 156 – 162

Sommige aspekte van glukose-homeostase is by *Bufo gutturalis* ondersoek en beskryf. Normale bloedglukose- en lewerglikogeenvlakke is bepaal en glukose-toleransietoetse is op diere wat by laboratoriumtoestande geakklimatiseer het, uitgevoer. Die gemiddelde bloedglukosekonsentrasie by sulke diere, wat vir 48 h sonder kos gelaat is, was 2,94 ± 0,65 mmol/l en 8,8 ± 2,62% van die nat massa van die lewer het uit glikogeen bestaan. Die snelheid van glukose-verwydering uit die bloed is afhanklik van die grootte van die dosis wat toegedien word; hoe groter die dosis hoe vinniger die verwyderingsnelheid. Die nierdrempel vir glukose is tussen 3,22 en 5,64 mmol/l bloedglukose.

S.-Afr. Tydskr. Dierk. 1981, 16: 156 – 162

A. Vawda*, A.L. Smit
Department of Zoology, University of Durban-Westville,
Private Bag X54001, Durban 4000

and F.J. Burger
Department of Human Physiology and Physiological Chemistry,
University of Durban-Westville, Durban
*To whom correspondence should be addressed

Received 17 November 1980; accepted 30 March 1981

Reports on normal blood glucose levels in amphibians are varied. For example, the normal blood glucose level for *B. arenarum* has been reported as 1,44 mmol/l (Penhos & Lavintman 1964) and 2,11 mmol/l (von Lawzewitsch 1963) and that for *Rana catesbiana* is only 0,75 mmol/l (Wright 1959). In general, there are considerable variations in blood glucose levels amongst different species of amphibians (Marvin & Frye 1974). The significance of these variations is difficult to evaluate in view of different experimental conditions and methods of analysis employed by investigators.

There is a great disparity in the number of investigations on sugar tolerance tests in ectotherms and endotherms. The number of studies in the latter group far exceed the number in the former group. Amongst ectotherms, sugar tolerance tests have been performed on bullfrogs (Wright 1959), salamanders (Marvin & Frye 1974) and snakes (Prado 1947, Houssay & Penhos 1960). The disparity is probably due to the problems encountered when performing tolerance tests on ectotherms. For example, it is difficult to collect pure urine samples at the desired times; in many cases the animals cannot withstand continuous blood sampling due to a small blood volume; often the tests have to be performed under anesthesia and it is usually difficult to determine the optimum quantity required.

In view of the varied reports on blood glucose levels in amphibians, investigations into this field were thought necessary. It also seemed necessary that an attempt be made to overcome the problems involved in amphibian sugar tolerance tests and to add to the information available on amphibians.

Materials and Methods

Collection and care of animals

Toads were collected in and around Durban (29°49′S; 30°56′E) from three basic habitats; *viz.*, short, wet grass with scattered trees and shrubs, ponds of water, and wet roads during and immediately after thunderstorms.

The captured toads were housed in a large laboratory terrarium (4,83 × 1,80 × 3,58 m) in which the natural habitat was simulated as closely as possible. The mean temperature in the terrarium was 23,56 ± 2,24°C during summer and 20,99 ± 1,8°C during the winter months. The relative humidity averaged 67,24 ± 8,18% in summer and 31,60 ± 4,34% during winter. The toads were fed

daily on live insects. All experimental animals were acclimatized to laboratory conditions for at least one month and fasted for 48 h before any experimentation.

Glucose analysis

The toads were pithed through the brain and spinal cord. The heart was then exposed, blood was drawn from the truncus arteriosus in a non-heparinized syringe and 0,1 ml of the sample was added to 1 ml 0,33 N perchloric acid. This mixture was centrifuged and the supernatant collected for glucose analysis.

Glucose analysis of the blood was performed on a spectrophotometer at 340 nm, by means of the glucose hexokinase method (Epple, Jorgensen & Rosenkilde 1966, Gater & Balls 1977). In order to determine variations between experimental batches, a standard glucose solution (5,55 mmol/l double distilled water) was run with each series of analyses.

The present study demanded the availability of a fresh and pure urine sample for glucose determination at specified intervals during the glucose tolerance test. Preliminary investigations into experimental techniques indicated that the toads voided urine during pithing. As a result all experimental animals were pithed over a beaker and sufficient urine collected. In a few instances where the toads did not micturate during pithing, the bladder was exposed and urine collected directly from it. All urine samples were tested immediately for glycosuria with Ames Labstix.

Glycogen determination

The method used to determine glycogen involved the digestion of the liver, followed by the precipitation of glycogen and its subsequent hydrolysis to glucose (Pfleiderer 1963). The glucose was then enzymatically determined by the glucose hexokinase method. The glycogen content is expressed as a percentage of the wet mass of the liver.

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. The student's t-test with n_1+n_2-2 degrees of freedom was calculated to compare the means of two groups. Probabilities $\leqslant 0{,}05$ were accepted as significant.

In order to compare the relative variability between different sets of experimental data, the coefficient of variation (cv) was calculated from the standard glucose solutions (5,55 mmol/l) according to the following formula:

$$cv = \frac{\text{standard deviation (SD)}}{\text{mean } (\bar{x})}$$

Results

Normal values

In order to determine the normal blood glucose level and liver glycogen content of *B. gutturalis*, 28 toads were acclimatized to laboratory conditions for one month and then analysed for blood glucose and liver glycogen. These results were then compared with an unacclimatized group and with a group of 28 toads fasted for 48 h (Table 1).

B. gutturalis has a mean blood glucose level of 2,94 ± 0,65 mmol/l and 8,8 ± 2,62% of the wet mass of the liver consists of glycogen. The average haematocrit is 35,6 ± 4,35. In addition there is no statistical difference between the blood glucose levels of acclimatized and unacclimatized toads. Nevertheless, the glycogen content in the acclimatized toads is approximately four times greater than the glycogen content of unacclimatized toads.

The cv was calculated to be 4,28%. This cv of 4,28%, compared to a sample variation of about 22% clearly indicates that variations in the results can be regarded as actual biological variations and not experimental errors.

Glucose tolerance tests

Toads fasted for 48 h were selected at random from the terrarium and weighed. The required number of controls were immediately killed and sampled. The remaining toads were divided into groups, each group representing one sampling time. Subsequently glucose was administered either intramuscularly or via stomach tube. The toads were then placed individually in glass tanks containing some water and left until the desired sampling time (*eg.* $\frac{1}{2}$ and 1 h after glucose administration), after which they were killed and blood and urine samples collected for glucose analysis.

Glucose dosages had to be varied continuously in order to determine the optimum dosage required to perform a glucose tolerance test on *B. gutturalis*. This was especially necessary since there appears to be no conformity with regard to glucose dosages in glucose tolerance tests on amphibians in particular and ectotherms in general. In bullfrogs, for example, sugar dosages for glucose tolerance tests were calculated to raise the blood sugar level by 5,5 mmol/l on the basis of body mass and were

Table 1 Blood glucose and liver glycogen in *B. gutturalis*

Group	Mass (grams) $\bar{x}$ ± SD	Haematocrit $\bar{x}$ ± SD	Blood glucose (mmol/l) $\bar{x}$ ± SD	Liver glycogen % wet mass of liver $\bar{x}$ ± SD
Acclimatized for 30 days	23,3 ± 6,97	35,6 ± 4,35	2,94 ±0,65	8,8 ±2,62
Unacclimatized	34,8 ±16,8	34,54 ± 4,28	2,55 ±0,77	2,5 ±1,45
Unacclimatized, fasted for 48 hours	34,4 ±11,01	36,75 ± 4,33	2,35 ±0,82	2,6 ±1,70

administered in 0,1% saline solution (Wright 1959). Salamanders, on the other hand, were given intravenous injections of 0,5 g glucose/kg body mass as a 10% solution (Marvin & Frye 1974), and snakes received as much as 0,1 to 0,3 g/100 g body mass (Houssay & Penhos 1960).

The glucose dosage used for human glucose tolerance tests formed a basis for initial tolerance tests on *B. gutturalis*. It should be pointed out that it was not possible to perform an entire tolerance test on a single toad because each animal had to be sacrificed before a blood or urine sample could be collected. Consequently several groups of toads were used in any one test and each group represented one sampling point in the test.

Tables 2 & 3 summarize experimental conditions for the various tolerance tests.

In an initial attempt to perform a glucose tolerance test (Experiment 1), eighteen toads were selected at random from the terrarium, fasted for 48 h and then divided into six groups of three toads each. One group served as the controls and was sampled immediately after the fast. The remaining toads were given 1,5 g glucose/kg body mass as a 5% solution via a stomach tube. The toads were then sampled in groups after $\frac{1}{2}$, 1, $1\frac{1}{2}$, 2 and $2\frac{1}{2}$ h.

The results of this experiment (Figure 1) indicated no correlation between glucose concentration and time. The fitted statistical model gave a linear relationship between glucose concentration and time, thereby indicating the lack of a tolerance response. An important fact, however, was that none of the toads exhibited glycosuria.

From the foregoing results it was assumed that the glucose concentration was not high enough to elicit a tolerance response within the toads. Furthermore it can be argued that since this toad does not drink water (Cloudsley-Thompson 1967) the administration of a 5% glucose solution may have introduced a large quantity of fluid into the stomach thereby creating an abnormal situation. Consequently the glucose concentration was increased from 5% to a 20% solution (Experiment 2) and introduced into the toads via a stomach tube.

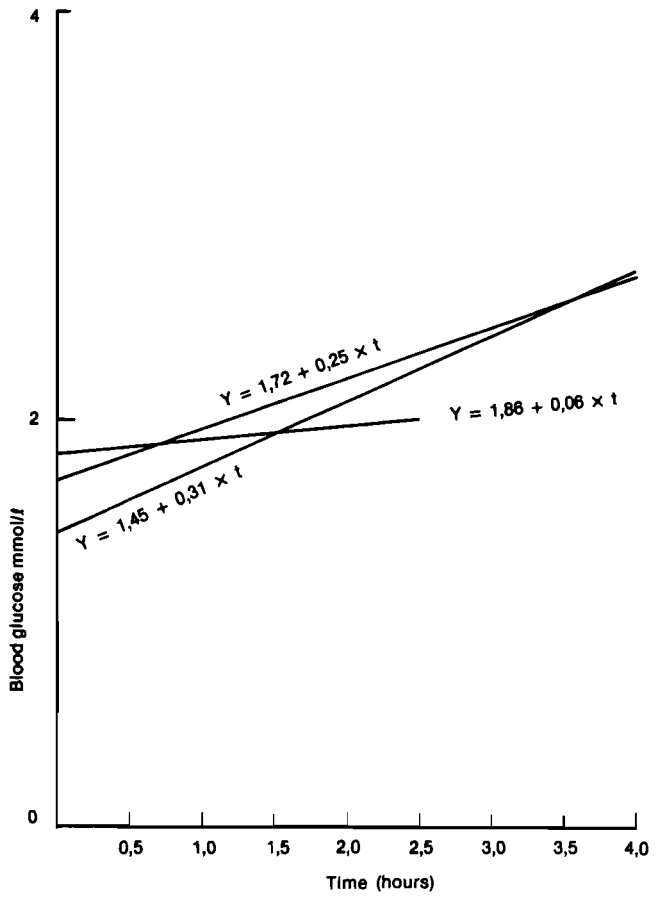

Figure 1 Glucose tolerance in *B. gutturalis* after oral administration of glucose.

Here again there was no significant tolerance response (Figure 1), and the fitted statistical model is a straight line increasing with time. There was again no evidence of glycosuria. The results, however, indicate a slight increase in glucose concentration with time. Therefore the increase in glucose concentration from a 5% solution (Experiment 1) to a 20% solution (Experiment 2) had the desired effect

Table 2 Glucose tolerance tests on *B. gutturalis* (stomach tube method)

Experiment No.	Glucose dosage g/kg body mass	Concentration	Sampling times hours after glucose administration	No. of animals
1	1,5	5% in distilled water	0,$\frac{1}{2}$,1,$1\frac{1}{2}$,2,$2\frac{1}{2}$	18
2	1,5	20% in 0,9% saline	0,$\frac{1}{2}$,1,$1\frac{1}{2}$,2,3,4	21
3	3,0	20% in distilled water	0,$\frac{1}{2}$,1,$1\frac{1}{2}$,2,$2\frac{1}{2}$,4	49

Table 3 Glucose tolerance tests on *B. gutturalis* (intramuscular method)

Experiment No.	Glucose dosage g/kg body mass	Concentration	Sampling times hours after glucose administration	No. of animals
4	3,0	40% in 0,9% saline	0,$\frac{1}{2}$,1,$1\frac{1}{2}$,2,$2\frac{1}{2}$	18
5	1,5	20% in 0,9% saline	0,$\frac{1}{2}$,1,$1\frac{1}{2}$,2,$2\frac{1}{2}$,$3\frac{1}{2}$	19
6	0,75	20% in 0,9% saline	0,$\frac{1}{2}$,1,$1\frac{1}{2}$,2,3,4	49
7	0,3	20% in 0,9% saline	0,$\frac{1}{2}$,1,$1\frac{1}{2}$,2,3,4	49

but it was thought that the dosage was not high enough to produce a significant tolerance response. Consequently the dosage was now increased to 3 g glucose/kg body mass and the concentration maintained at 20% (Experiment 3).

The fitted statistical model for Experiment 3 is a straight line increasing with time, with no indication of a tolerance response (Figure 1). There was much random variation in the experimental data and no peak in glucose concentration was readily discernible.

From the foregoing experiments it was concluded that a glucose tolerance test can not be successfully performed on *B. gutturalis* if the glucose is introduced into the toad via a stomach tube, irrespective of the dosage and concentration used. Because administration by stomach tube was not successful, it was decided to administer the glucose intramuscularly in subsequent tolerance tests. The glucose solutions were injected into the triceps femoris muscle in the hindlimb. Figure 2 summarizes the results of Experiment 4. The fitted statistical model is accepted at the 1% level of significance. The peak in glucose concentration (15,71 mmol/l) occurs at 50 min. It is clear from the curve (Figure 2) that a sharp increase and a subsequent decrease occurs in the glucose concentration from 0 to 50 min and from 50 min to 4 h respectively. The rate of removal of glucose from the blood is approximately 9 mmol/l/h.

In view of the following observations, the curve in Figure 2 cannot be accepted as an ideal glucose tolerance curve. First, it is apparent that the blood-system is overloaded with glucose (some 15,71 mmol/l). Secondly, there appears to be a secondary rise in blood glucose after 2 h, and this cannot be readily explained at this stage. Thirdly, the blood glucose concentration does not return to the control level after $2\frac{1}{2}$ h. Finally, all the toads except the controls, exhibited glycosuria. Consequently this dosage could not be accepted as the ideal dosage for a glucose tolerance test on *B. gutturalis*. However, this experiment had indicated that intramuscular administration of glucose produces a tolerance response in the toads.

In order to obtain a more acceptable glucose tolerance curve with no glycosuria, the dosage and concentration of glucose used in the previous test were halved (Experiment 5). The results of Experiment 5 are summarized in Figure 3. The regression and peak prediction of the tolerance curve are significant at the 5% level. The peak in glucose concentration (8,44 mmol/l) occurs at 1 h 11 min.

It should be noted that the tolerance curve is now more 'physiological' when compared with the previous curve (Figure 2) although glycosuria is still evident. The peak glucose concentration (8,44 mmol/l) is almost half that reported in Figure 2 (15,71 mmol/l) and the rate of removal of glucose is now 2 mmol/l/h. In addition the

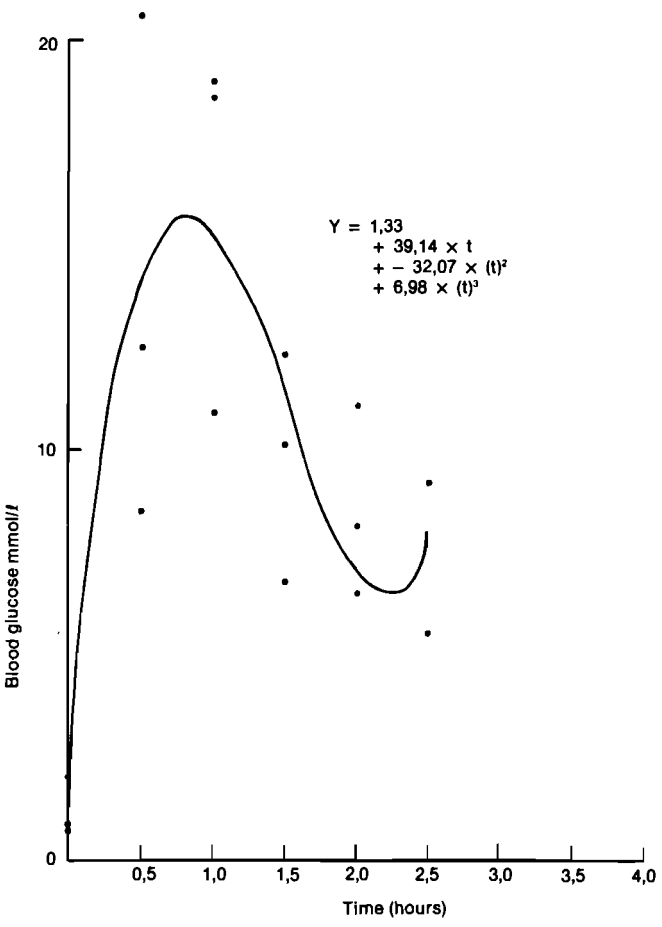

Figure 2 Glucose tolerance in *B. gutturalis* using 3 g glucose/kg body mass as a 40% solution (I.M.).

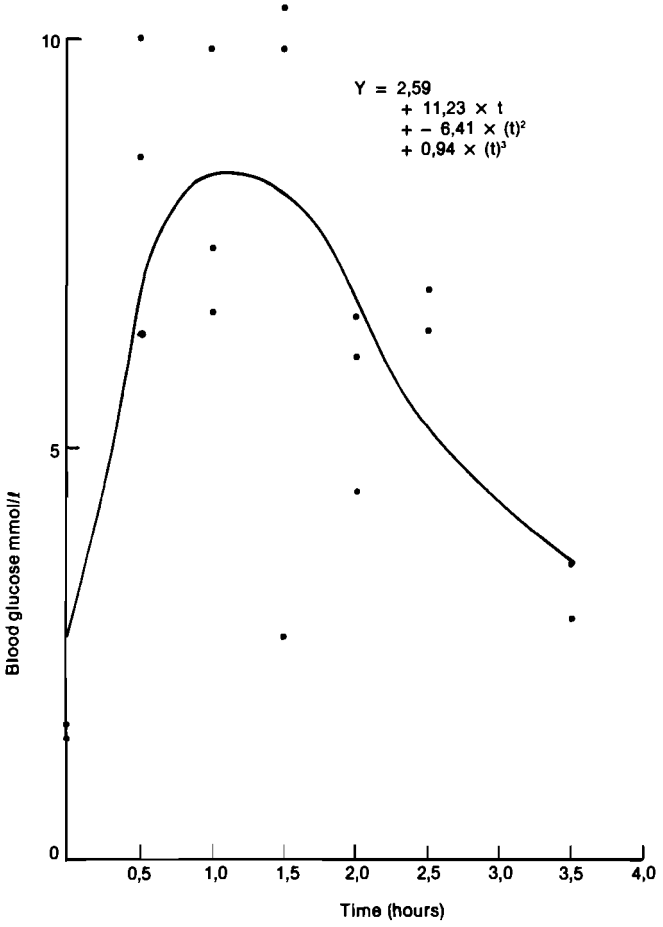

Figure 3 Glucose tolerance in *B. gutturalis* using 1,5 g glucose/kg body mass as a 20% solution (I.M.).

glucose concentration at the last sampling point (3 ½ h) is now closer to the basal value than it was previously, although the concentration does not reach the control value. In view of these observations the glucose dosage of 1,5 g/kg was consequently halved in an attempt to obtain a more acceptable tolerance curve (Experiment 6).

The fitted statistical model for Experiment 6 is accepted at the 1% level of significance (Figure 4). The glucose concentration peak (6,11 mmol/l) occurs at 1 h 8 min. Physiologically this appears to be an ideal tolerance curve, except that approximately 48% of the toads exhibited glycosuria. However, the glucose was present only in trace quantities. Immediately after the administration of glucose, the blood glucose concentration commences to increase steadily from the control level (Figure 4). Within an hour the blood glucose level reaches a peak of about 6,11 mmol/l, with a subsequent decrease in concentration over the next 2 ½ h. At about this time (3 ½ h) the glucose concentration drops below the control level and then increases to approximately the original control level. The rate of removal of glucose is in the order of 1,6 mmol/l/h. In order to eliminate the glycosuria evident in this test, the foregoing dosage was reduced to 0,3 g glucose/kg body mass (Experiment 7).

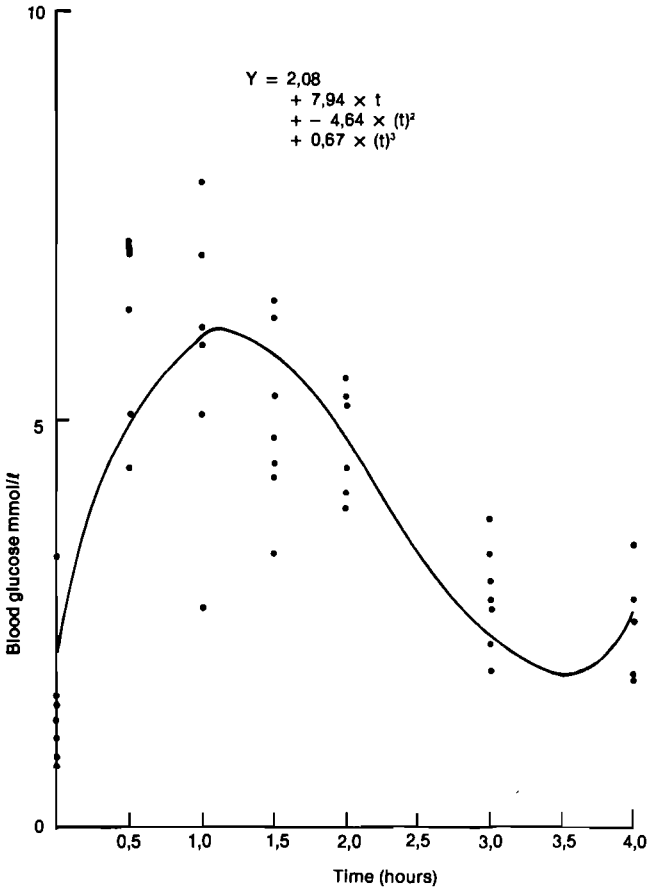

Figure 4 Glucose tolerance in *B. gutturalis* using 0,75 g glucose/kg body mass as a 20% solution (I.M.).

This experiment presented data with much inherent random variation and made statistical analysis difficult (Figure 5). However a peak in glucose concentration of 4,94 mmol/l (mean at ½ h) occurred at ½ h and there was no evidence of glycosuria in the experimental

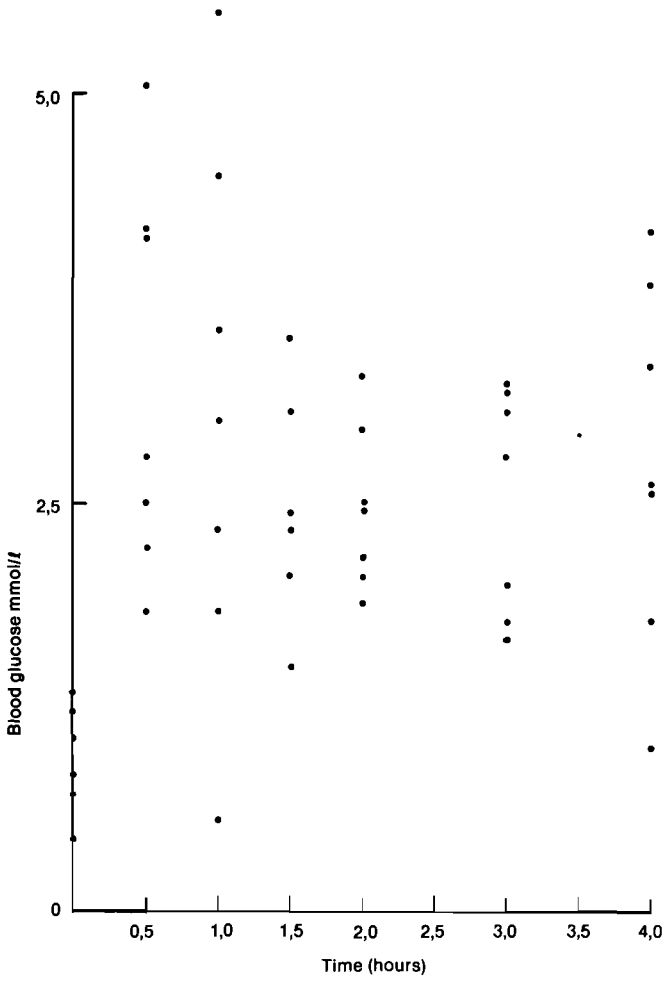

Figure 5 Glucose tolerance in *B. gutturalis* using 0,3 g glucose/kg body mass as a 20% solution (I.M.).

animals. Furthermore, this dosage appears to fall on the 'border line' between dosages eliciting a response and those not eliciting a response. Therefore 0,3 g glucose/kg body mass administered intramuscularly as a 20% solution in 0,9% saline appeared ideal to demonstrate any abnormality in glucose metabolism in *B. gutturalis*.

Renal threshold for glucose

Glucose tolerance tests on *B. gutturalis* gave an indication of the renal threshold for glucose. From the results of these tests it was concluded that the renal threshold for glucose in *B. gutturalis* is in the range 3,22 to 5,64 mmol/l blood glucose.

Discussion

Although many studies have dealt with normal blood glucose levels in amphibians, there seems to be little conformity in the results. This appears to be owing to different experimental conditions, different species of amphibians and different analytical methods. However, since the present study deals with a single species of toad, a closer look at results on the Anura is appropriate. Miller (1960) has reported a blood glucose range of 0 to 4,16 mmol/l for this group. The normal blood glucose level of 2,94 mmol/l in the present study falls within this range. In addition it compares favourably with reports on related species (McMillian & Wilkinson 1972; Smith 1954; von Lawzewitsch 1963).

When blood glucose levels in acclimatized toads were compared with glucose levels in unacclimatized toads, it was found that there was no statistical difference between the two groups. However, the acclimatized toads contained four times more glycogen than the unacclimatized toads. This indicates that the former group of toads was better fed than the latter group and it appears as though feeding in the field is a 'chance operation' depending on the availability of food which in turn is dependent on environmental factors like temperature, rainfall and humidity. Consequently it can now be concluded that the blood glucose level in *B. gutturalis* does not give a true indication of the nutritional state of the animal. This therefore confirms the report by Wright (1959) that there is no correlation between amphibian blood glucose levels and the apparent nutritional state of the animal.

In initial glucose tolerance tests on *B. gutturalis*, three different glucose dosages were administered by stomach tube with no satisfactory tolerance response (Figure 1). It appears as though glucose is not readily absorbed by the toad. The reasons for this are speculative but may, to a large extent, be owing to the relatively carbohydrate-free diet of this species. Nonetheless, specialized biochemical studies on glucose absorption in the toad are necessary before any conclusion can be reached.

Glucose administration by intramuscular injection produced definite tolerance responses. In Experiment 4 the blood glucose concentration peaked at 15,71 mmol/l within 50 min with excessive glycosuria. Immediately after the peak the homeostatic system of the toad began to stabilize the blood glucose concentration. However, the blood glucose did not reach the control level within $2\frac{1}{2}$ h. In addition there was a secondary upsurge in concentration at 2 h. This secondary increase is difficult to explain. It may be due to a secretion of glucagon in response to the hyperinsulinism initiated by the hyperglycemia. This idea is further supported by Kumar & Khanna (1978) who showed that insulin administration in *Rana tigrina* resulted in an initial hyperglycemia which they attributed to an immediate and huge secretion of glucagon under a contra-insulin mechanism to counteract the injected insulin. From these observations it may be argued that glucagon secretion from the pancreas is stimulated both by hypoglycemia and hyperinsulinism.

In a subsequent tolerance test (Figure 3) a smaller quantity of glucose was administered and the tolerance curve appeared more physiological although glycosuria was still evident and the blood glucose concentration did not reach the control level within 3,5 h.

When the glucose dosage was reduced even further (Figure 4), an ideal glucose tolerance curve was obtained and the fasting glucose level was regained in $3\frac{1}{2}$ h. However, glucose was present in the urine of some of the toads.

The increase in blood glucose concentration between 3,5 and 4 h shown in Figure 4 is probably due to glucagon secreted in response to the subnormal glucose concentration between 3 and 3,5 h.

The oral administration of glucose in salamanders gave slow and variable results (Marvin & Frye 1974). Similar observations were made in the present study. However the intravenous administration of 0,5 g glucose/kg body mass in salamanders produced a peak in blood glucose concentration within 10 min and the concentration reached the control level after about 29 h. It therefore appears as though salamanders may have a relatively poor 'insulin apparatus' if glucose removal in salamanders is totally insulin dependent. In snakes on the other hand, 0,5 g glucose/kg body mass injected into the coelom gave a peak in concentration after 3 h and the concentration dropped to the control level after 36 h (Prado 1947). Here it appears as though the peak is delayed because the administration was intracoelomic and the assimilation of the injected glucose was prolonged because of the relatively infrequent feeding habit and the slower digestion and absorption in snakes.

One of the major drawbacks in the present investigation was the lack of facilities for insulin and glucagon assay. The determination of the insulin content of the blood would have been extremely useful in explaining the anomalies encountered in some of the tolerance curves. Although glucagon assay would have been difficult in the toad because of the lack of suitable antibodies, Gater & Balls (1977) have shown that amphibian immunoreactive insulin can be detected by using a radioimmunoassay employing anti-porcine insulin antibodies. Studies on insulin assay during tolerance tests on *B. gutturalis* are definitely required to complement the present work.

The description of a new technique, which is more suitable than those previously published (Ewer 1950), for collecting pure urine samples at specified times and information on maintaining *B. gutturalis* in captivity can now open up new avenues of physiological research on this toad. Some parameters requiring further study include the question of how the tissues of this toad are adapted to function at a low extracellular glucose level; why the administration of glucose via stomach tube does not induce a tolerance response; and whether glucagon is secreted both in response to hypoglycemia and hyperinsulinism.

Acknowledgements

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

References

CLOUDSLEY-THOMPSON, J.L. 1967. Diurnal rhythm, temperature tolerance and water relations of the African toad, *Bufo regularis*. *J. Zool. Lond.* 152: 43 – 54.

EPPLE, A., JORGENSEN, C.B. & ROSENKILDE, P. 1966. Effect of hypophysectomy on blood sugar, fat, glycogen and pancreatic islets in starving toads *Bufo bufo* (L.) *Gen. Comp. Endocr.* 7: 197 – 202.

EWER, R.F. 1950. The effect of pituitrin injection on the water balance of *Bufo regularis* Reuss. *J. exp. Biol.* 27: 40 – 49.

GATER, S. & BALLS, M. 1977. Amphibian pancreas function in long-term organ culture: Control of insulin release. *Gen. Comp. Endocr.* 33(1): 94 – 102.

HOUSSAY, B.A. & PENHOS, J.C. 1960. Prancreatic diabetes and hypophysectomy in the snake. *Xenodon merrimii*. *Acta. Endocr.* 35: 313 – 323.

KUMAR, S. & KHANNA, S.S. 1978. Insulin administration in the frog *Rana tigrina*: Studies on the blood glucose and the histology of the endocrine pancreas. *Gegenbaurs morph. Jahrb.*, Leipzig. 124: 893 – 904.

MARVIN, H.B. & FRYE, B.E. 1974. Blood glucose regulation in larval and adult salamanders. (*Ambystoma tigrinum*). *J. exp. Zool.* 190: 175 – 183.

McMILLIAN, J.E. & WILKINSON, R.F. 1972. The effect of pancreatic hormones on blood glucose in *Ambystoma annulatum*. *Copeia* 4: 664 – 668.

MILLER, M.R. 1960. Pancreatic islet histology and carbohydrate metabolism in amphibians and reptiles. *Diabetes* 9: 318 – 323.

PENHOS, J.C. & LAVINTMAN, N. 1964. Total pancreatectomy in toads: effect of hypophysectomy and glucagon. *Gen. Comp. En - docr.* 4: 264 – 270.

PFLEIDERER, G. 1963. Glycogen. In: Methods of enzymatic analysis, Section B: estimation of substrates. ed. Bergmeyer, H.U. Academic Press, New York and London.

PRADO, J.L. 1947. Glucose tolerance test in Ophidia and the effect of feeding on their glycemia. *Rev. Canad. Biol.* 5: 564 – 569.

SMITH, C.L. 1954. The relation between seasonal hyperglycemia and thyroid activity in the frog *Rana temporaria*. *J. Endocr.* 10: 184 – 191.

VON LAWZEWITSCH, I. 1963. A histologic study of pancreatic regeneration after pancreatectomy in the toad *B. arenarum* Hensel. *Acta. Physiol. Latinoam.* 13: 195 – 197.

WRIGHT, P.A. 1959. Blood sugar studies in the bullfrog *Rana catesbeiana*. *Endocrinology*. 64: 551 – 558.