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# The influence of nutritional factors on biopterin excretion in laboratory animals*

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*Summary:* Nutritional influences on urinary total biopterin levels in rats and pigs were investigated. During the first nights in metabolic cages with food deprivation a significant increase in biopterin values was found in rats. This could be diminished either by feeding, adaptation to food deprivation or by oral glucose application. With food deprivation under normal housing conditions, this increase could not be found. Rats that were fed a cellulose preparation without metabolizable energy had no increase in biopterin excretion. The circadian rhythm of biopterin excretion was influenced by food deprivation as well as by cellulose. Alterations in water intake and urinary output had no effect on biopterin levels related to creatinine. Remarkable changes in biopterin excretion are more likely due to hormonal functions and regulations related to stress than to nutritional factors. More investigations into these problems are being performed.

*Zusammenfassung:* Ernährungsbedingte Einflüsse auf die Gesamtbiopterinwerte im Urin von Ratte und Schwein wurden untersucht. Während der ersten Nächte in Stoffwechselkäfigen bei gleichzeitigem Futterentzug zeigte sich ein signifikanter Anstieg der Biopterinausscheidung im Urin der Ratte. Dieser konnte entweder durch Fütterung, vorhergehende Gewöhnung an den Futterentzug oder durch orale Glukosegaben gesenkt werden. Bei Futterentzug unter normalen Haltungsbedingungen konnte dieser Anstieg nicht gefunden werden. Der tageszeitliche Rhythmus der Biopterinausscheidung wurde sowohl vom Futterentzug als auch von der Cellulosefütterung beeinflußt. Veränderte Wasseraufnahme und -ausscheidung hatten keine Auswirkung auf die Biopterinausscheidung bezogen auf die Kreatininwerte. Die auffallenden Veränderungen sind eher mit streßbedingten hormonellen Funktionen und Regulationen als mit ernährungsbedingten Faktoren in Zusammenhang zu bringen. Weitere Untersuchungen zu diesen Problemen sind in Bearbeitung.

*Schlüsselwörter:* Biopterin, Ratte, Glukose, Cellulose, Fasten

*Key words:* biopterin; rats; glucose; cellulose; fasting

*Abbreviation index:* $BH_4$ = tetrahydrobiopterin, B = biopterin, GTP = guanosine triphosphate, PKU = phenylketonuria

* In memoriam Prof. Dr. Hermann Zucker

## Introduction

Pterins are a group of pyrazino-pyrimidine compounds occurring in many different organisms; they are present in almost all tissues and body fluids (10, 11).

L-erythro-5,6,7,8-tetrahydrobiopterin ($BH_4$) is one of the major unconjugated pterins present in mammalian urine. It is synthesized de novo from GTP. A possible pathway has been proposed and most of the steps involved are proven. Its role as a cofactor in the hydroxylation of the aromatic amino acids phenylalanine, tyrosine, and tryptophan is well established. During this reaction tetrahydrobiopterin is reduced to quinonoid dihydrobiopterin, which is regenerated to $BH_4$ by dihydropteridine reductase (9).

The biosynthesis of important biogenic amines such as catecholamines and indolamines depends on the presence of this cofactor. Deficiency causes progressive neurological signs seen in the hereditary atypical form of PKU (2).

However, biosynthesis also takes place in tissues lacking either of these three enzymes, suggesting further metabolic functions of $BH_4$ that are still under investigation (1, 8, 14).

Earlier studies we performed on physiological biopterin values in rats showed that the concentrations of biopterin excreted in urine seemed to change under different housing conditions (7, 12). The aim of this work was to rule out some nutritional factors that could influence biopterin levels in the urine of laboratory animals.

## Materials and methods

*Animals*

In experiment I, 39 German Landrace pigs (20 gilts; 19 male castrates) with average body weight of 28 kg at the beginning of experiments were housed in groups in pens at constant temperature (18 °C) and relative humidity (50–60 %), and fed standard diet. They had free access to water.

In experiment II pigs were of the breeds Pietrain, German Landrace, and their crossbreds (24 gilts and 16 castrates). Conditions were identical.

Specific pathogen-free male Sprague-Dawley rats weighing 100–120 g were used for all rat experiments. The animals were housed singly at controlled temperature (24 °C), relative humidity (50 %), and light cycle (0700–1900 hours; light:dark). They were maintained on standard diet (Altromin) and received water ad libitum.

*Collection of specimens*

Urine of the pigs was spontaneous.

Urine samples of rats were collected in round, wire-bottomed metabolic cages (104 $cm^2$ × 15,5 cm), where the animals were kept during the time of sample collection (12 h over night) or in whole-wire housing cages where they were kept throughout the experiment and samples were collected every 3 h. Samples were protected from light.

*Sample preparation*

Pterins in urine are present in their different oxidation states. To measure the total amount present they are converted to the fully oxidized form by acidic oxidation. All of the following operations are carried out in dim light to protect

pterins from photo-oxidation. 1 ml of urine is acidified with 300 µl of 1 mol/l HCl and oxidized with 200 µl of 0.5 % I/1 % KI (in 0.1 mol HCl). After incubation in the dark for 1 h, 200 µl of ascorbic acid are added to reduce the excess iodine. The sample is centrifuged for 10 min. The oxidized pterins are purified by Dowex $H^+$ chromatography (Dowex 50 WX8, 100–200 mesh) on a 30 × 5 mm column, as described by Fukushima and Nixon (3). The purified pterins are determined by reverse-phase high performance liquid chromatography. Recovery of pterins added as an internal standard was more than 95 %. Chemicals were suprapure (Merck, Darmstadt, and Sigma, Heidelberg, FRG).

To account for physiological variations in urine excretion, we related biopterin concentrations to urinary creatinine concentrations that were quantified by colorimetric Jaffé determination.

*HPLC-procedure*

The instrumentation consists of a high-pressure pump, a degasser, an automatic sample injector, and a fluorescence detector (Biotronik, Maintal, F.R.G.). Monitoring excitation is at 350 nm, emission at 450 nm. Chromatographic elution was performed either with a 1.5 mmol/l aqueous phosphate buffer (pH 4), containing 7 % methanol and 1 % acetonitril on a silica cartridge (500 × 4.6 mm; Grom, Spherisorb ODS II), or a 3.5 mmol/l potassium hydrogenphosphate (pH 5.8) containing 3 % methanol on a ready-to-use column (244 × 4 mm; Merck, Superspher 100 RP 18), using precolumns in both cases. Column temperature was 20 °C. Chemicals were of HPLC-grade (Merck, Darmstadt). External standards were from Dr. B. Schircks Laboratories (Jona, Switzerland).

*Statistical analysis*

Values of pigs are mean values ± SEM. Data were analyzed using Student's *t*-test for comparison of groups. Differences of $p < 0.05$ were considered to be significant.

Biopterin levels of the rats are mean values ± SD. The Wilcoxon, Mann and Whitney test was performed for statistical comparison of differences in experiments and controls (significant, $p < 0.05$).

## Results

During earlier investigations in pigs (5) concerning stress susceptibility and its effects on urinary pterin levels there was a suggestion that the total biopterin values could be influenced by different kinds of diets. Two groups of pigs were fed standard diet either with a fish-oil compound (5 %) or with cocoa oil (5 %). The biopterin values were 0.36 ± 0.033 µmol/mmol creatinine ($n = 33$) in the first group, and 0.443 ± 0.025 µmol/mmol creatinine ($n = 40$) in the second group ($p < 0.05$).

In another experiment two groups of pigs were fed either ad libitum or on restricted diet (24 h food:24 h food deprivation, alternating). Biopterin levels of the first group were higher than those of the second. As creatinine concentrations depended on the breed, the total biopterin levels were not related to creatinine levels in this case. Considering the creatinine levels of the alternately fed group of pigs of the same breed, they showed lower creatinine levels in the days without food. No significant difference between fed and fasted animals was found for biopterin values. Unfortunately, we could not measure total water intake and urinary output. Concerning these facts there must be an influence of food deprivation on biopterin concentrations related to creatinine.

We tried to rule out external influences by transferring these findings to an established rat model.

Housing of animals in metabolic cages for the time of urine collection caused a limited increase of biopterin in urine samples ($p < 0.001$). During the first days of sampling, values were about 300 % higher in the first night. Then they declined steadily until they reached a physiological mean value of $2.1 \pm 0.53$ µmol BP/mmol creatinine ($n = 90$) from the fifth night on (Fig. 1).

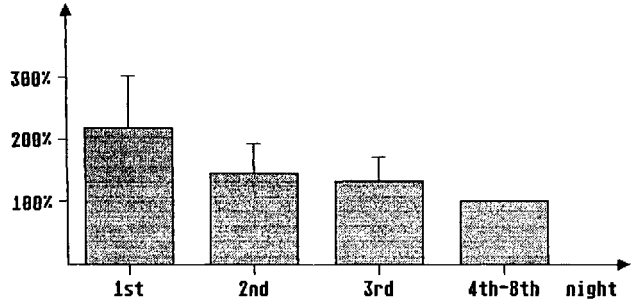


Fig. 1. Urinary B levels of rats with food deprivation ($n = 24$) (values from 4th–8th nights are 100 %).

Feeding in the metabolic cages diminished the biopterin increase, as well as adaptation to food deprivation resulting from a withdrawal of food for 12 h overnight in the normal housing cages 4 days before the experiment started (Fig. 2, 3).

Offering 25 ml of a glucose solution (4.8 %) instead of water (with food deprivation) the biopterin excretion nearly normalized, although the metabolizable energy of normal food intake was about 10-fold compared to glucose (Fig. 4).

If the glucose solution was offered 4 days before the rats were placed into the metabolic cages, the biopterin levels were even lower.

After an adaptation period of 9 days in the metabolic cages overnight (with food and water ad libitum), rats were given a combination of glucose solution (4.8 %) and food. Biopterin values fell significantly (Fig. 5).

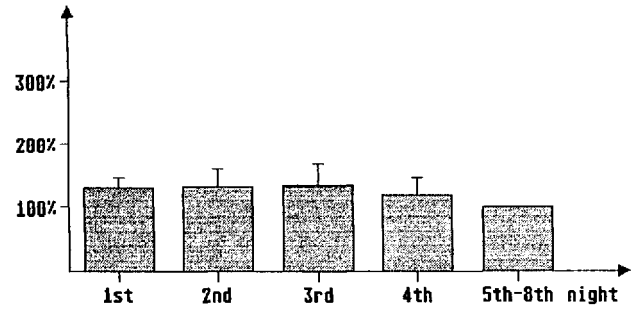


Fig. 2. Urinary B levels (in %) of rats with food ($n = 24$) (values from 5th–8th nights are 100 %).

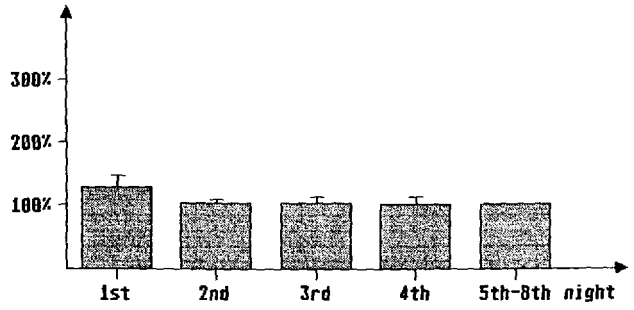


Fig. 3. B levels (in %) of rats with adaptation to food deprivation (n = 24) (values from 5th–8th nights are 100 %).

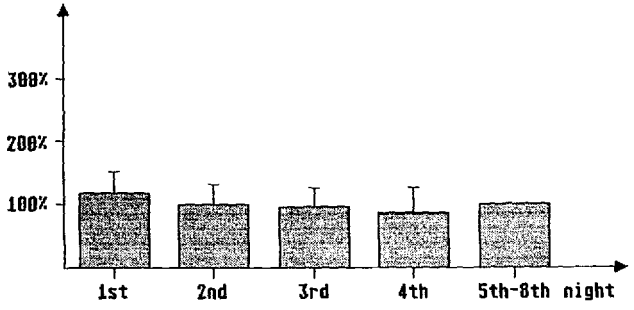


Fig. 4. B levels (in %) of rats with glucose solution (n = 24) (values from 5th–8th nights are 100 %).

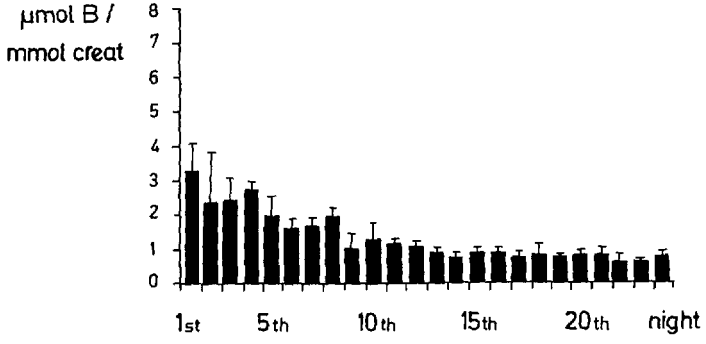


Fig. 5. B levels of rats (n = 8): days 1–8 food and water; days 9–24 food and glucose solution; significance between days 8 and 9: p = 0.02.

Depriving food, together with substitution of water by glucose solution under the conditions described above did not lead to the same increase in biopterin as was found with food deprivation and water (Figs. 6, 7).

Any experimental design offering glucose solution on different days and in different combinations (day 2–4; day 1–8, for example; with food deprivation) in metabolic cages led to diminished biopterin values vs controls.

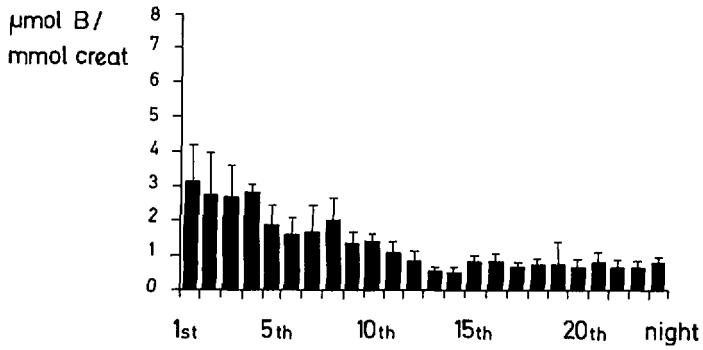


Fig. 6. B levels of rats (n = 8): days 1–8 food and water; days 9–24 glucose solution; significance between days 8 and 9: p = 0.04.

However, a solution of Na-saccharin instead of water (sweetness is about the same as in glucose solution; water intake as high as with glucose solution), as well as the application of 1.2 g glucose powder had no effect on biopterin excretion compared to control groups.

As it could be observed that the cage types, as well as the fact that the animals were confronted with new surroundings influenced testing, thus, we tried to eliminate these factors by using wire-bottomed housing cages. The rats were kept in these for an adaptation period of 2 weeks. Urine could be collected without disturbing the animals.

In addition, samples were collected for 3 days every 3 h as it showed that peaks in urinary biopterin levels often could not be detected in 12-h samples due to the short duration of these peaks.

There was no increase in biopterin excretion by starting sample collection in fed animals.

Depriving food once for 12 h did not elevate biopterin values. Even daily deprivation did not increase the levels. The rats in these experiments were used to handling and did not show any signs of stress.

The only experiment where a remarkable increase could be seen was an experiment with rats who were extraordinarily nervous and not used to

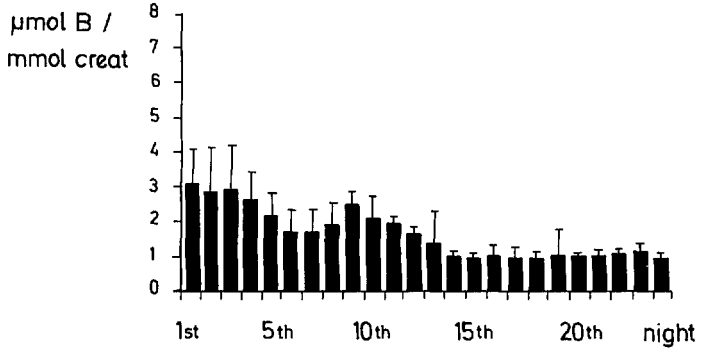


Fig. 7. B levels of rats (n = 8) in metabolic cages: days 1–8 food and water; days 9–24 food deprivation and water; significance between days 8 and 9: p = 0.03.

handling. Food deprivation together with an additional stress situation (weighing, opening cage, strange person) was associated with a remarkable increase in biopterin excretion.

Feeding cellulose for 12 h, twice during trial period, did not lead to significant changes in biopterin values in most of the animals. In this case most of the rats seemed to prefer the cellulose preparation to normal food. As there is no caloric effect, results could be compared to fasting, having ruled out the side effects of stress of food deprivation. We can confirm that diuresis during early fasting in rats is consistent with a recent report (13). Food deprivation leads to diuresis that lasts about 9 to 12 h. Rats did respond to food deprivation for 12 h daily with an effect of adaptation. Creatinine levels returned to normal during the second and third nights. There is no correlation between diuretic creatinine levels and changed biopterin levels.

Fasting for 12 h, as well as feeding cellulose obviously changed the circadian rhythm of biopterin to the opposite with an early onset of changes after 3–6 h. Normally, the highest physiological values are found in the evening (1900–2200 hours; under our specific environmental conditions), that being the beginning of the rats' activity period which decreases during the night and increases again during the day. With food deprivation the highest levels are found in the morning (0700–1000 hours); they then decline until dusk and rise again the next morning (Figs. 8, 9).

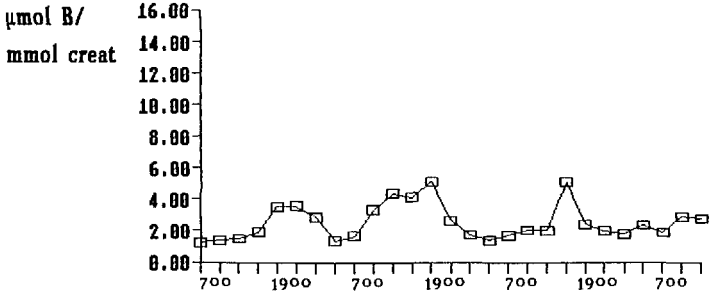


Fig. 8. B levels of a rat with food and water ad libitum.

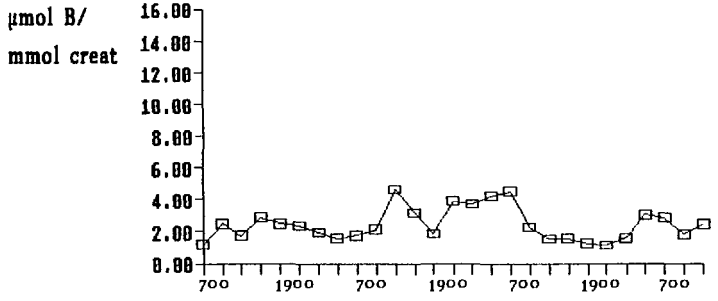


Fig. 9. B levels of a rat with food deprivation.

**Discussion**

Undoubtedly, there was an influence of feeding on biopterin levels, but the mechanisms underlying the observed changes were not obvious. Elevated biopterin concentrations in metabolic cages could be associated, either with changes in food or water intake, or with changes in environmental housing conditions.

From experiments that were performed in metabolic cages it looked as if food deprivation could be the main factor influencing biopterin levels. However, the studies in housing cages produced contradictory results. Two groups of rats did not express any influence of fasting on urinary biopterin levels. As they were weighed and handled daily, and were used to the environmental events related to urine collection (noise, opening cages), stress factors were minimized. In contrast to that, rats that seemed to be extremely sensitive to stressful situations and who were not used to the conditions outlined above showed a significant increase in biopterin excretion.

Furthermore, two- to threefold elevated concentrations in urinary biopterin only 3 h after food deprivation could not be explained just by caloric effects. After this time food was still found in the rats' stomach. These findings are in agreement with the studies of other authors (6).

As changes in the diurnal variations also appear just a few hours after food deprivation or after the feeding of cellulose they can hardly be attributed to fasting alone. A more likely explanation for the observed phenomenon may be a low short-term elevation at the beginning of food deprivation caused by an unexpected change in environmental conditions. The altered feeding conditions, which are followed by a change of circadian activity pattern, are reflected by an additional change in diurnal biopterin rhythm.

It is possible that unfed animals develop hypoglycemia, that inducing stress reactions and hormonal regulations. A glucose solution that had only about one-tenth of metabolizable energy of normal food intake was able to restore elevated biopterin values back to normal or to even lower values. This could be due to hormonal effects or alterations in water intake or output.

The high water intake did not lower the elevated amounts of urinary biopterin, as was shown with the application of saccharin solution.

Investigating the influence of fatty acids, differences were not only seen in biopterin values, but also in parameters like daily weight gain or creatin kinase. However, it must be taken into consideration that animals that were fed on the fish-oil compound diet had a lower food consumption. Further investigations are necessary to solve this problem. The differences in pigs that were fed ad libitum and those on a restricted diet again showed the importance of reference parameters like creatinine. After determining the values per ml, it looks like a restricted diet would significantly lower biopterin concentrations due to the altered metabolic situation. In the present study, however, water intake during starvation would be of equal interest, as well as urinary output. During early fasting, both are increased in humans and rabbits, whereas the rat shows a different behaviour.

Looking at our rat experiments, we could confirm the diuresis in early fasting in rats, as has been suggested (13). Additionally, we can say that

there is an effect of habituation because creatinine concentrations improved rapidly after 9–12 h and did not deviate from the reference range during repeated food deprivation. Apparently, this fasting diuresis seemed to be a possible explanation for the high biopterin levels in the first night in metabolic cages. But by evaluating excretion in housing cages in a great number of animals and in short-term periods there was no correlation in biopterin and diuresis. Diuresis was also found in animals in response to feeding cellulose, indicating that there is, in fact, no metabolizable energy.

In conclusion, the effects of food, food deprivation, and nutritional factors investigated up to now are not the main factors influencing biopterin levels. The data suggests that they are more likely to be attributed to stress and its hormonal regulations and reactions, as we described elsewhere (5, 7, 12). To provide answers to these questions further studies in physiological biopterin metabolism have to be carried out.

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