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To cite this version:
Alexander Breidenbach, Christina Schlumbohm, Johein Harmeyer. Peculiarities of vitamin D and of the calcium and phosphate homeostatic system in horses. Veterinary Research, BioMed Central, 1998, 29 (2), pp.173-186. <hal-00902522>

HAL Id: hal-00902522
https://hal.archives-ouvertes.fr/hal-00902522
Submitted on 1 Jan 1998
Peculiarities of vitamin D and of the calcium and phosphate homeostatic system in horses

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(Received 28 October 1997; accepted 5 December 1997)

Abstract — The aim of the present study was to investigate the importance of putative regulatory factors of the calcium (Ca) and inorganic phosphate (Pi) homeostatic system in the horse. The concentrations of Ca, Pi, vitamin D metabolites, parathyroid hormone (PTH), the activity of the alkaline phosphatase (AP) and the concentration and binding properties of vitamin D binding protein (DBP) were measured in the plasma. In addition, the ability of the renal cortex to hydroxylate calcidiol into 24,25(OH)2D3 and 1,25(OH)2D3 was evaluated in vitro. The plasma concentration of Ca (3.2 ± 0.15 mmol · L−1, N = 100) showed no significant differences between different horse breeds and was not influenced by Ca intake, exercise or by indoor maintenance. The concentration of plasma Pi which ranged from 0.58 to 1.99 mmol · L−1 was negatively correlated with age and positively correlated with the P content of the feed. AP activities in plasma ranging from 131 to 852 U · L−1 were also negatively correlated with age and tended to be higher in horses than in other domestic animals. Plasma concentrations of calcidiol and 24,25(OH)2D were much lower than in most other mammals and birds. The concentration and binding properties of DBP to calcidiol were not markedly different from those of other mammals. The mean plasma concentration of calcitriol (55 ± 24 pmol · L−1, N = 19) was much lower than in other mammals. The plasma concentration of PTH was 218 ± 181 ng · L−1. In renal cortex homogenates, only 25-hydroxycholecalciferol-24-hydroxylase activity could be detected (Vmax: 0.42 ± 0.11 pmol · min−1 · mg−1 protein; Km: 373 ± 263 nmol · L−1). In conclusion, this study provided evidence that in contrast to other species, vitamin D does not appear to play a key role in regulating Ca and Pi homeostasis in horses. © Inra/Elsevier, Paris

Original article

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horse / vitamin D / calcium homeostasis / phosphate homeostasis / renal 25-hydroxycholecalciferol-hydroxylases

Résumé — Particularités du système homéostatique de la vitamine D, du calcium et du phosphate chez le cheval. Le but de cette étude était de déterminer l'importance des facteurs potentiels intervenant dans la régulation du système homéostatique du calcium (Ca) et du phosphate inor-

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ganique (P_i) chez le cheval. Nous avons mesuré, dans le plasma, les concentrations en Ca, P_i, en métabolites de la vitamine D, en hormone parathyroïdienne (PTH), ainsi que l’activité de la phosphatase alcaline (AP) et la concentration et les propriétés ligantes de la protéine liant la vitamine D (vitamin D binding protein, DBP). De plus, nous avons déterminé in vitro, la propriété du cortex rénal à hydroxyler le calcidiol en 24,25(OH)_2D et en 1,25(OH)_2D. La concentration plas-lique en Ca (3,2 ± 0,15 mmol · L^{-1}, n = 100) n’était pas significativement différente entre les différents races équines et n’était pas modifiée par la prise orale de Ca, l’exercice ou le maintien en écurie. La concentration plas-ique en P_i variait entre 0,58 et 1,99 mmol · L^{-1} et était corrélée négativement avec l’âge mais positivement avec la concentration en P dans la nourriture. L’acti-vité de l’AP dans le plasma variait entre 131 et 852 U · L^{-1}, était corrélée négativement avec l’âge et apparaissait plus importante chez les chevaux que chez les autres animaux domestiques. La concentration plas-ique en calcidiol et en 24,25(OH)_2D est beaucoup plus faible que chez la majo-rité des autres mammifères et oiseaux. En revanche, la concentration en DBP et les propriétés ligantes de la DBP au calcidiol n’étaient pas différentes de celles des autres mammifères. La concentration plas-lique en 1,25(OH)_2D (55 ± 24 pmol · L^{-1}, n = 19) était plus faible que chez les autres mammifères. La concentration plas-lique en PTH était de 218 ± 181 ng · L^{-1}. Dans des homogénats de cortex rénal, est seule mesurable l’activité 25-hydroxycholecalciferol-24-hydroxylase. Cette étude apporte donc de nouvelles preuves que, chez le cheval, et contrairement aux autres espèces, la vitamine D ne semble pas jouer un rôle primordial dans la régulation de l’homéosta-sie du calcium et du phosphate. © Inra/Elsevier, Paris

cheval / vitamine D / homéostasie du calcium / homéostasie du phosphate / 25-hydroxycholecalciferol-hydroxylases rénales

1. INTRODUCTION

The equine calcium (Ca) and inorganic phosphate (P_i) homeostatic system differs in many aspects from that of most mammalian species. Although the Ca concentra-tion in horse blood is regulated as accu-rately as in the blood of other species including non-reproducing birds [1, 2] its concentration is remarkably high, ranging from 2.75 to 3.25 mmol · L^{-1}. This range must be regarded as hypercalcemic when compared with other species in which the concentration ranges from 2.3 to 2.5 mmol · L^{-1}. Such high Ca concentra-tions are found in blood of different breeds, e.g. thoroughbreds [22], warm-bloods [14] and ponies [12]. The high Ca concentration is paralleled by a remark-ably high concentration of ionized cal-cium (Ca^{2+}) [32]. This high plasma Ca concentration, however, is not the only peculiar feature of the equine Ca and P_i homeostatic system. The high Ca level is complemented by an unusually low concentra-tion of P_i in plasma which ranges from 0.7 to 1.7 mmol · L^{-1} [6, 22, 32]. In addition, hormonal control of intestinal absorption of Ca appears to be poor. In contrast with other species, elevation of Ca in feed leads to an almost linear increase in intestinal absorption of Ca [27].

If one looks at the three hormones, calcitriol, parathyroid hormone (PTH) and calcitonin, which are responsible for regu-lating Ca and P_i metabolism, it is inter-esting to note that the circulating concentra-tion of calcidiol in horse plasma is also remarkably low (~ 10 nmol · L^{-1}) [26, 30] when compared with other mammalian species, including humans (50 to 115 nmol · L^{-1}) [4, 16]. This is worth men-tioning since the calcidiol concentration in plasma usually functions as a sensitive and reliable indicator of the vitamin D sta-tus [25]. The low concentrations of cal-cidiol, usually present in horses would be considered rachitogenic if present in other mammals. Despite this fact, spontaneous development of rickets or osteomalacia is rare in horses, if not completely absent. El Shorafa et al. [11] have demonstrated
that it was very difficult to induce rickets experimentally in horses by either vitamin D or sunlight deprivation or by both means.

In view of these peculiarities, it was the aim of the present study to further investigate the regulatory factors of the equine Ca and Pi homeostatic system with particular emphasis to the physiological role of vitamin D (lack of an identifying subscript indicates both vitamins D$_2$ and D$_3$) in regulating the Ca and Pi metabolic system.

2. MATERIALS AND METHODS

2.1. Animals

2.1.1. Field studies

Plasma samples were collected from 100 horses, which came from 21 different farms. The age of the animals ranged from 1 to 30 years. Daily intakes of Ca, phosphate and vitamin D were estimated from their concentrations in feeds. These values were obtained from common feed tables or from the listings which were attached to the feed products. Mean daily intake of vitamin D in the horses of this study was $0.65 \pm 0.29 \mu$mol ($N = 85$) but reached a value of 5 and 8.7 $\mu$mol in some cases ($N = 4$). Samples of 40 mL of heparinized blood were collected from each horse by venipuncture prior to the morning feed. The samples were immediately cooled on ice, then centrifuged and the plasma was stored at $-20^\circ$C for further analyses.

2.1.2. Vitamin D metabolites

Volumes of 20 mL of plasma were collected from 13 horses and 11 ponies, which were randomly selected with respect to breed, age (0.8 to 20 years) and sex (11 females, 10 geldings and 3 stallions). The animals were fed pure hay and had free access to water and paddocks. The heparinized blood was cooled on ice, centrifuged and the plasma was stored at $-20^\circ$C.

2.1.3. 25-Hydroxycholecalciferol-hydroxylases

Six ponies (3 females and 3 geldings) from 6 to 18 years of age and 147 to 310 kg body weight were used for the experiments. Two of the ponies were fed pure hay and four ponies were given a mixture of 10% hay and 90% concentrate. Daily intakes of Ca and phosphate were 10 and 5 g per 100 kg BW (pure hay group) and 11 and 10 g (hay-concentrate group), respectively. The animals were killed by stunning and bleeding 5 h after the morning meal. The left kidneys were removed from the abdominal cavity 3 min after the end of bleeding.

2.1.4. Vitamin D binding protein

Plasma was collected from three horses. The animals had been brought to the clinic for horses at the School of Veterinary Medicine in Hannover because of orthopedic disorders.

2.1.5. Parathyroid hormone

Plasma samples were collected from three horses on six occasions throughout the year. In all samples, the Ca$^{2+}$ concentration was measured (ISE-Analyzer 987S, AVL, Bad Homburg, Germany).

2.2. Analytical procedures

2.2.1. Calcium, inorganic phosphate and activity of alkaline phosphatase (AP) in plasma

Ca, Pi, and activity of AP were photometrically determined by use of test kits (Boehringer, Mannheim, Germany). Determination of AP activity was included in this study because of the unusually low concentrations of calcidiol and calcitriol in the plasma. All determinations were carried out in duplicate. If the two values differed by more than 6%, the measurement was repeated again in duplicate.

2.2.2. 25OHD and 24,25(OH)$_2$D in plasma

Aliquots of stored frozen plasma (1 to 4 mL) were used for the assays. The procedure
consisted of two purification steps, followed by radiometric quantification of individual vitamin D metabolites. First, a liquid/liquid partition (extraction) according to a modified procedure outlined by Hollis and Frank [15] was carried out, followed by extraction of the supernatants. These were further purified in a two-step solid phase extraction on porous Bond-Elut® C18 and Bond-Elut® silica cartridges (ICT-ASS Chem GmbH, Bad Homburg, Germany). The extraction procedure yielded three eluate fractions containing 250HD, 24R,25(OH)2D and 1α,25(OH)2D. 250HD and 24R,25(OH)2D were quantified by radioimmunoassay using an antibody raised in sheep against calcitriol-semicarbazide. This antibody cross-reacted with both metabolites. For details see Clemens et al. [7]. Concentrations of the metabolites were calculated by use of standard curves which were set up with metabolites kindly provided by Hoffmann-La Roche (Basel, Switzerland).

To determine the concentration of 1α,25(OH)2D in the plasma, a receptor binding assay based on the calf thymus vitamin D receptor (VDR) was used. Preparation of the receptor and the assay followed essentially the method outlined by Horst et al. [17]. The concentrations of calcitriol in plasma were calculated by use of standard curves.

2.3. Renal 25-hydroxycholecalciferol-hydroxylase activities

Measurement of calcidiol hydroxylation in vitro followed the procedure described by Winkler et al. [33]. In brief, supernatants of renal cortex homogenate were incubated with increasing amounts of calcidiol (final concentrations between 29 and 2445 nmol · L⁻¹) and 3.7 kBq of 25-hydroxy-[26,27-methyl-3H]-cholecalciferol (Amersham Buchler, Braunschweig, Germany) for 10 min in a shaking water bath (37 °C). The reaction was stopped by adding of 6 mL of methanol. Samples were extracted using the method of Bligh and Dyer [5]. The efficiency of metabolite extraction was controlled by adding of 0.8 kBq of labeled calcidiol and calcitriol to extra aliquots of supernatant in which hydroxylation of calcidiol was prevented by adding methanol at the start of the experiment. Next, 50 ng each, of unlabeled 24,25(OH)2D3, 25,26(OH)2D3 and 1,25(OH)2D3 were added to the labeled hydroxylation products of calcidiol to mark the position of labeled products on the UV-chromatogram. This mixture was separated by HPLC (Waters Millipore, Eschborn, Germany) under straight phase conditions on a Zorbax-Sil® column with 5 µm particles (Knauer, Bad Homburg, Germany). Eluate fractions were counted by liquid scintillation (Tri-Carb 460C, Canberra Packard, Frankfurt/Main, Germany).

In two samples from two horses, radioactivity and UV-absorption of the eluate were continuously recorded by simultaneous UV- and radioactive flow through detection (LB 507A, Berthold, Wildbad, Germany) with the two detectors mounted in series (figure 4).

The quantity of newly hydroxylated products was calculated from the amount of radioactivity and its specific radioactivity in the eluate. It was expressed as pmol of product formed per min and per mg of protein. Recovery of the vitamin D metabolites after extraction was taken into account. Michaelis-Menten constants (Km) and maximal turnover rates (vmax) of calcidiol hydroxylations were calculated from plots according to methods outlined by Lineweaver and Burk [24].

2.4. Vitamin D binding protein

The binding capacity and dissociation constant of DBP were determined according to Kaune et al. [21]. In brief, a 250 µL amount of diluted plasma was combined with increasing amounts of 250HD3 (from 1.56 to 801 nmol · L⁻¹) and with 66.7 Bq of labeled 250HD3. After mixing and equilibrating in polypropylene tubes for 18 to 24 h at 4 °C, the tracer bound to DBP was separated by adsorption of the unbound fraction to activated charcoal. After centrifugation, the radioactivity was counted in 250 µL aliquots of supernatant by liquid scintillation. Total radioactivity (T), non-specific binding (BN) and zero binding (B0) were measured in quadruplicate. Specific binding of 250HD3 to DBP (Bsv, pmol · L⁻¹) was calculated by subtracting BN from the radioactivity measured in each supernatant. The maximal number of binding sites (Bmax) and the dissociation constant (Kd) for 250HD3 were computed after plotting Bsv versus free 250HD3 (F). F was calculated by subtracting B0 from total unlabeled 250HD3 concentration. The data were fitted to a double rectangular hyperbola (GRAPH PAD, San Diego, CA, USA).
2.5. Parathyroid hormone

A two-sited immuno-radiometric assay based on two different polyclonal antibodies from goats raised against human intact PTH (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA) was used to determine the concentration of intact PTH in horse plasma. Concentrations of intact PTH were calculated by the use of standard curves which were set up concurrently with the samples. The results obtained are relative values due to the use of a heterologous PTH for the standard curve.

2.6. Chemicals

All chemicals and reagents, unless indicated otherwise, were purchased from Merck (Darmstadt, Germany). Activated charcoal was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Labeled calcidiol and 24,25(OH)₂D₃ were from Amersham-Buchler (Braunschweig, Germany).

2.7. Statistics

Data are expressed as arithmetic means (X̄) and standard deviations (SD) unless indicated otherwise. Linear regression analysis was performed to i) test the influence of phosphate in feed or calcitriol concentration in plasma on Pᵢ concentration in plasma, ii) test the relationship of Ca content in feed with plasma Ca concentration and iii) examine the influence of PTH concentration in plasma on ionized plasma Ca. Nonlinear regression analysis was used to test the influence of age on Pᵢ concentration and on AP activity in plasma, respectively.

3. RESULTS

3.1. Plasma concentration of calcium (Ca), phosphate (Pᵢ) and activity of alkaline phosphatase (AP)

The Ca concentration in plasma was 3.2 ± 0.15 mmol · L⁻¹. Despite great vari-
ability Ca content in feed (from 0.39 to 1.1 % in dry matter (DM)) and in vitamin D uptake, no relationship was observed between the Ca concentration in plasma and the Ca content in feed or vitamin D uptake. The age of the animals also had no significant influence on plasma Ca concentration.

The concentration of Pᵢ in the plasma ranged from 0.58 to 1.99 mmol · L⁻¹ and decreased significantly with age (figure 1). There was a positive correlation (y = 1.02x + 0.71 mmol · L⁻¹, r = 0.23, P < 0.05) between its concentration in the plasma and its content in the feed (from 0.16 to 0.39 % in DM). Plasma Ca concentration was unaffected by the P content in feed. Activity of AP in plasma showed great variability (from 131 to 852 U · L⁻¹) but was found to be negatively related to age (figure 2). Horse breed had no influence on Ca, Pᵢ or activity of AP in plasma (table I).

3.2. Calcidiol (25OHD), 24,25(OH)₂D₃, calcitriol (1,25(OH)₂D) and vitamin D binding protein (DBP)

Plasma concentrations of calcidiol were very low and often (N = 15) below the lower detection limit of the method (4.7 to 22 nmol · L⁻¹), which varied according to the plasma volume used for the assay (table II). Examination of 1,25(OH)₂D concentrations in 19 horses by the receptor assay yielded plasma concentrations from 26 to 106 with a mean of 55 ± 24 pmol · L⁻¹ (table II). Vitamin D metabolite concentrations were not different between horses and ponies and showed no correlation with plasma Ca, Pᵢ (figure 3), breed, sex or age of the animals (data not shown).

In 13 of the 22 plasma samples, 24,25(OH)₂D concentration was near the lower end of the calibration curve and could not be quantified accurately. In the
remaining samples the concentration ranged from 2 to 34 nmol \cdot L^{-1} (table II).

3.3. Renal 25-hydroxycholecalciferol-1\alpha\text{-hydroxylase and 25-hydroxycholecalciferol-24-hydroxylase activities}

Renal cortex homogenates from six ponies showed no 1\alpha\text{-hydroxylase activity when incubated with labeled calcididiol as precursor for 10 min. No radioactively labeled 1,25(OH)_{2}D_{3} could be detected in the lipid extracts prepared from the tissue homogenates after separation on HPLC. This was true for both sexes.

Crude renal tissue converted, however, labeled 25OHD_{3} into 24,25(OH)_{2}D_{3} indicating the presence of an active 24-hydroxylase. Depending on the substrate concentration, 1.5 to 19\% of the labeled precursor was converted into 24,25(OH)_{2}D_{3}.

V_{\text{max}} and the K_{\text{m}} of the renal 24-hydroxylase were 0.42 \pm 0.11 pmol \cdot min^{-1} \cdot mg^{-1} protein and 373 \pm 263 nmol \cdot L^{-1}, respectively. Type of diet (hay or concentrate) had no effect on 24-hydroxylase activity and there was also no influence exerted by the sex of the animal.
Figure 2. Relationship between AP activity in plasma and age of horses.

Table I. Plasma concentration of total calcium and inorganic phosphate and activity of alkaline phosphatase in breeds of horses examined in the field study (x ± SD).

<table>
<thead>
<tr>
<th>Breed</th>
<th>Ca (mmol · L⁻¹)</th>
<th>Pᵢ (mmol · L⁻¹)</th>
<th>AP (U · L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Warmblood</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Hannover Breed) (N = 52)</td>
<td>3.18 ± 0.15</td>
<td>1.07 ± 0.28</td>
<td>267 ± 122</td>
</tr>
<tr>
<td>Other warmblood breeds</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(N = 23)</td>
<td>3.19 ± 0.13</td>
<td>0.90 ± 0.29</td>
<td>285 ± 134</td>
</tr>
<tr>
<td>Cold blooded horses</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(N = 7)</td>
<td>3.20 ± 0.07</td>
<td>0.85 ± 0.14</td>
<td>237 ± 61</td>
</tr>
<tr>
<td>Thoroughbreds</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(N = 7)</td>
<td>3.27 ± 0.10</td>
<td>0.87 ± 0.15</td>
<td>229 ± 66</td>
</tr>
<tr>
<td>Ponies</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(N = 11)</td>
<td>3.08 ± 0.16</td>
<td>0.99 ± 0.22</td>
<td>253 ± 58</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(N = 100)</td>
<td>3.20 ± 0.15</td>
<td>0.99 ± 0.27</td>
<td>265 ± 114</td>
</tr>
</tbody>
</table>
The HPL-radio- and -UV-chromatograms, simultaneously recorded after separation on a Zorbax-Si column, showed a peak at the position of 24,25(OH)2D3 but no signal at position of 25,26(OH)2D3 or 1,25(OH)2D3 (figure 4).

3.4. Vitamin D binding protein

The binding capacities of DBP for calcidiol in plasma (B\text{max}) of the three horses were 3.5, 5.5 and 10.1 \( \mu \text{mol} \cdot \text{L}^{-1} \), and the corresponding dissociation constants (K\text{d}) of the binding protein were 0.12, 0.15 and 0.18 pmol \cdot L^{-1}.

3.5. Parathyroid hormone

In 18 plasma samples, the mean PTH concentration was 218 ± 181 ng \cdot L^{-1}. The corresponding mean Ca\text{2+} concentration was 1.59 ± 0.07 mmol \cdot L^{-1} (adjusted to pH 7.4). Figure 5 illustrates a negative correlation between PTH and Ca\text{2+}.

\[ y = -0.001x + 1.19 \]
\[ r = 0.07 \]

Figure 3. Correlation between concentrations of calcitriol and P\text{\textsubscript{i}} in plasma of 19 horses and ponies.
4. DISCUSSION

4.1. Ca, Pi and AP in plasma

The plasma Ca concentration of 3.2 mmol · L⁻¹ found in this study underlines the view that the concentration of extracellular Ca in horses and ponies is higher than in all other domestic animals. Little, if any, influence was exerted on plasma Ca by breed, by intake of Ca, vitamin D or by maintenance conditions, such as exercise or exposure to sunlight. This agreed with findings of others. No effect on plasma Ca concentration (\( \bar{x} = 2.88 \text{ mmol} \cdot \text{L}^{-1}, N = 4 \)) was found in growing ponies fed diets containing 1.5, 0.8 and 0.15 % of Ca [27]. Deprivation of dietary vitamin D and/or sunlight did not result in unphysiological concentrations of plasma Ca (\( \bar{x} = 3.09 \text{ mmol} \cdot \text{L}^{-1} \)) in 12 young Shetland ponies [11]. Thus, it is likely that the lack of correlation in our study between plasma Ca concentration and Ca intake was probably not primarily due to the uncertainties associated with the estimation of Ca concentrations in feed. It is still unknown which factors or mechanisms are responsible for the control of the plasma Ca in horses. Though quite unresponsive to many influencing factors it shows much variation and does not appear to be as precisely controlled as in other species. The negative correlation

<table>
<thead>
<tr>
<th>Animal number</th>
<th>25OHD (nmol · L⁻¹)</th>
<th>24,25(OH)₂D (nmol · L⁻¹)</th>
<th>1,25(OH)₂D (pmol · L⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pony 1</td>
<td>&lt; 8</td>
<td>&lt; 8</td>
<td>32</td>
</tr>
<tr>
<td>Pony 2</td>
<td>&lt; 8</td>
<td>&lt; 8</td>
<td>40</td>
</tr>
<tr>
<td>Pony 3</td>
<td>&lt; 8</td>
<td>&lt; 8</td>
<td>85</td>
</tr>
<tr>
<td>Pony 4</td>
<td>90</td>
<td>19</td>
<td>48</td>
</tr>
<tr>
<td>Pony 5</td>
<td>&lt; 6.5</td>
<td>7</td>
<td>102</td>
</tr>
<tr>
<td>Pony 6</td>
<td>&lt; 6.2</td>
<td>&lt; 0.7</td>
<td>66</td>
</tr>
<tr>
<td>Pony 7</td>
<td>52</td>
<td>&lt; 0.6</td>
<td>51</td>
</tr>
<tr>
<td>Pony 8</td>
<td>&lt; 22</td>
<td>29</td>
<td>26</td>
</tr>
<tr>
<td>Horse 1</td>
<td>&lt; 4.5</td>
<td>&lt; 0.7</td>
<td>65</td>
</tr>
<tr>
<td>Horse 2</td>
<td>26.5</td>
<td>2</td>
<td>36</td>
</tr>
<tr>
<td>Horse 3</td>
<td>&lt; 7.5</td>
<td>&lt; 0.5</td>
<td>n.a.</td>
</tr>
<tr>
<td>Horse 4</td>
<td>21</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>Horse 5</td>
<td>&lt; 7.7</td>
<td>&lt; 0.7</td>
<td>n.a.</td>
</tr>
<tr>
<td>Horse 6</td>
<td>43</td>
<td>34</td>
<td>n.a.</td>
</tr>
<tr>
<td>Horse 7</td>
<td>&lt; 6</td>
<td>&lt; 0.6</td>
<td>n.a.</td>
</tr>
<tr>
<td>Horse 8</td>
<td>&lt; 11.2</td>
<td>&lt; 1.2</td>
<td>27</td>
</tr>
<tr>
<td>Horse 9</td>
<td>&lt; 15</td>
<td>&lt; 0.6</td>
<td>26</td>
</tr>
<tr>
<td>Horse 10</td>
<td>&lt; 15.5</td>
<td>11</td>
<td>32</td>
</tr>
<tr>
<td>Horse 11</td>
<td>&lt; 17</td>
<td>8.4</td>
<td>59</td>
</tr>
<tr>
<td>Horse 12</td>
<td>6.2</td>
<td>&lt; 0.7</td>
<td>55</td>
</tr>
<tr>
<td>Horse 13</td>
<td>&lt; 4.7</td>
<td>6.4</td>
<td>53</td>
</tr>
<tr>
<td>Horse 14</td>
<td>45</td>
<td>n.a.</td>
<td>62</td>
</tr>
<tr>
<td>Horse 15</td>
<td>13.2</td>
<td>2.2</td>
<td>106</td>
</tr>
<tr>
<td>Horse 16</td>
<td>7.5</td>
<td>&lt; 0.8</td>
<td>72</td>
</tr>
</tbody>
</table>

n.a., not assayed.
between P uptake and plasma Ca concentration which was reported earlier [28, 29] was not seen in this study. This might be due to less marked variation of P intake in our study as compared with the experiment of Schryver et al. [28, 29].

Plasma Pi concentrations behaved differently from plasma Ca. They responded to P intake [28, 29] and declined with age (figure 1). The decline appears to be characteristic for horses. The Pi levels in plasma, however, showed no relationship to vitamin D intake (figure 3). The concentrations were remarkably low when compared to other species [3, 9, 23]. This might have to do with the high Ca concentration present in plasma of these animals, since an inverse relationship has been shown to exist between plasma Pi and Ca, e.g. in pigs (own observations).

As with P, the activity of AP in plasma (ranging from 131 to 852 U · L⁻¹) was also negatively correlated with age (P < 0.0001, figure 2). It showed, however, no significant relationship with plasma Ca or
Pi concentrations. It is not clear at present whether the comparably high activity of AP in horses when compared with other species [8-10] is either related to the high Ca concentration in plasma or to the low concentrations of vitamin D metabolites (see below).

4.2. Vitamin D metabolites in plasma

The remarkably low concentrations of 25OHD which were found in 24 horses and ponies demonstrated that the physiological significance of vitamin D in these animals differed from that of other species. Clearly, the equine circulating level of calcidiol cannot serve as an indicator of vitamin D status when the standards derived from other species are applied. In other species, e.g. in humans, pigs, chicken, etc., such low concentrations of calcidiol would indicate a condition of serious vitamin D deficiency.

Calcidiol is the metabolic precursor for the biologically active form of vitamin D, i.e. calcitriol. Therefore, it was not too surprising that the concentration of calcitriol in horse plasma was also found to be unusually low. The calcitriol concentrations of 55 ± 24 pmol · L\(^{-1}\) were even

Figure 5. Correlation between concentrations of PTH and Ca\(^{2+}\) in plasma.
lower than those usually seen in a pig strain with an inherited defect of renal \(1\alpha\)-hydroxylase activity. These pigs, which suffer from pseudo vitamin D deficiency, type I rickets, have calcitriol concentrations in plasma of 72 ± 26 pmol \(\cdot\) L\(^{-1}\) [20]. This level is too low to protect the animals from developing florid rickets. But horses despite having even lower concentrations of calcitriol do not develop any symptoms of rickets. Nephrectomized humans have circulating calcitriol concentrations of up to 48 pmol \(\cdot\) L\(^{-1}\) [19]. Horses, however, are capable of maintaining supraphysiological Ca concentrations in their plasma irrespective of such low circulating concentrations of vitamin D hormone.

Since none of the horses suffered from any vitamin D related metabolic bone disorders, the question arises as to what the physiological significance of calcidiol and calcitriol in horses is.

### 4.3. Renal calcidiol hydroxylases

Renal \(1\alpha\)-hydroxylase activity could not be detected in vitro, even in the presence of substrate concentrations about 70 times higher than those present in plasma. Production of \(24,25(OH)_{2}D_{3}\), however, occurred always. It remains unanswered whether the lack of renal \(1\alpha\)-hydroxylase activity resulted from a down regulation of the enzyme due to the high circulating levels of plasma Ca or whether renal \(1\alpha\)-hydroxylase is virtually absent in equidae.

The small amounts of calcitriol which could be detected in the horse plasma could be explained by the presence of extrarenal hydroxylases. This has been deduced from studies with nephrectomized humans [19] and pigs [16] which showed similar concentrations of calcitriol in plasma as seen in our horses. The extrarenal synthesis of calcitriol, however, is not sufficient in humans and pigs to prevent them from developing rickets or osteomalacia.

### 4.4. DBP in plasma

Since the possibility of a reduced affinity of DBP to calcidiol could compensate for a low total calcidiol concentration in plasma it appeared important to examine the concentrations of its free and biologically diffusible forms. Our results for the \(B_{\text{max}}\) and \(K_{d}\) of DBP showed, however, that the free form of calcidiol must be as low as its total concentration. Both, \(B_{\text{max}}\) and \(K_{d}\) of DBP did not differ significantly from those of other species, such as piglets [21] or ruminants [31].

### 4.5. Parathyroid hormone

PTH concentrations appeared to be low in horses under basal conditions when compared with sheep [13] or cows [18] and exhibited a negative relationship with plasma Ca\(^{2+}\) (figure 5). The statistical significance of this relationship between PTH and Ca\(^{2+}\) concentration in plasma, should not be confounded by the method. The fact that only relative values of PTH were obtained by our method could perhaps affect the slope of the relationship but not its statistical significance. Thus, the finding suggests that the renal \(1\alpha\)-hydroxylase activity is probably down regulated, and indicates that, in horses, PTH is perhaps more closely involved in Ca and P\(_{i}\) homeostasis than it is the case in other animal species.

### 4.4. General conclusions

The present findings showed that the regulatory mechanisms in horses for keeping the plasma Ca and P\(_{i}\) concentrations constant differ markedly from most other
mammals, including all domestic animal species and birds. Evidence was provided that due to their low circulating concentrations, calcidiol and calcitriol are probably not required for maintaining the Ca and Pi concentrations in plasma at their physiological levels. Although many features of the vitamin D- and Ca-system differ between horses and other mammals, horses have also things in common. This includes the concentration and binding properties of DBP in plasma and the activity of renal 25-hydroxycholecalciferol-24-hydroxylase. It also includes the occurrence of soft tissue calcifications which can develop in horses following the administration of pharmacological doses of vitamin D. Further studies are required for better understanding of the regulatory factors which are responsible for controlling the Ca and Pi homeostatic system in horses.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the help of Dr M. Neunlist and Dr M. Venner in translating the abstract.

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