Discrimination in the metabolism of orally dosed ergocalciferol and cholecalciferol by the pig, rat and chick

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Vitamin D-deficient pigs, rats and chicks were repleted with four daily oral doses of crystalline ergocalciferol (vitamin D₂) and cholecalciferol (vitamin D₃) containing equal concentrations of each. At 24 h after the last dose, the plasma of each species was analysed for vitamin D and 25-hydroxyvitamin D by standard methods. The mean ($\pm$ S.D.) ratios of plasma cholecalciferol to ergocalciferol concentration were 1.5 ± 0.1 (pig), 1.7 ± 0.1 (rat) and 6.3 ± 1.2 (chick). The mean ratios of plasma 25-hydroxycholecalciferol to 25-hydroxyergocalciferol concentration were 4.0 ± 0.1 (pig), 0.4 ± 0.02 (rat) and 10.7 ± 3.4 (chick). The mean plasma cholecalciferol/ergocalciferol ratios for the 24,25-dihydroxy-, 25,26-dihydroxy- and 1,25-dihydroxy-derivatives in the pig were 2.6 ± 0.6 , 5.8 ± 1.3 and 5.8 ± 0.8 respectively. This is the first evidence that mammals other than the New World monkey, like birds, discriminate between ergocalciferol and cholecalciferol. These data, therefore, suggest that species discrimination between the different forms of vitamin D is probably a general phenomenon in mammals. Moreover, this is the first indication of a species (rat) that discriminates against a cholecalciferol metabolite in favour of an ergocalciferol metabolite. Species discrimination against particular forms of vitamin D may be important to the choice of experimental animal models for studying the regulation of vitamin D metabolism and may also be an important consideration in dietary vitamin supplementation.

Early studies with ergocalciferol (vitamin D₂) and cholecalciferol (vitamin D₃) indicated that these two prohormones are equally bioactive in mammals (Bethke *et al.*, 1946; Norman, 1979). Consequently, ergocalciferol has been freely substituted for cholecalciferol as a dietary supplement for humans and for most commercially important mammals (DeLuca, 1978; Norman, 1979). More precise recent

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Abbreviations used: 25(OH)D, 25-hydroxyvitamin D; 25(OH)D₂, 25-hydroxyergocalciferol; 25(OH)D₃, 25-hydroxycholecalciferol; 24,25(OH)₂D, 24,25-dihydroxyvitamin D; 24,25(OH)₂D₂, 24,25-dihydroxyergocalciferol; 24,25(OH)₂D₃, 24,25-dihydroxycholecalciferol; 25,26(OH)₂D, 25,26-dihydroxyvitamin D; 25,26(OH)₂D₂, 25,26-dihydroxyergocalciferol; 25,26(OH)₂D₃, 25,26-dihydroxycholecalciferol; 1,25(OH)₂D, 1,25-dihydroxyvitamin D; 1,25(OH)₂D₂, 1,25-dihydroxyergocalciferol; 1,25(OH)₂D₃, 1,25-dihydroxycholecalciferol; h.p.l.c., high-pressure liquid chromatography.

measurements have supported the efficacy of the substitution by demonstrating that in the rat low doses of ergocalciferol produce the same degree of intestinal calcium transport stimulation, bone calcium mobilization and calcification of rachitic cartilage as equal doses of cholecalciferol (Napoli *et al.*, 1979). Moreover, the metabolism of both forms, as presently known, proceeds through the same intermediates, with the possible exception that 25(OH)D 23,26-lactone may be produced from cholecalciferol alone (Horst *et al.*, 1979, 1981*a,b*). Chickens (Steenbock *et al.*, 1932; Windaus & Trautman, 1937; Chen & Bosmann, 1964) and New World monkeys (Hunt *et al.*, 1967), on the other hand, discriminate against ergocalciferol. This knowledge, plus the report that ergocalciferol is not as hypercalcaemic as cholecalciferol in the rat (Roborgh & Deman, 1960), prompted us to determine whether ergocalciferol metabolism is quantitatively as well as qualitatively similar to cholecalciferol metabolism in mammals. The de-

velopment of specific, sensitive and reproducible assays for ergocalciferol and cholecalciferol and their metabolites (Horst *et al.*, 1979, 1981a,b) and the chemical synthesis of [3α - ^3H]ergocalciferol has allowed a critical examination of this question.

This paper reports that metabolite biosynthesis from ergocalciferol and cholecalciferol is quantitatively dependent on the species studied when equal amounts of both vitamins are co-dosed to vitamin D-deficient animals.

Materials and methods

Sterols

Crystalline ergocalciferol and cholecalciferol were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Crystalline $25(\text{OH})\text{D}_2$ was a gift from the Upjohn Co., Kalamazoo, MI, U.S.A. [$1\alpha,2\alpha$ - ^3H]Cholecalciferol and $25(\text{OH})[23,24\text{-}^3\text{H}]\text{D}_3$ were purchased from Amersham, Arlington Heights, IL, U.S.A. Synthesis of [3α - ^3H]ergocalciferol was by the procedure of Barton & Patin (1976), with NaB^3H_4 substituted for NaBH_4 . Metabolites of [3α - ^3H]ergocalciferol were prepared *in vivo* in vitamin D-deficient rats and purified to homogeneity by h.p.l.c. (Horst *et al.*, 1981b). Preparation of $1,25(\text{OH})_2\text{D}_2$ was from $25(\text{OH})\text{D}_2$ by the procedure described by Jones *et al.* (1980).

Animals and diets

Pigs, rats and chicks were fed vitamin D-deficient diets (Miller *et al.*, 1964; Suda *et al.*, 1970; Knutson & DeLuca, 1974) from 7 days, 21 days and 1 day of age respectively. After 4–5 weeks of the vitamin D-deficient diet, animals of each species were given, orally, an equal mass of cholecalciferol and ergocalciferol (molar ratio 1.03) in propylene glycol daily for 4 days. Ten pigs were given $50\mu\text{g}$ (2000 i.u.) of each, whereas 20 chicks and 30 rats were given $2.5\mu\text{g}$ (100 i.u.) of each. The concentrations of ergocalciferol and cholecalciferol in each preparation were confirmed by h.p.l.c. before the experiments. Blood samples were taken from the rat and the chicken via heart puncture and from the pig via the jugular vein before administration of vitamin D and again on day 5, 24 h after the last dose was given. Blood samples were also collected from chicks and pigs receiving commercial maintenance diets for a comparison of vitamin D and vitamin D metabolite concentrations in normal animals with the experimental animals.

Assays

The amount of blood drawn was: rat, 3–6 ml; pig, 20 ml; chicken, 5–10 ml. Plasma from chickens and rats was combined to make a total of ten samples/species. Plasma was assayed for ergocalciferol and cholecalciferol and their metabolites as previously

Fig. 1. The elution of $1,25(\text{OH})_2\text{D}_2$ and $1,25(\text{OH})_2\text{D}_3$ from a Zorbax Sil silicic acid column developed in dichloromethane/propan-2-ol (19:1). The flow rate of the column was 2 ml/min.

described (Horst *et al.*, 1979, 1981a,b), except for the following modification. For separation of $1,25(\text{OH})_2\text{D}_2$ from $1,25(\text{OH})_2\text{D}_3$, the $1,25(\text{OH})_2\text{D}$ fraction used for competitive protein-binding analysis (Horst *et al.*, 1979, 1981a,b) was chromatographed by h.p.l.c. on a Zorbax Sil silicic acid column developed in dichloromethane/propan-2-ol (19:1, v/v). The u.v. absorbance profiles (Fig. 1) confirm the baseline resolution of $1,25(\text{OH})_2\text{D}_2$ from $1,25(\text{OH})_2\text{D}_3$.

Variance was analysed by using Student's *t* test or Duncan's multiple range test as described by Steel & Torrie (1960).

Results and discussion

Assays of plasma, prepared before repletion was started, demonstrated that vitamin D and vitamin D metabolite concentrations were not detectable in animals of any species; i.e., the animals were vitamin D-deficient.

The concentrations of circulating ergocalciferol, cholecalciferol (Fig. 2), $25(\text{OH})\text{D}_2$ and $25(\text{OH})\text{D}_3$ in the pig, rat and chick 24 h after the last dose of vitamin D are given in Table 1. Included in Table 1 are plasma values obtained from pigs and chicks on normal stock diets. The values obtained from animals in our experiment compare closely with values obtained in normal animals, which indicates that the vitamin D doses used were physiological. Also in Table 1 are data that demonstrate that, without exception, each species had higher plasma concentrations of cholecalciferol than of ergocalciferol. Likewise, the concentrations of $25(\text{OH})\text{D}_3$ were higher than those of $25(\text{OH})\text{D}_2$ in the pig and chick, but the level of $25(\text{OH})\text{D}_2$ was greater than that of $25(\text{OH})\text{D}_3$ in the rat. The plasma ratios of cholecalciferol/ergocalciferol, $25(\text{OH})\text{D}_3/25(\text{OH})\text{D}_2$, $25(\text{OH})\text{D}_2/\text{ergocalciferol}$ and $25(\text{OH})\text{D}_3/\text{cholecalciferol}$ are shown in Table 2. Consistent with the data of Table 1, the pig and the chick had cholecalciferol/ergocalciferol and

25(OH)D₃/25(OH)D₂ ratios greater than 1; in the rat the cholecalciferol/ergocalciferol ratio was also greater than 1, but the 25(OH)D₃/25(OH)D₂ ratio was less than 1. Furthermore, in the pig and chick the 25(OH)D₂/ergocalciferol ratios were lower than the 25(OH)D₃/cholecalciferol ratios ($P < 0.05$). In the rat, however, the plasma 25(OH)D₂/ergocalciferol ratio was greater than the 25(OH)D₃/cholecalciferol ratio ($P < 0.05$).

Concentrations of the cholecalciferol and ergocalciferol forms of 24,25(OH)₂D, 1,25(OH)₂D and

25,26(OH)₂D were measured in the pig (Table 3). Without exception, the plasma concentrations of the ergocalciferol metabolites were lower ($P < 0.05$) than those of their cholecalciferol counterparts. In addition, the cholecalciferol/ergocalciferol ratios of the dihydroxylated metabolites were higher than the cholecalciferol/ergocalciferol ratio. The data in Table 3 also indicate that 25(OH)D₂ and 25(OH)D₃ are equally effective for further hydroxylation.

These results demonstrate for the first time that when presented simultaneously with equal dietary quantities of ergocalciferol and cholecalciferol, the pig and the rat, like the chick, discriminate in their metabolism of the two forms of vitamin D. Discrimination by the pig is qualitatively similar to that observed in the chick. However, the data indicate that discrimination by the pig appears to be primarily at the vitamin D 25-hydroxylase level of vitamin D metabolism, whereas discrimination in the chick appears to occur before the metabolism of vitamin D to 25(OH)D. The higher 25(OH)D₃/25(OH)D₂ ratio than cholecalciferol/ergocalciferol ratio in the pig indicates either a preference for the hydroxylation of cholecalciferol over that of ergocalciferol or a more rapid clearance of 25(OH)D₂ than of 25(OH)D₃. If there was no differentiation at

Fig. 2. Structures of ergocalciferol and cholecalciferol. Cholecalciferol has the same side chain as cholesterol. Ergocalciferol has the ergosterol side chain.

Table 1. Concentrations of vitamin D and 25(OH)D in plasma of the pig, rat and chick

Each species was given orally a 1:1 (w/w) mixture of ergocalciferol and cholecalciferol for 5 days. Results are means $\pm$ S.D. and values in parentheses represent concentrations found in animals fed normal stock diet (see Horst *et al.*, 1981b). When means were compared by using Duncan's multiple range test, the concentrations of ergocalciferol were significantly different ($P < 0.05$) from concentrations of cholecalciferol (*) and the 25(OH)D₂ concentrations were significantly different ($P < 0.05$) from 25(OH)D₃ concentrations (**).

Sterol	Concentration (ng/ml)		
	Pig	Rat	Chick
Ergocalciferol	8.9 $\pm$ 1.6* (<1.0)	3.0 $\pm$ 1.1*	0.4 $\pm$ 0.2* (<1.0)
Cholecalciferol	12.9 $\pm$ 2.3 (10.2)	5.2 $\pm$ 1.7	2.6 $\pm$ 0.7 (2.3)
25(OH)D ₂	9.1 $\pm$ 0.5** (<1.0)	5.5 $\pm$ 1.1**	1.7 $\pm$ 0.6** (<1.0)
25(OH)D ₃	37.2 $\pm$ 2.7 (74.9)	2.3 $\pm$ 0.5	17.2 $\pm$ 2.3 (25.3)

Table 2. Relative abundance of the cholecalciferol and ergocalciferol forms of vitamin D and 25(OH)D in plasma of pigs, rats and chicks

The animals were dosed with a 1:1 (w/w) mixture of ergocalciferol and cholecalciferol for 5 days. Data are means $\pm$ S.D. for nine animals. When means were compared by Duncan's multiple range test, the values in each row with different superscripts were significantly different ($P < 0.05$), and the cholecalciferol/ergocalciferol ratio was different ($P < 0.05$) from the 25(OH)D₃/25(OH)D₂ ratio for the values underlined.

Sterols	Ratio of concentrations		
	Pig	Rat	Chick
Cholecalciferol/ergocalciferol	1.4 $\pm$ 0.1*	1.7 $\pm$ 0.1*	6.5 $\pm$ 1.2**
25(OH)D ₃ /25(OH)D ₂	4.1 $\pm$ 0.1**	0.4 $\pm$ 0.02*	10.1 $\pm$ 3.4†
25(OH)D ₂ /ergocalciferol	1.0 $\pm$ 0.2*	1.8 $\pm$ 0.4**	4.2 $\pm$ 0.3†
25(OH)D ₃ /cholecalciferol	2.9 $\pm$ 0.6**	0.4 $\pm$ 0.1*	6.6 $\pm$ 1.0†

Table 3. Concentrations of cholecalciferol and ergocalciferol forms of vitamin D metabolites in pigs given cholecalciferol and ergocalciferol orally (2000 i.u. of each) for 5 days

The data are means $\pm$ S.D. for nine animals. When means were compared using Duncan's multiple range test, the ergocalciferol metabolite concentrations were significantly lower ($P < 0.05$) than the corresponding cholecalciferol metabolite concentrations. The ratios with different superscripts were significantly different ($P < 0.05$).

Sterol	Concentration of indicated form (ng/ml)		Ratio of cholecalciferol/ ergocalciferol form
	Cholecalciferol	Ergocalciferol	
Vitamin D	12.9 $\pm$ 2.3	8.9 $\pm$ 1.6	1.4 $\pm$ 0.1**
25(OH)D	37.2 $\pm$ 2.7	9.1 $\pm$ 0.5	4.1 $\pm$ 0.1††
24,25(OH) ₂ D	2.7 $\pm$ 0.3	1.2 $\pm$ 0.2	2.2 $\pm$ 0.6**†
25,26(OH) ₂ D	1.20 $\pm$ 0.2	0.28 $\pm$ 0.1	4.3 $\pm$ 1.3††
1,25(OH) ₂ D	10 ⁻³ $\times$ (212 $\pm$ 28)	10 ⁻³ $\times$ (40 $\pm$ 6.5)	5.3 $\pm$ 0.8††

these points in the metabolic scheme, the cholecalciferol/ergocalciferol and 25(OH)D₃/25(OH)D₂ ratios would be similar. The fact that the 25(OH)D₂/ergocalciferol ratio in the pig was only one-third the size of the 25(OH)D₃/cholecalciferol ratio also supports the hypothesis that discrimination by the pig begins at the vitamin D 25-hydroxylase level. In the chick, however, there was no significant difference ($P < 0.05$) between the plasma cholecalciferol/ergocalciferol ratio and the plasma 25(OH)D₃/25(OH)D₂ ratio. These data indicate that in chicks the major point of discrimination between oral ergocalciferol and cholecalciferol is before their metabolism to 25(OH)D. The apparent discrimination against ergocalciferol in the chick may be a result of less efficient absorption, or, alternatively, circulating ergocalciferol may be more efficiently cleared from blood.

Jones *et al.* (1975) have shown that 24 h after an intravenous dose of 7.5 i.u. of [³H]ergocalciferol to chicks, the plasma ergocalciferol concentrations were higher than the 25(OH)D₂ concentrations [the 25(OH)D₂/ergocalciferol ratio was approx. 0.6]. In our experiments, the chicks received 100 i.u. of ergocalciferol orally and the opposite condition arose; i.e., plasma ergocalciferol concentrations were lower than the 25(OH)D₂ concentrations [the 25(OH)D₂/ergocalciferol ratio was 3.8 $\pm$ 0.3]. Therefore, our observation of 25(OH)D₂/ergocalciferol ratios remaining above 1.0 when ergocalciferol is given orally as opposed to intravenously is evidence that ergocalciferol is less efficiently absorbed than cholecalciferol. Alternatively, vitamin D from an oral dose could be more rapidly presented (in cyclomicrons) to the liver than would an intravenous dose (protein bound). If liver catabolizes ergocalciferol faster than cholecalciferol, then our results could be explained by more rapid clearance of ergocalciferol from the circulation. This phenomenon was not apparent in the previous work of Drescher *et al.* (1969), Imrie *et al.* (1967) or Jones

et al. (1975). All of these previous experiments suggested that the point of discrimination against ergocalciferol by the chick was at the 25(OH)D level. The apparent discrepancy lies in the experimental design. These previous studies were performed with radioisotopes given as a single intravenous dose of the radioactive vitamin D preparations. Thus intestinal absorption was effectively eliminated as a possible point of discrimination. Our experiments, however, were done by using the more physiological approach of chronic simultaneous oral administration of non-radioactive preparations of ergocalciferol and cholecalciferol. We also determined that discrimination beyond 25(OH)D is not evident in the pig (Table 3).

The discrimination between ergocalciferol and cholecalciferol by the pig came as a surprise because work with growing pigs (Bethke *et al.*, 1946) has suggested that ergocalciferol and cholecalciferol are similar in their ability to maintain normal plasma calcium and phosphorus contents and maintain normal bone strength. However, this indirect biological evidence may not accurately reflect quantitative differences in metabolism between ergocalciferol and cholecalciferol.

The differences we observed between the pig and rat were of great interest. In contrast with the pig, the rat, on the one hand, preferred to circulate cholecalciferol rather than ergocalciferol, but, on the other hand, preferred the presence of 25(OH)D₂ over that of 25(OH)D₃. These differences are not fully understood or easily explained, but we can speculate about them. Rats evolved as nocturnal animals, consuming grains (which contain predominantly ergocalciferol) and animal carcasses. The rat, therefore, probably evolved with ergocalciferol as its major source of vitamin D. The pig, however, evolved as a grazing animal that consumed fresh green plants of low vitamin D content. These animals, therefore, had opportunity for long periods of exposure to sunlight than enabled them to utilize

fully the photochemical conversion of 7-dehydro-cholesterol into cholecalciferol. The pig, therefore, probably evolved with cholecalciferol as its major source of vitamin D. Evolution could have provided means for each species to more efficiently process the form of vitamin D to which it was exposed.

We have shown that the pig and rat, as well as the chick, discriminate between ergocalciferol and cholecalciferol when these vitamins are co-dosed orally. Metabolic discrimination between ergocalciferol and cholecalciferol in mammals other than the New World monkey has heretofore been undescribed. These data suggest that mammals in general may metabolize D vitamins differently, quantitatively if not qualitatively. These findings may have interesting ramifications concerning the choice of a D vitamin to fortify diets. It is quite possible that, for mammals like the pig, which have evolved with significant exposure to sunlight, dietary supplementation with ergocalciferol would be less efficient than dietary supplementation with cholecalciferol particularly if the animals were exposed to cholecalciferol at the same time. On the other hand, perhaps some caution is appropriate in using the rat as a mammalian model for study of quantitative differences in metabolism of cholecalciferol and ergocalciferol.

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