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Changes with malnutrition in the concentration of plasma vitamin D binding protein in growing rats

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The work presented here examines the possible effects of nutritional deficiencies on the characteristics of the plasma transport protein for vitamin D and its metabolites (vitamin D binding protein, DBP) in the growing rat. Deficiencies in both dietary protein intake and dietary energy intake may decrease the concentration of DBP in the circulation, although plasma DBP was not affected by dietary Ca deficiency. None of the dietary factors examined appears to influence the affinity of DBP for its major ligand, 25-hydroxycholecalciferol ($25(OH)D_3$). Protein-deficient rats seemed to have difficulty in maintaining adequate concentrations of 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$) in the circulation. The sensitivity of DBP to dietary protein and energy intake may constitute a novel mechanism that may help to explain the observed associations between malnutrition and the development of metabolic bone disease, through alterations to the cellular availability of vitamin D ligands to DBP.

Plasma vitamin D binding protein: Cholecalciferol: Malnutrition

Developmental bone diseases such as rickets and dyschondroplasia are characterised by defects in cartilage maturation and/or bone calcification. Abnormalities in the formation, metabolism, utilisation or action of vitamin D and its metabolites are thought to be involved in the pathogenesis of both rickets and dyschondroplasia, although the mechanisms remain unclear.

There have been few investigations of a possible role for the plasma vitamin D binding protein (DBP) in developmental bone disease. This protein, which is secreted by the liver, binds vitamin D and its metabolites, most notably 25-hydroxycholecalciferol ($25(OH)D_3$) and 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$), in a specific, high affinity manner (Rikkers & DeLuca, 1967; Bouillon *et al.* 1996). DBP has evolved from an ancestral gene that also gave rise to plasma albumin and α-fetoprotein (Lichenstein *et al.* 1994). It is not clear whether the protein is involved in specific delivery of its ligands to target cells in a manner previously suggested for other steroid hormone binding proteins (Siiteri *et al.* 1982; Kuhn, 1988; Rosner, 1990; Blomhoff *et al.* 1991), or whether its ligands obey conventional equilibrium kinetics, being available to cells only in their free state (Mendel, 1989). In either case, it is clear that the properties of DBP, and its concentration in plasma have the potential to influence the availability of its ligands to the intracellular space, and thus to influence the biological activity of these ligands.

The effects of relative deficiencies of dietary protein and energy on liver function, and in particular on protein metabolism have been well documented (Kirsch & Saunders, 1972). Furthermore, several, albeit conflicting, associations have been documented between the incidence of malnutrition and that of developmental bone disease (Soliman *et al.* 1996; Walter *et al.* 1997; Akpede *et al.* 1999; Lulseged & Fitwi, 1999). Dietary Ca is known to influence the kinetics of vitamin D metabolite turnover in the circulation, although the mechanism by which this occurs has been disputed (Halloran *et al.* 1986; Clements *et al.* 1987; Reinholz & DeLuca, 1998).

In the studies presented here, the effects of dietary constituents on the plasma concentration and binding kinetics of DBP are examined in the growing rat. Dietary protein, energy and Ca are known to be important in the pathogenesis of metabolic and developmental bone diseases. It is hypothesised that the plasma concentration of DBP and its affinity for its ligands may be altered in response to limitations to these dietary factors.

Abbreviations: $1,25(OH)_2D_3$, 1,25-dihydroxycholecalciferol; $25(OH)D_3$, 25-hydroxycholecalciferol; DBP, vitamin D binding protein.

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Experimental methods

Rats on dietary calcium restriction

Male Sprague-Dawley rats were obtained as weanlings at 3 weeks of age and were maintained on a synthetic purified diet containing 6·4 g Ca and 5·4 g P/kg. After 10 d, half of the rats were placed on a similar diet containing only 0·2 g Ca/kg and were maintained on this diet for a further 10 d. Following this, the vitamin D content of all diets was reduced (to produce a vitamin D-deficient intake) without altering the mineral composition. Rats were killed 1 week later, and plasma Ca, vitamin D metabolites, and DBP concentrations were determined.

Rats on dietary protein and energy restriction

Male Sprague-Dawley rats were obtained as weanlings and were acclimatised over 1 week to a semisynthetic diet, containing 250 g vitamin-free casein/kg as the major protein source (Sakuma *et al.* 1987). Rats were then divided into three groups of sixteen. Group 1 was fed the 250 g protein/kg diet *ad libitum* (control group), group 2 was fed the same diet in restricted amounts, which varied from 60–80 % of the feed consumption of rats in group 1 over the previous 24 h period (energy-deprived group). The diet of the third group of rats was changed to one in which the casein content was decreased to 30 g/kg by addition of dextrin and sucrose (protein-deprived group). This protein-deficient diet has been demonstrated to limit the hepatic production of albumin (Sakuma *et al.* 1987). Rats were weighed daily, and their feed consumption was recorded for 14 d (this period is termed the 'restricted period'), by which time profound differences in growth rate and body weight were observed between the groups. Ten rats from each group were then killed, their livers weighed, and blood plasma collected and stored at −20°C for later analysis. The remaining six rats in both the protein-restricted and energy-restricted groups were then rehabilitated on the 250 g casein/kg *ad libitum* (control) diet, and together with the control rats were maintained on this complete diet for a further 1 week (this period is termed the 'rehabilitation period'). After this time, recoveries in body weight and growth rate were observed, and the rats were killed, blood plasma was prepared, and liver weights were recorded.

The relationship between body weight and liver weight was determined in control rats (combining results from the restricted and rehabilitation periods) by linear regression of liver weight against body weight. This relationship was then used to transform all liver weight data to the weights they would assume at mean body size, eliminating the effect of body weight on liver weight. These data were expressed as a liver weight index for each group of rats.

Chemicals

All solvents used were analytical reagent grade, and all chemicals analytical grade. 25-Hydroxy[26,27-methyl-^{3}H]-cholecalciferol ([^{3}H]25(OH)D$_3$), 1α,25-dihydroxy[26,27-methyl-^{3}H]cholecalciferol ([^{3}H]1,25(OH)$_2$D$_3$), Sephadex LH20 and Dextran T20 were obtained from Amrad Biotech (Boronia, New South Wales, Australia). Non-radioactive 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ were donated by Hoffman La-Roche (Basel, Switzerland), and were dissolved in spectroscopic ethanol. Activated charcoal was purchased from Merck (Kilsyth, Victoria, Australia), and scintillation fluids were obtained from Canberra Packard (Mt Waverly, Victoria, Australia). Serum standards for plasma Ca determination were obtained from Roche Diagnostics (Castle Hill, New South Wales, Australia).

Plasma binding protein analyses

The concentration of DBP in plasma and its affinity for 25(OH)D$_3$ were calculated from competitive protein binding assay, a modification of a method described elsewhere (Mason & Posen, 1977; Woloszczuk, 1985). Briefly, the plasma for DBP analysis was diluted in barbital acetate buffer (pH 8·6) to a concentration of 0·15 μl/ml. Solutions containing increasing concentrations of [^{3}H]25(OH)D$_3$ in ethanol from 0·1–12·0 nmol/l (specific activity of approximately 6 kBq/pmol) were added to successive tubes containing the diluted plasma, so that the final volume of ethanol in each tube was less than 10 % of the total volume. The association of ligand and protein was allowed to reach equilibrium at 4 °C for 2 h, after which a dextran–charcoal suspension was added, and the tubes were centrifuged to remove the unbound ligand. Radioactivity (representing the bound fraction of [^{3}H]25(OH)D$_3$) was estimated in the supernatant fraction.

The maximum binding (and hence the DBP concentration, assuming a single class of specific binding sites and a stoichiometric ratio of protein to ligand of 1:1) and the dissociation constant (K_D) of specific binding sites for 25(OH)D$_3$ were determined by plotting total bound [^{3}H]25(OH)D$_3$ on the *y*-axis, and total competing 25(OH)D$_3$ on the *x*-axis. It has previously been demonstrated that most species possess a single specific binding protein (Hay & Watson, 1976; Licht, 1994), and no species has yet been identified to possess a DBP with multiple vitamin D ligand-specific binding sites. Curves describing total binding were created by fitting a non-linear regression equation to the data using the Marquardt-Levenberg algorithm in a commercial graphics software package (Sigma Plot, version 3.0; Jandel Scientific, Hearne Scientific Software, Melbourne, Australia) by the method of Swillens (1995). The regression equation that describes the non-specific binding as a linear function and the specific binding using a hyperbolic curve was chosen because a relatively high fraction of ligand is bound in the assay procedure (thus violating the rules of standard binding regression analysis).

Plasma biochemistry

The concentration of 25(OH)D$_3$ in plasma was measured by competitive protein binding analysis, a modification of a method described previously by Mason & Posen (1977). Human plasma diluted in barbital acetate buffer (pH 8·6) was used as a source of binding protein. The plasma concentration of 1,25(OH)$_2$D$_3$ was determined by

a competitive protein binding assay as described by Reinhardt & Hollis (1986) with modifications as described by Seshadri *et al.* (1985). Plasma Ca concentration was measured using the Arsenazo III method (Trace Scientific, Melbourne, Victoria, Australia) using a discrete biochemical analyser (Roche Cobas Mira, Castle Hill, New South Wales, Australia). Total plasma protein concentration was measured using the Bradford method (Bradford, 1977), and plasma albumin concentration by the Bromocresol Green method, using a commercial kit (Trace Scientific).

Statistical analysis

Results are presented as the mean values and standard deviations. Where data approximated a normal distribution and variances were not significantly different, variation between groups was determined using ANOVA. Pairwise comparisons were made using the method of least significant difference. Where the assumptions of ANOVA were not satisfied, non-parametric tests were employed: Mann-Whitney tests were used to determine significant differences in distribution between pairs. Statistical analyses were performed using the Minitab software package, version 12.21 (State University, Philadelphia, PA, USA).

Test statistics are provided for ANOVA (F), Mann-Whitney (W), and Student's t test (t). Subscripted values indicate degrees of freedom. Where no test statistic is provided, differences were investigated using the method of least significant difference. Significance was inferred where $P<0{\cdot}05$.

Results

Rats on dietary calcium restriction

There was no difference in mean growth rate or in mean final body weight between the two groups of rats. Rats on the 0·2 g Ca/kg diet had a significantly lower plasma total Ca concentration (Table 1) than those on the 6·4 g Ca/kg diet (t_{13} 6·67, $P<0{\cdot}0001$). Furthermore, rats on the 0·2 g Ca/kg diet had a significantly higher concentration of circulating $1,25(OH)_2D_3$ (Table 1) than rats on the 6·4 g Ca/kg diet (W 84·0, $P=0{\cdot}0015$). All the rats were found to be vitamin D deficient at the end of the experiment, as judged by a circulating $25(OH)D_3$ concentration (Table 1) less than the lower limit of detection of the assay (6 nmol/l). However, the lipid extract of plasma from rats on the 0·2 g Ca/kg diet was significantly less able to displace $[^3H]25(OH)D_3$ from specific binding sites in a solution of human plasma than that of rats on the 6·4 g Ca/kg diet (t_{14} 4·87, $P=0{\cdot}0002$). This would suggest that the rats on the low-Ca diet had a lower concentration of plasma $25(OH)D_3$ than those on the high-Ca diet. No significant differences could be detected between the two groups of rats in either the concentration of plasma DBP or its affinity for $25(OH)D_3$ (Table 1), nor were there any differences between the groups in either total plasma protein, or in plasma albumin concentration.

Dietary energy and protein restriction in rats

Rats on the control diet demonstrated a steady increase in body weight over the entire 21 d experimental period (Fig. 1). Those on the protein-deficient diet demonstrated an obvious decrease in growth rate after 1 week and showed no growth during the second week of the restricted period. Energy-deprived rats showed a similar decrease in body-weight gain, although they did continue to grow throughout the experimental period. When rehabilitated, rats on the energy-restricted diet showed a rapid recovery. Their body weights increased to match the control rats within 2 d, from which point growth rate was not discernibly different from controls. Protein-deprived rats likewise demonstrated an immediate increase in growth rate during the rehabilitation period, however, although their growth rate became similar to that of the control rats, their mean body weight remained significantly lower than that of control rats over the remainder of the experimental period.

The ANOVA indicated that the liver weight index varied

Table 1. Effects of differing dietary calcium concentrations on various biochemical variables in growing rats†

(Mean values and standard deviations for sixteen rats per group)

	0·2 g Ca/kg diet		6·4 g Ca/kg diet	
	Mean	SD	Mean	SD
Plasma DBP (μmol/l)	2·49	0·40	2·64	0·35
K_D $25(OH)D_3$ (nM)	0·13	0·010	0·20	0·15
Total plasma protein (g/l)	78·9	3·91	76·8	4·21
Plasma albumin (g/l)	26·9	2·76	25·9	3·19
Plasma total calcium (mmol/l)	1·95*	0·16	2·65	0·24
Plasma $1,25(OH)_2D_3$ (pmol/l)	1044*	180	290	40
Bound $[^3H]25(OH)D_3$ (kBq)‡	1·533*	0·100	1·335	0·057

DBP, vitamin D binding protein; K_D $25(OH)D_3$, dissociation constant of DBP for 25-hydroxycholecalciferol; $1,25(OH)_2D_3$, 1,25-dihydroxycholecalciferol; $[^3H]25(OH)D_3$, 25-hydroxy[26,27-methyl-^{3}H]cholecalciferol.

Mean values were significantly different from those of the 6·4 g Ca/kg group *$P<0{\cdot}05$.

† For details of diets and procedures, see p. 134.

‡ Bound $[^3H]25(OH)D_3$ is an indicator of the ability of $[^3H]25(OH)D_3$ to compete with $25(OH)D_3$ in the plasma sample, a high value indicating lower plasma $25(OH)D_3$ concentrations, and is used instead of $25(OH)D_3$ concentration as these values were below the detection limit of the assay employed.

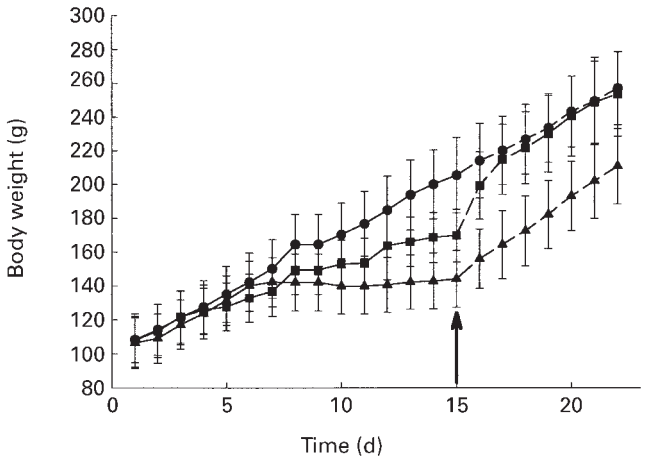

Fig. 1. Body weights of rats (sixteen per group) fed on a control diet (●), energy-restricted diet (■) or protein-restricted diet (▲). For details of diets and procedures, see p. 134. Rats were weighed daily from the time that the experimental diet was started. ↑, Rats fed the energy- and protein-restricted diets were put back on the rehabilitation (control) diet.

significantly with diet ($F_{2,42}$ 5·57, $P=0·007$), and between restricted and rehabilitation periods ($F_{1,42}$ 97·69, $P<0·001$). There was also a significant interaction between these factors ($F_{2,42}$ 5·97, $P=0·005$). At the end of the restricted period, the liver weight index of both energy-deficient ($P<0·001$), and protein-deficient ($P<0·05$) rats was significantly lower than that of control rats (Table 2), although the difference was greater for energy-deprived animals, in which mean liver weight index was also significantly lower than that of protein-deficient rats ($P<0·02$). In all three groups, the liver weight index increased significantly by the end of the rehabilitation period ($P<0·001$). The liver weight index of control rats was no longer significantly different from energy-deprived rats, although the difference between control rats and protein-deprived rats after the rehabilitation period approached significance ($P<0·1$).

The ANOVA indicated significant variation in total plasma protein with regard to diet ($F_{2,42}$ 7·90, $P=0·001$) and between the restricted and rehabilitation periods ($F_{1,42}$ 10·24, $P=0·003$). A significant interaction between the factors was also observed ($F_{2,42}$ 3·86, $P=0·029$). A similar variation in plasma albumin concentration was indicated due to diet ($F_{2,42}$ 9·26, $P<0·001$) and to period ($F_{1,42}$ 18·14, $P<0·01$), with a similar interaction between the factors ($F_{2,42}$ 5·23, $P=0·009$).

Total plasma protein concentration and plasma albumin concentration were both significantly depressed in rats fed the protein-restricted diet when compared with control and energy-restricted rats ($P<0·01$). However, after the rehabilitation period, the total protein concentration and plasma albumin concentration of the protein-deprived rats was significantly greater ($P<0·01$) compared with those during the restricted period, and was no longer significantly different from control rats, or rehabilitated energy-deprived rats.

The ANOVA indicated a significant variation in plasma DBP concentration (Table 2) due to time ($F_{1,42}$ 16·15, $P<0·001$), while the interaction between diet and time approached significance ($F_{2,42}$ 3·09, $P=0·056$). At the end of the restricted period, plasma DBP concentration was significantly lower in protein-deficient rats when compared with control rats ($P<0·01$). Energy-deprived

Table 2. Effect of dietary energy and protein on various biochemical variables and on liver weight as corrected for body weight (liver weight index) in growing rats during dietary protein or energy restriction (restricted), and during a return to a control diet (rehabilitation)§

(Mean values and standard deviations)

		Energy deprived			Protein deprived			Control		
		Mean	SD	*n*‖	Mean	SD	*n*‖	Mean	SD	*n*‖
DBP (μmol/l)	Restr	4·03	0·71	10	3·63*	0·69	10	4·53	0·86	10
	Rehab	5·04‡	0·67	6	5·00‡	0·47	6	4·66	0·58	6
K_D 25(OH)D_3 (nM)	Restr	0·074	0·20	10	0·064	0·023	10	0·078	0·019	10
	Rehab	0·089	0·012	6	0·080	0·019	6	0·096	0·015	6
LWI	Restr	129*	25	10	162*†	40	10	199	30	10
	Rehab	273‡	31	6	236‡	19	6	271‡	46	6
Total plasma protein (g/l)	Restr	43·5	2·4	10	35·7*†	7·5	10	44·9	2·4	10
	Rehab	45·5	0·8	6	44·2‡	1·5	6	45·9	3·1	6
Albumin (g/l)	Restr	29·0	2·5	10	23·1*†	4·7	10	30·1	1·7	10
	Rehab	30·7	0·4	6	30·4‡	1·2	6	31·7	2·4	6
Plasma 25(OH)D_3 (nmol/l)	Restr	12·1	2·1	10	16·3	3·6	9	13·8	5·8	10
	Rehab	12·3	2·2	6	14·0	3·0	6	10·7	2·5	6
Plasma 1,25$(OH)_2D_3$ (pmol/l)	Restr	604	318	9	259*†	99	9	549	147	10
	Rehab	480*	165	6	438*	53	4	802‡	269	6
Plasma total calcium (mmol/l)	Restr	2·4*	0·12	10	2·5	0·11	9	2·6	0·21	10
	Rehab	2·5*	0·3	6	2·5*	0·14	6	2·2‡	0·08	6

DBP, vitamin D binding protein; Restr, restricted-feeding period; Rehab, rehabilitating-feeding period; K_D 25(OH)D_3, dissociation constant of DBP for 25(OH)D_3; 25(OH)D_3, 25-hydroxycholecalciferol; 1,25(OH)D_3, 1,25-dihydroxycholecalciferol; LWI, liver weight index.

Mean values were significantly different from those of the control group: *$P<0·05$.

Mean values were significantly different from those of the energy-deprived group: †$P<0·05$.

Mean values were significantly different from those during the restricted feeding period: ‡$P<0·05$.

§ For details of diets and procedures, see Table 1 and p. 134.

‖ Number of samples analysed; in some cases limited availability of plasma restricted the number of analyses performed on each sample.

rats demonstrated a plasma DBP concentration somewhat intermediate between the other two groups, but not significantly different from either.

Both protein- and energy-deprived rats had a significantly greater DBP concentration after rehabilitation when compared with non-rehabilitated rats killed 1 week earlier ($P<0{\cdot}01$). There was no difference in plasma DBP concentration between control rats at either sampling period, nor were there differences in DBP concentration among the three groups after the rehabilitation period. Although the ANOVA indicated a significant variation in K_D for $25(OH)D_3$ (Table 2) due to time ($F_{1,42}$ 8·38, $P=0{\cdot}006$), there was no interaction between the two factors, and no significant differences were detected within each dietary regimen nor within each time period.

The ANOVA indicated that there was no significant variation in plasma $25(OH)D_3$ concentration (Table 2) among rats on different diets, nor between the two different time periods. However, there was significant variation in plasma $1,25(OH)_2D_3$ concentration (Table 2) due to diet ($F_{2,38}$ 8·17, $P=0{\cdot}001$), and a significant interaction for this variable between diet and time ($F_{2,38}$ 3·44, $P=0{\cdot}042$).

Protein-deprived rats at the end of the restricted period had a significantly lower $1,25(OH)_2D_3$ concentration than rats fed either of the other two diets ($P<0{\cdot}01$). Control rats demonstrated a significantly higher plasma $1,25(OH)_2D_3$ concentration at the end of the experiment than at the end of the restricted period ($P<0{\cdot}05$). After rehabilitation, the mean plasma $1,25(OH)_2D_3$ concentration of protein-restricted rats was no longer significantly different from that of the rats that had been rehabilitated from the energy-restricted diet.

The ANOVA did not indicate any significant variation in plasma total Ca (Table 2) due to either diet, or time, although there was a significant interaction between these two factors with regard to this variable ($F_{2,41}$ 0·75, $P<0{\cdot}001$). At the end of the restricted period, the plasma Ca concentration of the control rats was significantly higher than that of the energy-deprived rats ($P<0{\cdot}02$). While there was a significant decline in the plasma Ca concentrations of the control rats during the rehabilitation period ($P<0{\cdot}01$), such that the control rats demonstrated a significantly lower plasma total Ca concentration than either of the rehabilitated rats ($P<0{\cdot}01$), there were no significant differences in the plasma Ca concentrations before or after rehabilitation in either energy- or protein-deficient rats.

Discussion

Growing rats fed a Ca-deficient diet demonstrated an increased plasma $1,25(OH)_2D_3$ concentration and decreased plasma concentration of $25(OH)D_3$ as previously reported (Clements *et al.* 1987), when compared with rats fed a complete diet. However, there was no evidence to support the hypothesis that increased $1,25(OH)_2D_3$ influenced either the concentration of the plasma DBP, or its affinity for $25(OH)D_3$. These results support previous observations that the concentration of DBP is unaltered during the course of various diseases of mineral homeostasis (Jacobs & Ray, 1968; Bouillon *et al.* 1977*a*; Brissenden & Wilson Cox, 1978), and suggest that $1,25(OH)_2D_3$ is not likely to act as a regulator of the properties of this plasma protein. Any changes in the clearance rate of $25(OH)D_3$ from the circulating pool are therefore more likely to be the result of altered enzyme activity, as previously proposed (Halloran *et al.* 1986; Clements *et al.* 1987; Reinholz & DeLuca, 1998).

On the other hand, plasma concentrations of DBP, as with those of total protein and albumin, were found to be sensitive to dietary protein deficiency. The relative proportions (by mass) of total plasma protein represented by DBP and albumin were the same in control and protein-deprived animals, suggesting that the influence of protein deprivation on the circulating concentrations of DBP are not specific, but likely to be the result of a generalised effect on plasma protein. Amino acid supply is the most important known determinant of plasma albumin concentration (Hoffenberg, 1972; Rothschild *et al.* 1975; Waterlow *et al.* 1978; Sainz *et al.* 1986), although the effects of dietary protein deficiency on plasma protein concentration are known to vary for different plasma proteins (Hoffenberg, 1972; Tavill, 1972; Waterlow *et al.* 1978; Straus, 1994).

The effects of acute starvation on plasma protein are less clear, as, surprisingly, plasma albumin concentration is maintained in energy-deficient children (Waterlow *et al.* 1978). A similar finding was made in the current study in the rats fed on restricted amounts of the control diet. However, there is some suggestion that energy deficiency might influence the concentration of DBP in the plasma. There was a significant increase in plasma DBP concentration in energy-deprived rats associated with their rehabilitation on a control diet, even though the DBP concentration of energy-deprived and control rats did not differ significantly. However, unlike the case with dietary protein restriction, any decrease in plasma DBP associated with energy restriction is not accompanied by a similar effect on either total plasma protein or plasma albumin concentration, suggesting that the effect of energy restriction on DBP concentration might be more selective.

Previous studies have reported that limitations to the rate of hepatic protein production due to dietary protein restriction are more severe when dietary energy is adequate (Coon & Iob, 1964; Sakuma *et al.* 1987; Kaysen, 1988). It is likely that an altered balance between dietary energy and protein is responsible for the metabolic patterns regulating plasma protein turnover, and that energy supply can be rate limiting in hepatic protein turnover, although this effect might be specific to particular proteins.

The mechanisms by which limitations in the dietary supply of protein and energy influence the circulating concentration of DBP are unknown, and should be the focus of further study. Alterations to the concentration or stability of the DBP transcript may be involved, or perhaps alterations to ribosomal concentration or activity, as previously demonstrated for decreased albumin production in response to malnutrition (Waterlow *et al.* 1978; Sakuma *et al.* 1987). Nutritionally-influenced endocrine factors such as thyroid hormone, growth hormone and glucocorticoids, known to play a permissive role in maintaining basal albumin synthesis rates (Morgenthaler & Nydegger, 1984; Whicher & Spence, 1987; Kaysen, 1988), may also be involved in

regulating DBP synthesis. The case for increased plasma clearance is less convincing. It has been suggested that molecular alteration, and cellular recognition of these changes might be involved in the clearance of the plasma proteins (Tavill, 1972; Gregoriadis, 1975). Post-translational differences in the DBP have been shown to influence its functions (Fraser & Emtage, 1976; Yamamoto *et al.* 1996), so the unchanged affinity of DBP for $25(OH)D_3$ with protein and energy deficiencies suggests that changes in cellular recognition due to post-translational alteration are unlikely. An increased activity of non-specific proteolytic enzymes (Waterlow *et al.* 1978) should preserve the relative rates of albumin and DBP clearance. As DBP is ordinarily cleared more rapidly from the blood than albumin, a more profound effect would be expected on the plasma concentration of DBP than albumin (Haddad *et al.* 1981), yet their ratios remained the same in protein-deficient rats as in control rats, and while DBP concentration was affected by energy deficiency, albumin concentration was not.

Whatever the mechanisms for the altered concentration of DBP in the plasma of malnourished animals, the resulting effects on the physiology of vitamin D and on Ca homeostasis are likely to be significant. Protein-deficient rats had decreased ability to maintain elevated concentrations of $1,25(OH)_2D_3$ in Ca deficiency when compared with other rats, and this ability appeared to return after rehabilitation on the control diet. The reason for this is not known. One possibility is that the activity of the 1-hydroxylase enzyme is diminished in these animals. This may be a direct result of malnutrition, or a consequence of decreased growth rate as Ca requirements diminish. However, it is unlikely that this is the entire explanation for the observed effect, as the plasma concentrations of $1,25(OH)_2D_3$ in energy-deprived rats were not decreased compared with controls, despite a suppressed growth rate. The lower circulating concentrations of DBP in protein-deprived rats is likely to be contributing to the lower $1,25(OH)_2D_3$ concentrations. With less DBP in the blood, the lower number of specific binding sites would allow a greater proportion of free $1,25(OH)_2D_3$ to be available for cellular uptake, and hence a faster clearance rate of the metabolite from the blood. A similar mechanism has been proposed in the pathophysiology of vitamin D toxicity, in which the biological effects of the toxic overload are brought about by displacement of $1,25(OH)_2D_3$ by an absolute increase in $25(OH)D_3$ (Pettifor *et al.* 1995; Safadi *et al.* 1999). It is possible that this mechanism contributes to the decreased availability of $1,25(OH)_2D_3$ to the developing skeleton, which would lead to a predisposition to developmental bone diseases.

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