Calcium-binding protein and vitamin D metabolism in experimental protein malnutrition

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1. Intestinal and renal vitamin D-dependent calcium-binding protein (CaBP) activity and cholecalciferol metabolism were investigated in the protein-deficient rat (40 g casein/kg diet) and in control animals (200 g casein/kg diet). Compared to control animals, 3 weeks of protein deprivation resulted in consistently reduced intestinal CaBP activity, while renal CaBP activity was not significantly altered.

2. Intestinal CaBP activity was greatly reduced in rats fed on diets deficient in both protein and vitamin D. CaBP activity was doubled by cholecalciferol administration, but did not reach control values. The rate of conversion of intravenously injected [³H]cholecalciferol to 25-hydroxycholecalciferol (25-HCC) and the disappearance rates of plasma 25-HCC were similar in the two groups of animals.

3. It is concluded that in the protein-deficient rat: (*a*) intestinal CaBP activity is reduced; (*b*) coexistent vitamin D deficiency reduces intestinal CaBP activity still further, but the intestinal mucosa retains the potential to respond to administered cholecalciferol: (*c*) hepatic and probably renal metabolism of cholecalciferol appear to be normal; (*d*) reduced CaBP is likely to be the result of reduced CaBP synthesis as a consequence of deficient amino acid substrate.

Subnormal bone growth and mineralization are frequently found in human protein-energy malnutrition (PEM) in infants, but histological and radiological rickets is an uncommon association (Higginson, 1954; Reichman & Stein, 1968). Little is known about calcium metabolism in human PEM, while in animal models of the disorder the results of investigations have been conflicting (Platt & Stewart, 1962; Jha, Deo & Ramalingaswami, 1968; Shenolikar & Narasinga Rao, 1968). The protein-deficient rat has reduced intestinal Ca absorption, 'Ca pool' and Ca accretion by bone (Le Roith & Pimstone, 1973).

Vitamin D-dependent Ca-binding protein (CaBP) (Wasserman & Taylor, 1966) has been isolated from most organs involved in Ca homoeostasis: intestinal mucosa cells (Taylor & Wasserman, 1970), kidney (Taylor & Wasserman, 1972) and parathyroid glands (Oldham, Arnaud & Jowsey, 1973), but not bone. As intestinal CaBP has been shown to be intimately associated with Ca absorption (Taylor & Wasserman, 1969; MacGregor, Hamilton & Cohn, 1970), reduction of intestinal CaBP levels in protein deficiency could explain some of the abnormalities in calcium kinetics observed in the protein-deficient rat model cited above. This possibility was investigated in the present work.

* For reprints.

MATERIALS AND METHODS

Animals and diets. The protein-deficient rat model used in these experiments is described in detail elsewhere (Stead & Brock, 1972). Male, weanling (21-d-old, 25–35 g) Wistar rats were fed *ad lib.* on either low-protein (40 g casein/kg) or control (200 g casein/kg) diets for 21–25 d. Additional carbohydrate was substituted for the protein in the diets of the malnourished animals; the diets were otherwise identical, isoenergetic and adequate in minerals and vitamins (Harper, 1959). Animals fed on both diets usually received adequate amounts of vitamin D, 50 μg cholecalciferol equivalent/kg diet. However, in certain groups of animals on both diets, vitamin D was withheld and these animals were housed in the dark. Four diets were used: protein-deficient and control, both given with and without supplementary cholecalciferol.

CaBP experiments

Expt 1. Five paired groups of three to six animals each, fed on either the protein-deficient or control diet, were studied after 21–25 d. All diets contained adequate amounts of cholecalciferol. CaBP activities of intestinal and renal extracts from each group of animals were estimated.

Expt 2. A further three paired groups of six rats were fed on either the protein-deficient or control diet, but in addition, both diets were deficient in vitamin D. After 18 d, the rats given both low-protein and control diets were subdivided into groups of three each; 100 μg cholecalciferol in propylene glycol was administered by intraperitoneal injection to one subgroup of protein-deficient rats and one subgroup of control rats; the remaining three rats in each subgroup were untreated.

After 3 d the intestinal CaBP activity was determined in all four subgroups as described below. The estimates of CaBP activity in both experiments were expressed as a percentage of the CaBP activity of the extracts of intestine from the rats fed on the control cholecalciferol-supplemented diet.

Estimation of calcium-binding protein (CaBP). On the day of the experiment, groups of rats were anaesthetized with diethyl ether and bled to death. CaBP-rich extracts of intestinal mucosa or kidney or both were prepared using the method described by Wasserman, Corradino & Taylor (1968). The first anatomical loop of the small intestine (about 100 mm in the protein-deficient rats, 180–200 mm in the control rats) was excised; the intestine was immediately flushed with ice-cold buffer (0·0137 M-Tris, 0·12 M-NaCl, 4·74 mM-KCl and 1mM-mercaptoethanol, pH 7·4), slit open and the mucosa stripped from the muscle layers with a glass slide. The mucosa from each group of animals was pooled and weighed, and after the addition of 5·0 ml buffer/g mucosa, the mixture was homogenized at very high speed for 30 s. The kidneys were similarly pooled, weighed and homogenized. The homogenates from both intestine and kidney were centrifuged at 38000 g for 20 min in a refrigerated ultracentrifuge. Heat-labile proteins in the supernatant fractions were precipitated by heating to 60° for 10 min in a water bath; the partially 'purified' cooled supernatant fraction was then again centrifuged for 20 min at 38000 g. The CaBP activity in both the resultant intestinal and kidney supernatant-fraction extracts was assayed

by an 'ion-exchange' procedure (Wasserman *et al.* 1968). The test sample (1 ml) was pipetted into a small tube, 0·2 ml of the Ca-binding resin (Chelex 100, Bio-Rad Laboratories, Richmond, California 94804) suspension in buffer (about 0·1 ml packed resin and 0·1 ml buffer) was added, followed by 0·1 ml ^{45}Ca in buffer (0·05–0·1 μCi; The Radiochemical Centre, Amersham). The tube was vigorously stirred for 15 s with a vortex mixer and then centrifuged for 10 min. Portions (0·2 ml) of the supernatant fraction were pipetted into 5 ml scintillation fluid (1,4-bis-(2-(methyl-5-phenyloxazolyl)) benzene (POPOP) 100 mg, 2,5-diphenyloxazole (PPO) 5 g and 'Bisolve' 200 ml, in 1 l toluene) (Beckman Instruments Inc., Fullerton, California), and counted in a Beckman liquid scintillation counter (Beckman Instruments Inc.). CaBP activity in each supernatant fraction was assayed in duplicate.

Ca-binding activity of the CaBP supernatants was expressed as:

$$\frac{^{45}\text{Ca in supernatant/mg protein}}{^{45}\text{Ca in resin}}$$

(Wasserman *et al.* 1968).

Analytical procedures. Serum albumin from the rats was estimated by the bromocresol green method (Doumas, Watson & Biggs, 1971) and the total protein content of the supernatant fractions was determined by the Folin method of Lowry, Rosebrough, Farr & Randall (1951), using crystalline bovine albumin standards. Serum Ca concentration was determined by a modification of the EDTA method of Bett & Fraser (1958).

Studies of cholecalciferol metabolism

These experiments were all done using vitamin D-deficient rats, either on low-protein or control diets. Animals of both groups were divided into subgroups of three to six and killed 2, 4, 8, 12 and 16 h after intravenous injection of [1,2-^{3}H]cholecalciferol (specific activity 540 mCi/mmol; New England Nuclear, Boston, USA) dissolved in 0·2 ml Intralipid (Vitrum Pharmaceuticals, Stockholm, Sweden). The mean dose of cholecalciferol was 1·3 pmol/g body-weight for the rats given the low-protein diets and 2·2 pmol/g body-weight for those given the high-protein diets. Groups of animals on each diet were bled to death and studied at the stated time periods after the injection. Blood and livers from each stage of the experiment were pooled and the livers homogenized. Plasma and liver lipids were extracted by the method of Bligh & Dyer (1959); the chloroform-lipid extracts were evaporated by rotary evaporation, and the remaining lipid was dissolved in redistilled hexane–chloroform (35:65, v/v) and chromatographed on a 700 mm × 9·0 mm column of LH Sephadex (Pharmacia Ltd, Uppsala, Sweden) slurried in the same solvent mixture (Holick & DeLuca, 1971). Fractions (4 ml) were collected, evaporated in a stream of air, and dissolved in scintillation fluid (PPO, 5 g, POPOP, 100 mg in 1 l toluene) and the radioactivity was counted in a liquid scintillation counter (Beckman Instruments Inc.) with an efficiency of 33 %.

The radioactivity in fractions corresponding to the chromatographed peaks was expressed as the percentage of the total injected dose/ml blood or /g liver, and radioactivity in the fraction corresponding to plasma [1,2-^{3}H]25-HCC (see p. 573) was

Table 1. *Body-weights, serum albumin and calcium concentrations of rats given isoenergetic diets containing* 200 *or* 40 g casein/kg *with or without supplementary cholecalciferol for* 21 d

(Mean values with their standard errors for six animals/group)

	Dietary protein content (g casein/kg)							
	200				40			
	+		−		+		−	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Initial body-wt (g)	30·0	1·4	30·0	0·5	29·8	1·1	30·5	0·5
Body-wt after 21 d on diet (g)	79·8	3·3	77·5	10·3	28·7	1·7***	30·0	0·6***
Serum albumin after 21 d on diets (g/l)	4·1	0·2	3·9	0·6	0·9	0·2***	0·7	0·2***
Serum Ca after 21 d on diets (mmol/l)	2·6	0·1	2·5	0·1	1·6	0·1***	1·5	0·2***

Values for rats given 40 g casein/kg diet were significantly different from those of rats given 200 g casein/kg diet: *** $P < 0.0005$; cholecalciferol supplement v. no cholecalciferol comparisons were not significantly different.

Table 2. *Expt* 1. *Calcium-binding protein (CaBP) activity in extracts of intestines and kidneys from vitamin D-sufficient rats given isoenergetic diets containing* 200 *or* 40 g casein/kg

(Mean values for six animals/group)

Group no.	Casein (g/kg diet)	CaBP activity			
		Intestinal		Kidney	
		Absolute*	% of control	Absolute*	% of control
1	200	6·42	100	1·62	100
	40	4·06	63·3	1·58	98·0
2	200	9·42	100	3·73	100
	40	6·25	66·4	3·55	95·3
3	200	2·99	100	—	—
	40	1·98	66·3	—	—
4	200	6·15	100	1·84	100
	40	4·51	73·3	2·09	113·6
5	200	3·12	100	2·13	100
	40	2·64	84·4	1·73	83·8

* For details of calculation of CaBP activity, see p. 571.

expressed as a percentage of the total extracted plasma radioactivity. Both were plotted semi-logarithmically v. time.

RESULTS

General features of the animal model. Rats fed on the low-protein diet failed to grow, became hypoalbuminaemic and hypocalcaemic (Table 1) with fatty infiltration of the liver. Some developed ascites. The animals fed on the control diet grew normally.

Table 3. *Expt 2. Calcium-binding protein (CaBP) activity in extracts of intestines from rats given vitamin D-deficient, isoenergetic diets containing* 200 *or* 40 g casein/kg *with or without cholecalciferol treatment*

Group no.	Casein (g/kg diet)	Cholecalciferol treatment*	CaBP activity	
			Absolute†	(% of control (200 + cholecalciferol))
1	200	+	13·22	100
		−	6·69	50·5
	40	+	4·45	33·7
		−	1·81	13·7
2	200	+	30·10	100
		−	15·37	51·1
	40	+	8·81	29·2
		−	3·25	25·6
3	200	+	12·68	100
		−	8·38	66·1
	40	+	3·25	25·6
		−	2·24	17·7

* 100 μg Cholecalciferol injected intraperitoneally 72 h before slaughter.
† For details of calculations of CaBP activity, see p. 571.
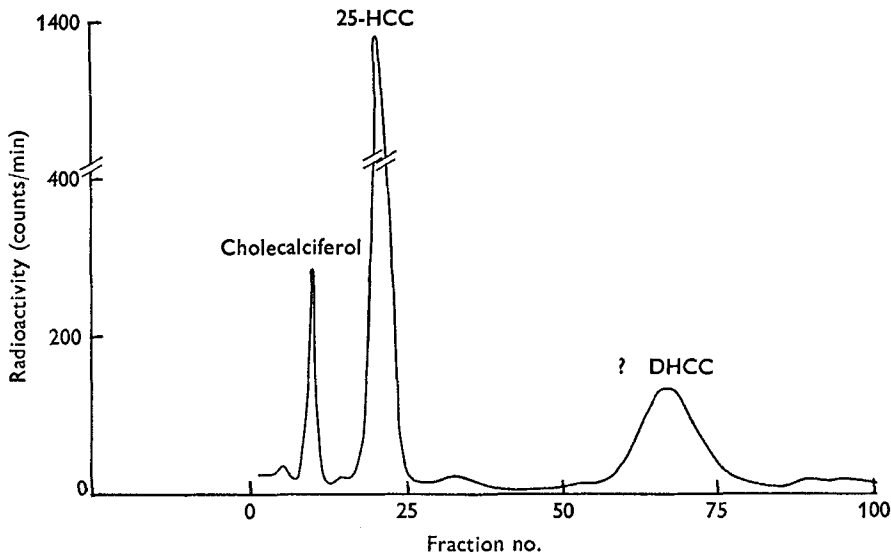
Fig. 1. Elution pattern, after fractionation on a LH Sephadex column, of [³H]cholecalciferol, [³H]25-hydroxycholecalciferol (25-HCC) and a third metabolite, presumed to be a dihydroxy metabolite (DHCC), in pooled plasma and liver lipid extracts from vitamin D-deficient rats given isoenergetic diets containing 200 or 40 g casein/kg after an intravenous injection of [1,2-³H]cholecalciferol (2·2 or 1·3 pmol/g body-wt for rats receiving 200 or 40 g casein/kg diet respectively).

Neither group of vitamin D-deficient animals showed rickets judged by the silver nitrate 'line test' (US Pharmacopeia, xv, 1955).

Expt 1. CaBP in the intestine and kidney of vitamin D-sufficient rats. CaBP activities of intestinal mucosal and renal supernatant fractions are shown in Table 2. The intestinal CaBP activities of the rats fed on the low-protein diet were consistently lower than
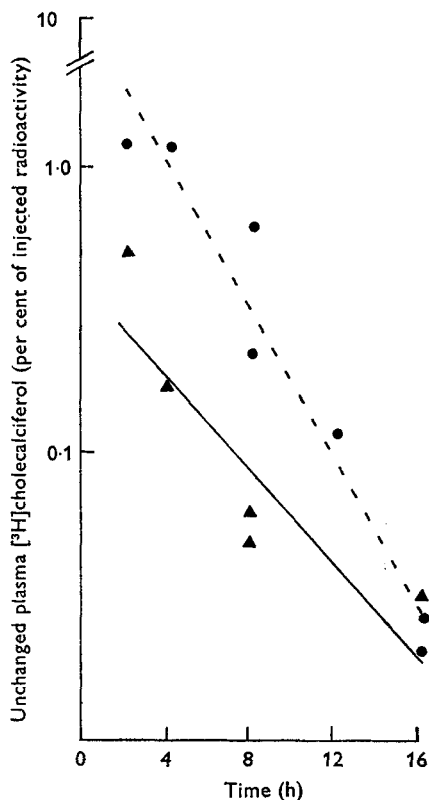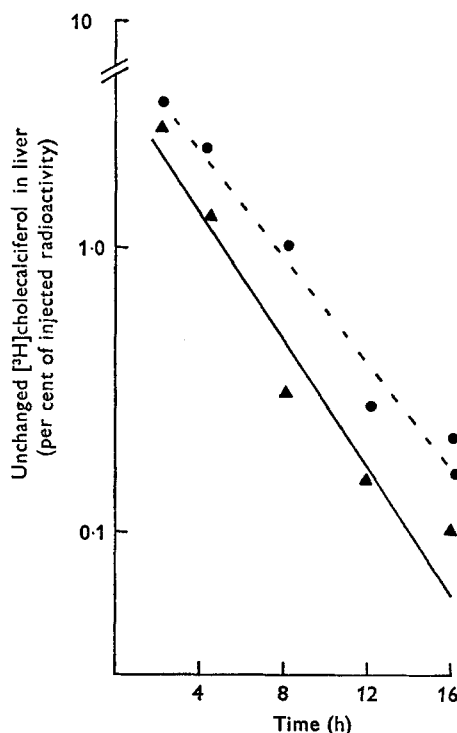
Fig. 2. Semilogarithmic plot of the disappearance of [³H]cholecalciferol from the pooled plasma from groups of three–six vitamin D-deficient rats given isoenergetic diets containing 200 (▲—▲) or 40 g casein/kg (●–––●), after an intravenous injection of [1,2-³H]cholecalciferol (2·2 or 1·3 pmol/g body-wt for rats receiving 200 or 40 g casein/kg diet, respectively) at 0 h.

Fig. 3. Semilogarithmic plot of the disappearance of [³H]cholecalciferol from the pooled livers from groups of three–six vitamin D-deficient rats given isoenergetic diets containing 200 (▲—▲) or 40 g casein/kg (●–––●) after an intravenous injection of [1,2-³H]cholecalciferol (2·2 or 1·3 pmol/g body-wt for rats receiving 200 or 40 g casein/diet, respectively) at 0 h.

those of the control animals; mean value ($\pm$ SE) for the five groups $70\cdot7 \pm 3\cdot4\%$ of the control value, when corrected for protein concentration.

There was no consistent difference between the CaBP activity in the kidney supernatant fractions from groups of low-protein and control animals in four experiments (Table 2).

Expt 2. The effect of cholecalciferol treatment on intestinal CaBP in vitamin D-deficient rats. Control rats fed on the vitamin D-deficient diet had a mean CaBP activity which was $55\cdot9\%$ of the value for the cholecalciferol-treated control group. Rats fed on the low-protein, vitamin D-deficient diets had a mean CaBP activity which was $15\cdot4\%$ of the control value, while after cholecalciferol supplementation CaBP activity increased to $29\cdot5\%$ of the control value. Thus the effect of cholecalciferol on CaBP activity in the vitamin D-deficient rats was similar in both protein-sufficient (81% increase in activity) and protein-deficient animals (96% increase in activity).
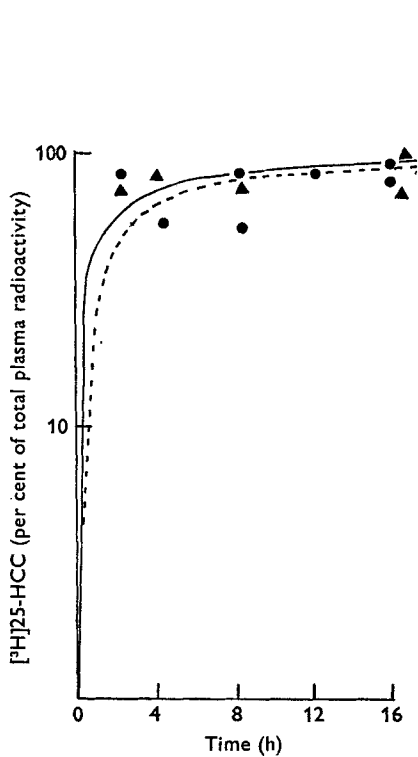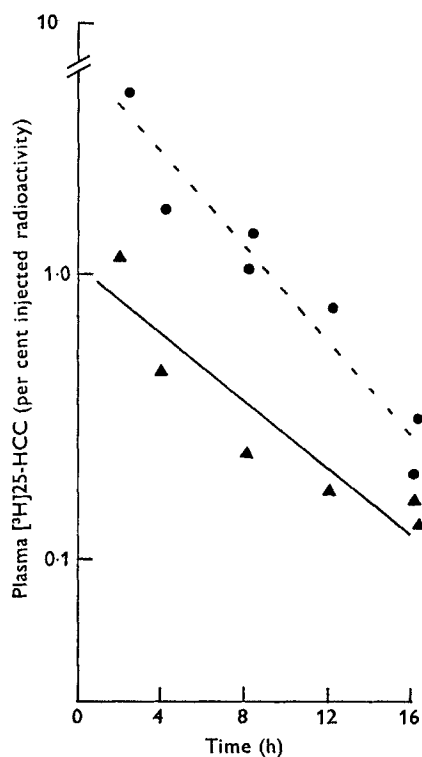
Fig. 4.   Fig. 5

Fig. 4. The percentage of total radioactivity chromatographing as 25-hydroxycholecalciferol (25-HCC) in pooled plasma from groups of three–six vitamin D-deficient rats given isoenergetic diets containing 200 (▲—▲) or 40 g casein/kg (● – – ●) after an intravenous injection of [1,2-³H]cholecalciferol (2·2 or 1·3 pmol/g body-wt for rats receiving 200 or 40 g casein/kg diet, respectively) at 0 h. Note the rapid appearance of this metabolite in both groups.

Fig. 5. Semilogarithmic plot of the disappearance of [³H]25-hydroxycholecalciferol (25-HCC) from the pooled plasma from groups of three–six vitamin D-deficient rats given isoenergetic diets containing 200 (▲—▲) or 40 g casein/kg (● – – ●) after an intravenous injection of [1,2-³H]cholecalciferol (2·2 or 1·3 pmol/g body-wt for rats receiving 200 or 40 g casein/kg diet, respectively) at 0 h.

Cholecalciferol metabolism. The elution volume on the LH Sephadex column of [³H]cholecalciferol was 50 ml and of [³H]25-HCC was 100 ml. When both plasma and liver lipid extracts were chromatographed, two major peaks were found which had elution volumes corresponding exactly to those of [³H]cholecalciferol and [³H]25-HCC, and were therefore presumed to be unchanged cholecalciferol and the metabolite 25-HCC respectively (Fig. 1).

The rates of disappearance from plasma and liver of unchanged cholecalciferol were exponential between 2 and 16 h. Plotted semilogarithmically, the computed best lines showed that the disappearance was no slower in the protein-deficient animals than in controls (Figs. 2, 3). 25-HCC rapidly became and remained the main circulating cholecalciferol metabolite, constituting over 80 % of plasma radioactivity at 2 h after injection in both the control and protein-deficient rats (Fig. 4).

The disappearance rates of plasma 25-HCC were likewise similar in both groups (Fig. 5).

Finally, a third peak of radioactivity appeared on chromatography of plasma extracts at an elution volume of 300 ml in both groups (Fig. 1). In the protein-deficient animals this peak was first evident at 8 h after injection, and at 12 h in the control rats. The nature of this peak has not yet been further explored.

DISCUSSION

After 3 weeks on the low-protein diets, the animals were severely protein-deficient, as evidenced by the hypoalbuminaemia and their failure to grow. Fatty infiltration of the liver, an indication of deranged hepatic physiology (Stead & Brock, 1972), was seen in many animals. Hypocalcaemia probably reflected the severe degree of hypo-albuminaemia (Le Roith & Pimstone, 1973). Animals fed on the control diet were normal in all respects.

Protein deprivation for 3 weeks in early-weaned rats resulted in significant diminu-tion of intestinal CaBP activity, confirming an earlier report (Reddy, 1972) and possibly accounting for the decreased Ca absorption found previously in a similar animal model (Le Roith & Pimstone, 1973). The slower turnover of renal proteins (Taylor & Wasserman, 1972) may explain the smaller and inconsistent reduction in renal CaBP seen in these animals after only 3 weeks on the diet.

In considering the cause of the low intestinal CaBP in cholecalciferol-treated, protein-deficient rats, it was important to exclude abnormalities of metabolism or action of exogenous cholecalciferol. When vitamin D deficiency coexisted with protein deficiency, the CaBP of intestinal mucosa extracts was very low indeed, but exogenous cholecalciferol was still capable of virtually doubling its activity; similarly in protein-sufficient, vitamin D-deficient rats CaBP activity was almost doubled after cholecalciferol administration. Thus the potential of the intestinal mucosa to respond to pharmacological doses of cholecalciferol appeared to be good in this model of protein depletion although the absolute levels of the CaBP did not reach normal levels.

Whether the intestinal CaBP activity of the protein-deficient rat was capable of increasing significantly after supplementation with physiological doses of cholecalci-ferol was not investigated. However, when physiological doses of [³H]cholecalciferol were administered intravenously its early (2–16 h) metabolic fate appeared to be similar in both the protein-deficient and control animals. Hydroxylation of the cholecalciferol to 25-HCC occurs in the liver (Horsting & DeLuca, 1969; Ponchon, Kennan & DeLuca, 1969) and in the present study was almost complete at 2 h after injection of cholecalciferol in both groups of animals. Furthermore, 25-HCC, normally the main circulating metabolite of cholecalciferol (Cousins, DeLuca & Gray, 1970), was found to be the main plasma metabolite for the duration of the study in both protein-deficient and control rats, although in many protein-deficient animals there was evidence of hepatic malfunction, hypoalbuminaemia, fatty infiltration and ascites.

Similarly the rapid disappearance of 25-HCC from the plasma of the protein-

deficient and control animals suggests, but does not prove, that the second (renal) hydroxylation of cholecalciferol (Fraser & Kodicek, 1970; Gray, Boyle & DeLuca, 1971; Lawson, Fraser, Kodicek, Morris & Williams, 1971) might also be normal in the protein-deficient animals. This second hydroxylation may even have been accelerated in the protein-deficient group, as implied by the earlier appearance in plasma of a third peak of radioactivity, presumably a dihydroxylated metabolite of cholecalciferol, possibly 1,25-dihydroxycholecalciferol. An identical peak appeared a little later in the control animals. We have no positive evidence for the identity of this metabolite as 1,25-dihydroxycholecalciferol.

Neither hepatic nor, inferentially, renal abnormalities in cholecalciferol metabolism appear to be implicated in the pathogenesis of the reduced intestinal CaBP activity in protein-deficient rats. However, because disorders of kidney metabolism are frequent in PEM (Klahr & Alleyne, 1973) the renal handling of vitamin D in protein deficiency needs further clarification once radioactive cholecalciferol metabolites become more readily available for study. A more likely explanation for the low intestinal CaBP activity in these animals is a shortage of appropriate amino acids. Intestinal mucosa cells have a high rate of turnover (Taylor & Wasserman, 1972) which may render them especially vulnerable to the effects of protein deprivation.

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