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EVIDENCE FOR VITAMIN D DEPENDENT γ-CARBOXYLATION IN OSTEOCALCIN RELATED PROTEINS

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SUMMARY: The content of γ-carboxyglutamic acid (gla) was determined in rat femoral bone and kidney cortex in rachitic and vitamin D treated animals. It was demonstrated that the level of gla is decreased in vitamin D depleted animals both in kidney cortex and femoral bone. Supplementation of vitamin D deprived animals with this vitamin resulted in an increase in the gla concentration to almost normal levels. Also the incorporation of ^{14}C $NaHCO_3$ in renal cortex microsomes from rachitic animals was blocked. It is suggested that the absence of gla resulted from the direct action of vitamin D on mRNA for the Ca-binding protein.

INTRODUCTION: It has been demonstrated that several tissues contain specific Ca^{++} binding proteins in which some residues of glutamic acid are γ-carboxylated ensuring thus the binding capacity for calcium ions and hence the biological function of these proteins (1-3). It has been demonstrated that such proteins are increased after long term feeding of high fat diet in rat aortae (atherocalcin) or kidney cortex (4). They are also present in bone (osteocalcin), dentin or calcified tendons (12,5). There appears to be minimum difference between the γ-carboxyglutamic acid (gla) containing proteins isolated from different tissues, at least in the neighbourhood of the γ-carboxylated residues (3).

By analogy with the traditional subject of γ-carboxylation studies, prothrombin, it was demonstrated that the γ-carboxylation reaction is vitamin K dependent (6). It was also proved that tetracyclines are capable of decreasing the detectable γ-carboxylate both in bone and kidney cortex (7).

The fact that vitamin D is known to play a crucial role in ossification processes prompted our search to reveal whether or not vitamin D might be involved in processing the γ-carboxyglutamate of osteocalcin like proteins. Methodological problems let us to test this hypothesis on the γ-carboxyglutamate containing protein from kidney cortex bearing in mind its structural similarity with osteocalcin (3).

MATERIAL AND METHODS:

Animal and diets. Male Wistar rats were housed six in a cage in a windowless animal room. Experimental animals were kept on a rachitogenic diet the composition of which is shown in Table I (8).

Controls were kept on a standard pelleted diet in a normal room. Both diets were available ad libitum.

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Rats just after weaning were put on the respective dietary regime. According to the dietary regime the animals were divided in four groups:

1) vitamin D deficient (rachitogenic) diet;
2) vitamin D deficient diet supplemented in the last two weeks before killing with 50 I.U./1 g body weight vitamin D_3 and 25 I.U./1 g body weight vitamin D_2;
3) normal diet;
4) normal diet supplemented throughout the experiment by the same doses of vitamin D as the 2. group.

At the end of the feeding period which was 30 days or one year, the animals were killed by decapitation and femoral bones and kidney cortices were used for further study. At this stage the animals kept on vitamin D deficient diet showed gross and histological evidence of rickets. The rachitic animals supplemented with vitamin D showed increased intestinal absorption of calcium.

Tissues investigated. Freshly dissected rat femoral bones (30-40 specimens) were frozen in liquid nitrogen and pulverized to pass a 210 nm sieve. This material was suspended in about 100 ml of double destilled water and the liquid was changed twice to remove fat and residues of adhering tissue (24 hours, 4°C). Then the suspension was dialysed against 0.5 M EDTA 500 ml (pH 8 at 4°C) for two weeks. The soluble nondialysable fraction was removed solids by centrifugation at 20 000 g for 20 min., the pellets were discarded and the supernatant was dialysed against 5 mM $NaHCO_3$. This material was used for the determination of γ-carboxyglutamic acid.

γ-Carboxyglutamate estimation. This was done as described previously; briefly, the corresponding samples were hydrolysed in 4 M NaOH at 100°C for 24 hours according to Hugli and Moore (9). Further procedure was identical with that described by Price et al. (10). An aminoacid analyzer (type 881 Microtechna Prague) with a column 0.28 x 33 cm, packed with Beckmann AA 30 resin was used for this purpose. The column of the amino acid analyzer was operated at 50°C with a stepped series of 0.2 M citrate buffers raging from pH 2.8 (0.6 M Na^+). The position of γ-carboxyglutamate in the aminoacid analyzer was identified by an authentic sample (Serva) and by prothrombin hydrolysate.

Microsomal study. In this part of our study four different groups of male Wistar rats were used. Similarly as in the first part of this study we started just after weaning, with the respective dietary regime, which was applied for 30 days.

1) vitamin D deficient (rachitogenic) diet,
2) vitamin D deficient diet supplemented in the last two weeks before killing with 50 I.U. vitamin D_3/1 g body weight and 25 I.U. vitamin D_2/1 g body weight,
3) vitamin K deficient diet (Nutritional Biochemical Company) supplemented with dicoumarol (800 mg/1 kg of the chow),
4) vitamin K deficient diet supplemented with vitamin K_1 (10 mg/1 kg of the chow).

Tests were also carried out with animals on standard chow supplemented two weeks before killing with vitamin D similarly to group 2. Microsomes were pre-

TABLE I
CONSTITUENTS OF RACHITOGENIC (VITAMIN D DEFICIENT) DIET

Ingredient	% Comp.
Cane sugar	35.00
Corn starch	15.00
Dextrose	20.00
Blood fibrin (alcohol extracted)	15.00
Brewers yeast	4.00
Calcium carbonate	3.00
Corn oil	7.00
Salt mix*	1.50

*By analysis this diet contained 1.17% Ca and 0.069% P.

Hydrogen peroxide. Cells were treated with H_2O_2 while in 0.15 M NaCl to avoid reactions of H_2O_2 with the culture medium. Cells suspended in 0.15 M NaCl were added to tubes containing various concentrations of H_2O_2. After 5 min, catalase was added (1350 units); after an additional 10 min, one-tenth volume of a tenfold concentrated solution of medium was added. Preliminary experiments showed that adding catalase as above was sufficient to remove essentially any residual H_2O_2 from the NaCl cell suspension. Growth of the cultures at 37°C was then followed spectrophotometrically.

Ozone. Ozone gas produced by a Welsbach generator was bubbled through a 0.15 M NaCl solution for 3 hr at 25°C to produce a saturated solution (0.8 mM). Aliquots (0.1 or 0.4 ml) of this solution were added to E. coli cultures suspended in 8 ml of 0.15 M NaCl. Ozone was allowed to react with the cells for 6 min at room temperature, after which tenfold concentrated medium was added and the growth of the culture was continued at 37°C.

Singlet oxygen. Rose Bengal (0.5 g) and silica gel G (4 g) were slurried in acetone and used to apply a thin coating of dye to the inside of a 30 x 2.5 cm Vigreaux distillation column. This column was illuminated by four 15-watt fluorescent bulbs in parallel, and oxygen was passed through the column at a rate of 150 ml/min. Gas flowing from the column was bubbled through teflon tubing into the suspensions of E. coli at 20-30 ml/min. Control cultures were treated simultaneously with oxygen. Aliquots from the cell suspensions were removed for viable cell counts at various times.

RESULTS AND DISCUSSION

The fatty acid composition of logarithmic stage cells supplemented with cyclopropane, oleic or linoleic acids is given in Table 1. Cultures that have grown for many generations on cyclopropane or linoleic acid still contain low levels of 16:1 and 18:1, presumably due to "leakiness" of the mutation in UFA

TABLE 1. Esterified fatty acid composition (mole % ± SE) of logarithmic E. coli cultures supplemented with cyclopropane, oleic and linoleic acids

	FATTY ACID SUPPLEMENT		
	Cy17:0	18:1	18:2
14:0	2.9 ± 0.6	3.1 ± 0.5	1.6 ± 0.1
16:0	51.8 ± 3.0	42.6 ± 0.9	50.6 ± 2.3
16:1	3.1 ± 0.4	3.2 ± 0.2	3.2 ± 0.6
Cy17:0	37.8 ± 4.2	1.9 ± 0.2	1.8 ± 0.1
18:1	4.0 ± 0.4	47.9 ± 0.6	4.6 ± 0.3
Cy19:0	n.d.	1.7 ± 0.3	n.d.
18:2	n.d.	n.d.	37.6 ± 1.2
Total % UFA	7.1	51.1	45.4

Abbreviations: n.d.: not detected. Cy17:0: cis-9,10-methylene hexadecanoic acid. Cy19:0: cis-9,10-methylene octadecanoic acid. UFA: unsaturated fatty acid.

TABLE III

VITAMIN D AND K DEPENDENT INCORPORATION OF ^{14}C $NaHCO_3$ IN KIDNEY CORTEX IN VITRO (RATS, 30 DAYS AFTER WEANING)

Sample	CPM per g wet tissue	
	Vitamin D	
	Supplemented two weeks before killing	Not supplemented
Crude homogenate		
Standard chow	1082	1049
Rachitogenic diet	1054	966
Microsomes		
Standard chow	1610	882
Rachitogenic diet	2730	872
	Vitamin K	
	Deficient diet, supplemented with 10 mg/kg of chow	Deficient diet, not supplemented
Crude homogenate	1082	1045
Microsomes	2627	499

Reaction volumes were 250 µl (150 µl microsomal suspension). Reaction conditions as described previously (Friedman and Shia 12).

that this incorporation is stimulated by the addition of vitamin D to the microsomal assay system of vitamin D deprived rats while only small increases are seen in crude homogenates. The extent of vitamin D stimulation is very similar to that observed with microsomal preparations of dicoumarol diet fed animals supplemented with vitamin MK_3 (2-methyl-3-farnesyl-1,4-naphthoquinone).

This clearly indicates that the synthesis of gla is strongly vitamin D dependent, though the target point must be different from that of vitamin K as long as the carboxylation reaction in prothrombin was not affected (data not shown). This statement is further illustrated in Fig. 1 where the elution profiles of hydrolysates from kidney microsomal fractions with or without vitamin D are compared. Total CPM in the neutralized hydrolysate were 18 110 (in the presence of vitamin D) and 1120 (in the absence of vitamin D), respectively. Aliquots (150 µl) representing 1322 CPM in vitamin D supplemented preparation and 82 CPM in vitamin D untreated sample were subjected to ion exchange chromatography which demonstrates the more intensive incorporation of the radioactivity in gla in the presence of vitamin D (64.2% of total loaded radioactivity in vitamin D treated and 40.2% in untreated samples).

Verification of the gla-peak identity was done by comparing the acid and alkaline hydrolysates of the incubation mixture (Fig. 2). Upon acid hydrolysis one γ-carboxyl is removed under the formation of glutamic acid. Theoretically the radioactivity of the glu peak should be one half of the gla peak. Our results correspond to this assumption - 930 CPM of gla and 478 CPM of glu. On

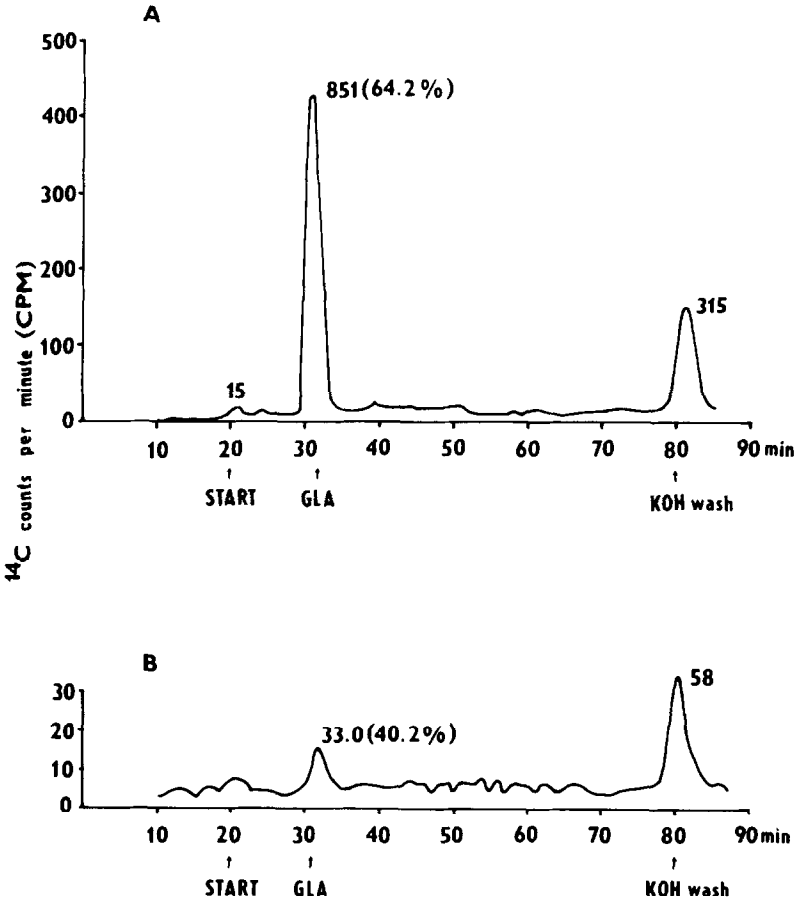

Fig. 1. Elution profile of radioactive products from kidney microsomal fraction fractionated on Microtechna Amino Acid analyzer. A) Vitamin D deficient diet supplemented in the last two weeks of the experiment with vitamin D; B) Rachitogenic diet.

the other hand comparison of total radioactivity in hydrolysates indicates that the presence of vitamin D has a general stimulatory effect on the ^{14}C incorporation in the microsomal fraction.

DISCUSSION: It has been demonstrated that the level of γ-carboxyglutamate is decreased in vitamin D depleted animals, both in kidney cortex and femoral bone.

As stated above, kidney cortices were used for a more detailed investigation because the microsomal fraction from this tissue was relatively easy to obtain. On the other hand it has been demonstrated before that there is a great deal of structural similarity between the γ-carboxyglutamic acid containing protein and osteocalcin (3).

The question whether or not all or some of the γ-carboxyglutamic acid containing proteins are vitamin D dependent has arisen already in the past (6,13). Such an effect can be easily monitored by assaying γ-carboxyglutamate which may

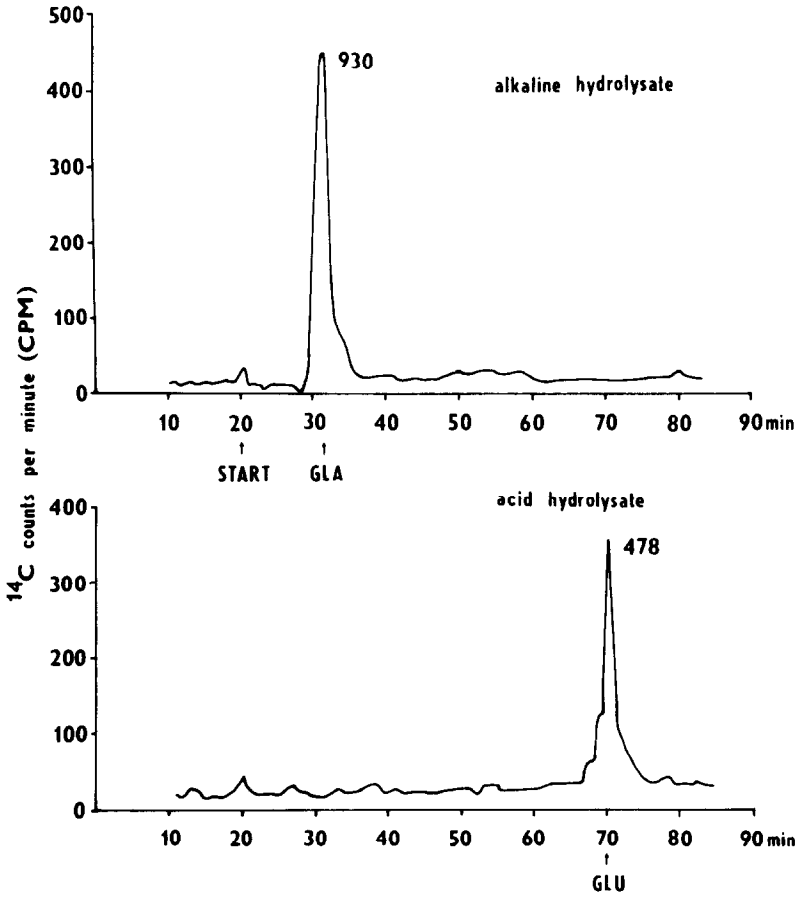

Fig. 2. Elution profiles of acid and alkaline hydrolysates of the incubation mixture from vitamin D supplemented animals (see Fig. 1A). Microsomes were subjected to alkaline hydrolysis and divided in four vials. Equal volumes of water or 12 M HCl were added to duplicate samples. The vials were flushed with nitrogen and those with 2 M HCl were heated to 100°C for 280 minutes. Then the samples were evaporated to dryness (three times under vacuo at 55°C). The residue was dissolved in 200 µl 0.2 M citrate buffer pH 2.2. 50 µl Aliquots were applied to the amino acid analyzer.

serve as marker amino acid. The dependency of γ-carboxyglutamic acid synthesis on the presence of vitamin K is well documented (3). The presence of vitamin K is necessary for the γ-carboxylation reaction to occur in proteins, γ-carboxyglutamate protein from kidney cortex and prothrombin have been investigated in this respect (3,14).

It has been demonstrated in the experimental part that vitamin D depletion leads to a similar decrease of γ-carboxyglutamate as can be observed in the absence of vitamin K. However, it is unlikely that vitamin D would be capable to interfere with the γ-carboxylation reaction. If it were so, increased bleeding would be expected in vitamin D deficiency. Such an effect was never observed. On the other hand it is well established that the regulatory effect of vitamin D

upon the intestinal Ca-binding protein resides in its direct action on mRNA for this protein (13). The presence of 1,25-dihydroxycholecalciferol in the nucleus results in an increase of calcium binding protein mRNA activity in polysomes. It seems feasable to assume that the regulatory effect of vitamin D upon the occurence of the γ-carboxyglutamic acid containing protein may be of similar nature.

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