REDUCTION OF CIRCULATING 25-HYDROXYVITAMIN D BY ANTIPYRINE

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1 Twenty-four Asian vegetarians had significantly lower 25-hydroxyvitamin D (25-OHD) levels and longer antipyrine half-lives than twenty white non-vegetarians ($P < 0.001$).

2 Treatment with oral antipyrine over 4 or 5 weeks in seven vegetarian Asians and five racially different non-vegetarians increased drug oxidation significantly in both groups as measured by a fall in antipyrine half-lives and a rise in serum γ -glutamyltranspeptidase levels and urinary 6β -hydroxycortisol/17-hydroxycorticosteroid ratios.

3 Antipyrine treatment produced a fall in circulating 25-hydroxyvitamin D of around 60% in all subjects in whom pretreatment levels could be measured, independent of race and diet.

4 In the Caucasian non-vegetarian group 1,25 dihydroxyvitamin D levels, the most active metabolite of vitamin D, were also measured and remained unaltered despite a substantial fall in 25-hydroxy substrate.

5 The acute fall in 25-hydroxyvitamin D concentration with a maintained level of 1,25 dihydroxyvitamin D may represent the early changes of drug-induced osteomalacia.

Introduction

Vitamin D₂ (ergocalciferol) is absorbed by the gut and vitamin D₃ (cholecalciferol) is synthesized in the skin by the action of ultraviolet light on 7-dehydrocholesterol. In Western society, the majority of circulating vitamin D originates from vitamin D₃ (Haddad & Hahn, 1973; Preece, Tomlinson, Ribot, Pietrek, Korn, Davies, Ford, Dunnigan & O'Riordan, 1975). Both forms are hydroxylated at the 25 position to 25-hydroxyvitamin D (25-OHD) the major circulating metabolite. In vitamin D depletion the majority of 25-OHD is converted in the kidney to the most biologically active form, 1,25-dihydroxyvitamin D. In vitamin D excess 25-OHD is converted mainly to 24,25 and 25,26 dihydroxy metabolites which are probably wastage pathways (Mawer, Backhouse, Hill, Lumb, De Silva, Taylor & Stanbury, 1975).

The concentration of 25-OHD in the serum reflects total body vitamin D status in patients with normal renal function. The hepatic 25-hydroxylase is present in the microsomal fraction (Battacharyya & De Luca,

1974) and recent animal evidence has shown it to behave like a cytochrome P-450-dependent monooxygenase (Delvin, Arabian & Glorieux, 1978; Madhok, Schnoes & De Luca, 1978). Hunt, O'Riordan, Windo & Truswell (1976) suggested that low levels of 25-OHD found in subgroups of British Asians were related to dietary factors and inadequate exposure to sunlight. The further possibility exists that Asian vegetarians have a lower rate of drug oxidation than non-vegetarians and that one of the additional factors limiting plasma 25-OHD concentration is its rate of formation in the liver by inducible microsomal enzymes.

Methods

Subjects

The study was divided into three parts. All subjects were informed about the aims of the study and gave their written consent. The study had the approval of the Research Ethics Committee of Hammersmith Hospital and the Royal Postgraduate Medical School. The Asian subjects all originated from the

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Indian sub-continent, the majority from the Punjab. No subjects were taking drugs known to induce liver microsomal enzymes.

Part 1

Healthy volunteers were recruited from a London food factory and a commercial bank. Twenty-four Asian vegetarians (7 males, 17 females; mean ages 34.2 years) and 20 white Caucasian non-vegetarians (10 males, 10 females; mean age 32.0 years) were studied. After an overnight fast, each subject took 600 mg antipyrine orally accompanied by 150 ml water. Saliva samples facilitated by the chewing of waxed film, were taken at 0, 3, 5, 8, 24 and 32 h. A blood sample was taken initially for 25-OHD level.

Part 2

Eleven vegetarian Asians volunteered for this part of the study. They were all mild hypertensives attending Hammersmith Hospital. All were receiving anti-hypertensive therapy but none were taking any drugs known to affect hepatic microsomal enzymes. There were eight males and three females (ages 19–71 years). An estimate of dietary vitamin D intake was obtained from each subject. Each subject had normal routine haematology, liver function tests, serum calcium and phosphorus and was negative for hepatitis B-surface antigen.

Subjects were randomly allocated to a 5 week course of antipyrine 600 mg daily or matched placebo; four subjects taking the placebo. All subjects were studied simultaneously in the early spring. A weekly serum sample was taken for 25-OHD and further supply of tablets dispensed at that time, allowing a weekly 'pill count' as a measure of compliance.

Antipyrine kinetics were investigated before and 3 days after the completion of the 5 week study. Following an oral dose of 600 mg antipyrine, blood samples were taken at 0, 3, 8 and 24 h. In addition, serum γ -glutamyltranspeptidase (γ -GT) and 24 h urinary 6 β -hydroxycortisol/17-hydroxycorticosteroid ratios were measured before and 3 days after the completion of the study.

Part 3

A similar protocol was followed with seven non-vegetarian volunteers. two subjects were randomized into the placebo group. There were four males and three females with an age range of 22 to 32 years. Plasma samples for antipyrine determination were taken at 0, 3, 7 and 10 h. The course was shortened to 4 weeks. A further 20 ml of blood was taken at 0, 2 and 4 weeks for plasma 1,25-dihydroxyvitamin D.

Once again, to minimize the effect of sunlight, all subjects were studied concurrently in early summer.

Analyses

Antipyrine in saliva and plasma was measured by gas chromatography (Fraser, Mucklow, Murray & Davies, 1976) and half-lives were calculated by least squares regression analyses. 25-hydroxyvitamin D was assayed by competitive protein binding assay using human osteomalacic serum diluted 1:2000 as binding protein and 25-[26-(27)-methyl-³H] hydroxy-cholecalciferol (Radiochemical Centre, Amersham) as tracer. Samples were first extracted using a chloroform/methanol mixture (Haddad & Chyu, 1971) dried down and dissolved in ethanol for assay. Two dilutions in triplicate were incubated overnight with tracer and binding protein in Tris buffer (pH 8.6). 25-hydroxyvitamin D bound to protein was separated from free using dextran-coated charcoal. Supernatant (500 μ l) was counted with 2,5-diphenyloxazole in toluene as scintillator (Greenberg, Hillyard, Galante, Colston, Evans & MacIntyre, 1974). 1,25-dihydroxyvitamin D levels were assayed by a modification of the method of Eisman, Hamstra, Kream & De Luca (1976). Plasma γ -GT was measured by an automated modification of the method of Rosalki & Tarlow (1974). Urinary 17-hydroxycorticosteroids were determined by the method of Sangvi, Taddeini & Wight (1973) and 6 β -hydroxycortisol obtained using a specific radioimmunoassay (Park, 1978).

Saliva, plasma and urine samples were stored frozen at -20°C until analyses. Statistics were carried out by Student's *t*-test for unpaired and paired values. Correlation coefficients were obtained by least squares regression analyses.

Results

Part 1

The antipyrine half-lives and 25-OHD levels in the two groups are plotted in Figure 1. There are highly significant differences ($P < 0.001$) in the antipyrine half-lives (Asians 16.3 ± 0.8 h; Caucasians 9.1 ± 0.5 h) and circulating plasma 25-OHD levels (Asians 4.8 ± 0.7 $\mu\text{g/l}$; Caucasians 13.7 ± 1.7 $\mu\text{g/l}$) between the Asian and Caucasian subjects.

Part 2

Assessment of vitamin D daily intake in all 11 subjects showed a mean figure of 3.0 $\mu\text{g/day}$ compared with recommended daily requirements of 10 μg (~ 400 i.u.). The antipyrine half-lives, serum γ -GT and 6 β -hydroxycortisol/17-hydroxycorticosteroid

Figure 1 Plasma concentration of 25-hydroxyvitamin D ($\mu\text{g/l}$) plotted against antipyrine $T_{\frac{1}{2}}$ (h) in 24 vegetarian Asians ($\circ$) and 20 non-vegetarian Caucasians ($\triangle$).

ratios before and after treatment with antipyrine are listed in Table 1. These results confirm induction in these seven subjects. Two controls failed to complete the study due to claimed placebo side effects. In the two remaining control subjects there was no change in any of the parameters studied.

Two of the seven subjects taking antipyrine had undetectable plasma 25-OHD levels throughout the study and were excluded from this analysis. Weekly plasma 25-OHD levels for the other five subjects are listed in Table 2. Only one subject had an initial plasma 25-OHD level within the normal range of the laboratory ($8\text{--}42 \mu\text{g/l}$). All showed a fall in 25-OHD from pre-treatment levels which was significant at 2, 3 and 4 weeks but not in the fifth week of the study. Both remaining placebo controls showed undetectable 25-OHD levels throughout the study.

Part 3

The parameters of hepatic enzyme induction in the five subjects taking antipyrine are listed in Table 3. There was a significant change in all three parameters measured. All five subjects showed a fall of 25-OHD

to subnormal values ($< 8 \mu\text{g/l}$) by the second week of the study. The fall was significant for the first 3 weeks of antipyrine treatment but not for the fourth week (Table 4). 25-OHD levels in the two controls showed no change over the 4 weeks of the study. Although the mean initial 25-OHD level in this group ($10.8 \pm 1.5 \mu\text{g/l}$) was higher than that in the Asian group ($6.4 \pm 1.3 \mu\text{g/l}$) the maximal percentage decrement over the period of antipyrine administration was about 60% in both groups (Figure 2). Plasma concentrations of 1,25-dihydroxyvitamin D levels in the five subjects taking antipyrine showed no significant change over the study period (Figure 3).

Discussion

Vegetarian Asians in Britain are known to have lower 25-OHD levels than non-vegetarians of both Asian and European origin (Preece *et al.*, 1975; Hunt *et al.*, 1976). This study has shown that vegetarian Asians with low circulating 25-OHD levels also metabolize antipyrine slowly. This may be consequent upon their vegetarian diet as Asians who eat meat have a higher antipyrine clearance than racially identical lactovege-

Table 1 Measurements of the parameters of liver microsomal enzyme activity before and after administration of 600 mg antipyrine for 5 weeks in seven Asian vegetarians (mean $\pm$ s.e. mean)

	Before	After	Significance
Antipyrine $T_{\frac{1}{2}}$ (h)	15.1 ± 1.2	11.0 ± 0.8	$P < 0.02$
γ -GT (i.u./l)	24.6 ± 3.8	42.3 ± 6.5	$P < 0.01$
6 β -hydroxycortisol %			
17-hydroxycorticosteroid	6.2 ± 0.8	9.9 ± 0.8	$P < 0.001$

Figure 2 Mean percentage change in plasma 25-hydroxyvitamin D levels in five vegetarian Asians (■) and five non-vegetarians (●) taking antipyrine 600 mg daily and four controls (○) taking matched placebo.

Figure 3 Mean plasma concentration ($\pm$ s.e. mean) of 1,25-dihydroxyvitamin D in five Caucasian non-vegetarians taking antipyrine 600 mg daily for 4 weeks.

Table 2 Weekly plasma levels of 25-hydroxyvitamin D (μ g/l) in vegetarian Asians during administration of 600 mg antipyrine for 5 weeks

Subject	Age (years)	Sex	Weeks						
			0	1	2	3	4	5	
LS	71	M	11.2	9.7	9.6	5.1	4.2	2.5	
GS	51	M	4.8	2.5	3.0	0.4	0.4	0.4	
NS	61	M	5.9	8.5	4.5	4.4	3.0	2.3	
KC	42	F	4.0	3.8	1.8	2.0	0.5	0.4	
VM	40	M	6.0	3.6	2.8	1.6	3.6	6.5	
Mean			6.4	5.6	4.3	2.7	2.3	2.4	
s.e. mean			1.3	1.5	1.4	0.9	0.4	1.1	
P				NS	<0.01	<0.01	<0.01	NS	

Table 3 Measurements of the parameters of liver microsomal enzyme activity before and after administration of 600 mg antipyrine for 4 weeks in five non-vegetarians (mean $\pm$ s.e. mean)

	Before	After	Significance
Antipyrine $T_{\frac{1}{2}}$ (h)	11.1 $\pm$ 1.1	6.1 $\pm$ 0.8	$P < 0.01$
γ -GT (i.u./l)	13.6 $\pm$ 1.6	18.4 $\pm$ 1.7	$P < 0.05$
$\frac{6\beta\text{-hydroxycortisol}}{17\text{-hydroxycorticosteroid}}$ %	2.9 $\pm$ 0.9	6.3 $\pm$ 1.4	$P < 0.02$

Table 4 Weekly plasma levels of 25-hydroxyvitamin D ($\mu\text{g/l}$) in non-vegetarians during administration of 600 mg antipyrine for 4 weeks

Subject	Age (years)	Sex	Weeks				
			0	1	2	3	4
FW	32	M	7.2	6.6	4.1	3.9	4.3
AB	29	M	10.0	5.6	4.0	3.8	5.6
MR	28	M	16.5	13.7	7.6	5.4	8.0
PB	32	M	10.0	8.6	3.7	3.0	1.5
CK	26	F	10.3	7.8	3.5	6.2	13.0
Mean			10.8	8.5	4.6	4.5	6.5
s.e. mean			1.5	1.4	0.8	0.6	1.9
P				<0.05	<0.01	<0.01	NS

tarians (Mucklow, Caraher, Henderson & Rawlins, 1979). These data might also indicate involvement of the same cytochrome P 450-dependent oxidizing enzyme in the liver endoplasmic reticulum in the metabolism of vitamin D and antipyrine. If the latter were the case, induction of mixed function oxidase activity by antipyrine might be expected to increase circulating 25-OHD. Despite a significant increase in antipyrine hydroxylation, however, there was a fall averaging 60% in 25-OHD in all subjects, in whom pretreatment 25-OHD could be measured, independent of race and dietary habit.

Plasma concentrations of 25-OHD are reduced in many epileptics on long term anticonvulsants (Hahn, Hendin, Scharp & Haddad, 1972). It has been suggested that this is due to induction of hepatic mixed function oxidases with precursor pool depletion but this remains unproven (Haussler & McCain, 1977). Phenytoin and phenobarbitone in toxic doses have been shown to inhibit liver 25-hydroxylase activity in the rat (Sulimovici & Roginsky, 1978) and at a high dose might have a similar effect in man. Inhibition of vitamin D hydroxylation by antipyrine or one of its metabolites would provide an explanation for the consistent fall in 25-OHD in this study but this seems unlikely at the low dosage of antipyrine administered.

Another explanation for the reduced 25-OHD levels would be that enzyme induction leads to increased metabolism of 25-OHD to inactive products. This could be conjugation, a pathway which is known to be induced by antipyrine (Orme, Davies & Breckenridge, 1974) or further hydroxylation to more polar metabolites (Silver, Neale & Thompson, 1974). It has recently been demonstrated

in rat liver microsomes that 25-OHD has a high affinity for cytochrome P-450 (Cinti, Glorieux, Delvin, Golub & Bronner, 1978). 25-OHD competitively inhibited aminopyrine demethylation and doubled cytochrome P-450 reductase activity suggesting the involvement of hepatic mixed function oxidases in further transformation of this metabolite. Although phenobarbitone treatment in the same study resulted in a fall of 40% of circulating 25-OHD in the rat, the concentration of the active hormone, 1, 25-dihydroxyvitamin D was unaffected. This is in keeping with our findings in man where, despite a substantial fall in the availability of the 25-hydroxy substrate, the levels of 1,25-dihydroxyvitamin D remained unaltered. Furthermore, patients on anticonvulsant drugs with low 25-OHD and abnormal bone biochemistry also had normal or increased 1,25-dihydroxyvitamin D, although none of these patients actually had histologically documented osteomalacia (Jubiz, Haussler, McCain & Tolman, 1977). Reduction in 25-OHD with normal 1,25-dihydroxyvitamin D levels are unlikely to explain the development of drug induced osteomalacia. Longer term studies, therefore, are required to assess the pathophysiological significance of these observations.

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Addendum

Since this paper was accepted for publication, three patients have been reported with advanced untreated nutritional osteomalacia with low concentrations of 25-hydroxyvitamin D and 24,25-dihydroxyvitamin D but normal concentrations of 1,25-dihydroxyvitamin

D. (Eastwood, J.B., de Wardener, H.E., Gray, R.W. & Lemann, J.L. (1979). Normal plasma 1,25-(OH)₂-vitamin-D concentrations in nutritional osteomalacia. *Lancet*, **i**, 1377–1378.)