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EFFECTS OF HEPATIC MICROSOMAL ENZYME INDUCERS ON THE ENDOGENOUS SUBSTRATES VITAMIN D_3 AND FOLATE IN RAT

TREVOR J. RISING
Hoechst Pharmaceutical Research Laboratories, Walton Manor, Walton, Milton Keynes, MK7 7AJ, Buckinghamshire, U.K.

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Abstract—A procedure for the estimation of possible effects of potential inducers of hepatic microsomal enzymes on vitamin D_3 metabolism has been devised. Together with methods for the determination of plasma folate and urinary formimino glutamic acid levels, it has been applied to a 43 day study in rats. Two anticonvulsants, phenobarbitone and pheneturide, were employed as inducers. Although both compounds had variable yet positive effects on enzyme induction, only the former had a significant action on vitamin D_3 metabolism and plasma folate levels. These results are discussed in relationship to results from previous work carried out, predominantly in man.

The high incidence of rickets observed in children and adults undergoing anti-convulsant therapy has been shown to be associated with a concomitant decrease in plasma vitamin D_3 (cholecalciferol) levels [1–4]. In addition, drug-treated epileptic patients are sometimes found to have folic acid deficiency which can lead to megaloblastic anaemia [5, 6]. It is now generally accepted that anti-convulsant osteomalacia is due to hepatic microsomal enzyme induction [7, 8] and there is a strong possibility that serum folate deficiency is also related to enzyme induction [9, 10]. Effects on these endogenous substrates in man only appear to manifest themselves following drug administration for long periods. As an earlier indication of any possible consequences, it might be advantageous to use laboratory animals and employ a dosing regimen which might produce induction and effect endogenous substrates at an early stage.

Vitamin D_3 is hydroxylated in the liver to 25-hydroxycholecalciferol [11], which in turn is hydroxylated to 1,25-dihydroxycholecalciferol, the presumed active form of the vitamin [12]. A number of analytical methods for the estimation of the 25-hydroxy metabolite have been reported, namely a protein binding radioassay [13], high pressure liquid chromatography [14] and a procedure employing silicic acid column chromatography and scintillation counting [15]. These methods are comparatively lengthy and are not ideal for any routine estimations of the effects of inducers on vitamin D_3 metabolism. Serum folate can be measured using a simple ^{125}I radioassay technique. However, since this procedure in small laboratory species involves the sacrifice of the animal, an alternative method could possibly be used. Such an alternative is by the measurement of urinary levels of formimino-1-glutamic acid (FIGLU), a substrate which requires tetrahydrofolic acid for its further metabolism [16].

A simple, routine method has been developed for vitamin D_3, and along with the serum folate and FIGLU methods, has been applied to a 43 day induction study in rats. The anti-convulsant compounds phenobarbitone and pheneturide were employed as inducing agents. The former is commonly used in rats as a positive enzyme inducer and the latter has been reported to be more active than phenobarbitone in man in increasing D-glucaric acid excretion and lowering serum folate levels [10].

MATERIALS AND METHODS

[4-^{14}C] Vitamin D_3 (sp. act. 36 mCi/m-mole) and 25-hydroxy [26 (27)-methyl-^3H] cholecalciferol (sp. act. 7.3 Ci/m-mole) were obtained from the Radiochemical Centre, Amersham. The folate ^{125}I radioassay kit was purchased from Bio-Rad Laboratories, Bromley, Kent. Ethyl morphine hydrochloride B.P.C. was obtained from May and Baker, Dagenham, Essex and scintillator NE 260 from Nuclear Enterprises Ltd., Edinburgh.

Dosing procedures. Male Sprague–Dawley rats, initially weighing approximately 75 g, were divided into 3 groups: A, B and C. Group A acted as controls, group B received phenobarbitone in their drinking water (0.5 mg/ml) and group C were dosed daily by gavage with pheneturide (0.5–1.0 ml of a 10 mg/ml aqueous suspension containing 3% (w/v) sodium carboxymethyl cellulose and 1% (w/v) Tween 85). The total body weights of the animals and dose levels of phenobarbitone and pheneturide were determined through the study. The average daily dose for phenobarbitone was 119 mg/kg at the commencement of dosing and decreased to 49 mg/kg at the end, whereas the daily dose for pheneturide averaged 40 mg/kg over the whole study. Rats were allowed water *ad libitum* and were fed the Charles River 4 R.F. diet which contained 0.52% (w/w) histidine, 3.3 mg/kg folic acid and 4250 I.U./kg vitamin D_3. The daily intake of food was also measured and was found not to vary between the 3 experimental groups. On days 7, 22 and 42 a number of rats from each

group were transferred to all-glass metabolism cages for the determination of the parameters given later.

Determination of effects on vitamin D_3. The rats were dosed intravenously (caudal vein) with [^{14}C]-vitamin D_3 (0.1 ml of an ethanolic solution of 35 μg/3.3 μCi/ml), and after 24 hr were bled by cardiac puncture to obtain plasma. The radioactive content of the plasma was determined by liquid scintillation counting. To the remainder was added 10 vol. of $CHCl_3$:MeOH (2:1) and the phases were separated, after hand shaking for 30 sec, by the addition of 2 vol. of water [17]. The chloroform phase, after drying over anhydrous Na_2SO_4, was evaporated under nitrogen at less than 50°. The residue was dissolved in hexane before being applied to a silica gel GF TLC plate. The plate was developed in the dark in an atmosphere of nitrogen using n-hexane:acetone (1:1 v/v) as solvent. [^{14}C]-vitamin D_3 and [^{3}H] 25-hydroxy cholecalciferol were chromatographed on the same plate. After drying, the standard compounds and radioactive components in the plasma were located by autoradiography and quantified by liquid scintillation counting of the segmented adsorbent layer.

Plasma folate levels. The concentration of folic acid in rat plasma samples was determined by quantitative radioassay [18, 19] using a commercially available kit. The ^{125}I measurements were carried out by liquid scintillation counting.

Urinary FIGLU levels. Urinary FIGLU levels were determined by the method of Chanarin and Bennett [20] using a crude chicken liver enzyme preparation. Since the daily intake of histidine (the precursor of FIGLU) was approximately 500 mg/kg, no extra loading was given except on the penultimate day of the study. On this occasion, the rats were maintained in metabolism cages for 48 hr. Following urine collection for 24 hr, an oral histidine loading of 250 mg/kg was given (corresponding to approximately 15 g administered to man) and urine was again collected for 24 hr.

Liquid scintillation counting. All samples were counted in a Packard 2450 or 3255 liquid scintillation counter after addition of NE 260 scintillator. For silica scrapings from TLC plates approximately 100 μl of water was added first to deactivate the silica.

Measurement of enzyme induction. Portions of liver (1 g) were homogenized in 3 ml HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid) buffer, 0.2 M, pH 7.4 [21], and centrifuged at 15,000 *g* for 10 min. The resulting supernatants were used as the microsomal source. All procedures were carried out at 4°. The experimental conditions employed for the determination of enzyme activity were basically those of Fouts [22]. The incubation medium contained NADP (0.25 mM), glucose-6-phosphate (1.25 mM), $MgCl_2$ (2.0 mM), enzyme source (approximately 3 mg protein), HEPES buffer (0.2 M) pH 7.4 and substrate to give a final volume of 1.0 ml. The substrates used were *p*-nitroanisole (0.4 mM) for *O*-demethylation, ethyl morphine (0.4 mM) for *N*-demethylation and aniline (10 mM) for ring hydroxylation. The samples were incubated at 37° in an atmosphere of oxygen for periods sufficient to allow initial reaction rates to be measured. The degree of ring hydroxylation and *O*-demethylation was determined using the methods of Kato and Gillette [23], except that di-ethyl ether was replaced by di-iso propyl ether (NaOH washed) in the former analytical procedure. Formaldehyde production from *N*-demethylation of ethyl morphine was measured according to Nash [24]. In addition, cytochrome P-450 levels were determined by the method of Schoene *et al.* [25] and ethyl isocyanide difference spectra were obtained as described by Imai and Sato [26]. Both analyses were carried out using a Perkin Elmer 356 Spectrophotometer. Protein concentrations were determined according to Lowry *et al.* [27].

RESULTS

Validation of the vitamin D_3 method. The following aspects of the 'vitamin D_3' method were considered before the main induction study was initiated. 1. The use of ethanol (0.1 ml) as dosing vehicle for vitamin D_3 rather than a propylene glycol/rat plasma mixture as used by others [28] had no observable effects when administered to rats in a tolerance study. 2. The 24 hr period between radiolabelled vitamin D_3 intravenous administration and plasma collection for metabolite analysis had been found by other workers to be satisfactory [28]. 3. Determination of the radioactive content of the aqueous phase after chloroform/methanol extraction showed it to contain less than 5 per cent of the total radioactivity. 4. Of the

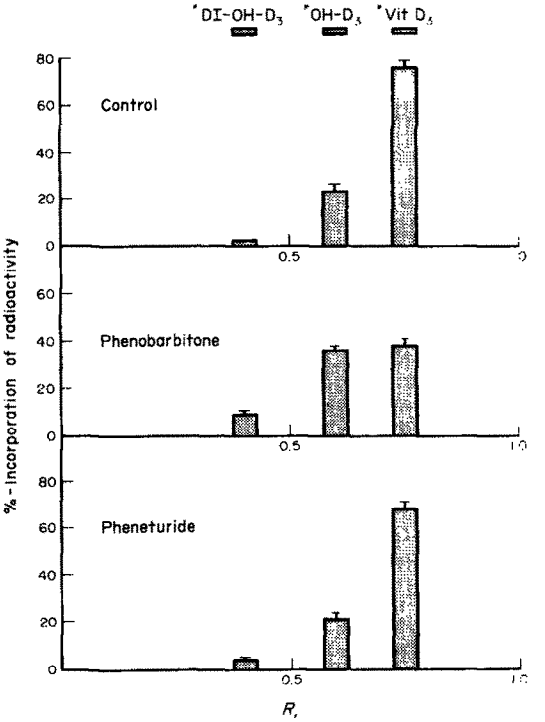

Fig. 1. Effects of phenobarbitone and pheneturide on plasma levels of [^{14}C] vitamin D_3 and its metabolites following oral administration for 43 days in rats. For experimental details see the Methods section. Results are the mean values ± S.E.M. for 4 rats per group.

* Vit D_3, vitamin D_3; OH-D_3, 25-hydroxycholecalciferol; DI-OH-D_3, a mixture of dihydroxy derivatives of cholecalciferol.

Table 1. Effects of phenobarbitone and pheneturide administration on rat liver weights

Group	Relative liver weights		
	Day 8	Day 22	Day 44
A	5.26 ± 0.04	4.24 ± 0.42	4.05 ± 0.13
B	6.05 ± 0.04*	6.24 ± 0.30*	4.67 ± 0.21*
C	4.71 ± 0.20	5.03 ± 0.25	4.11 ± 0.26

Results are expressed as mean values ± S.E.M. of relative liver weights (weight in grams per 100 g body weight) for 3 or 4 rats per group.
* $P < 0.05$ or better.

Table 3. Hepatic microsomal enzyme activity on day 44

Group	Microsomal enzyme activity		
	N-demethylation	*O*-demethylation	Ring hydroxylation
A	4.54 ± 0.81	0.510 ± 0.087	0.094 ± 0.005
B	6.26 ± 0.87*	0.799 ± 0.102*	0.141 ± 0.015*
C	6.17 ± 0.80*	0.783 ± 0.058	0.106 ± 0.003

Results are expressed as mean values ± S.E.M. of nmoles/mg protein/min for 4 rats per group.
* $P < 0.05$ or better.

various TLC solvent systems examined, n-hexane: acetone (1:1) was chosen for routine analysis because of the rapid and excellent separation of vitamin D_3, 25-hydroxycholecalciferol and other vitamin D_3 metabolites (Fig. 1). These other vitamin D_3 metabolites, which chromatographed as a single band, were thought to include various dihydroxy derivatives, including 1,25-dihydroxycholecalciferol.

Effects on rat whole body and liver weights. There were no differences observed in the total body weights over the duration of the study. However, the relative liver weights of the phenobarbitone group were significantly higher than those of the controls on all three occasions on which they were measured (Table 1).

Effects on microsomal enzyme activity. The results in Table 2 indicate that whereas phenobarbitone had no effect on total microsomal protein levels over the duration of the study, pheneturide tended to lower them, with the decrease being significant at day 22. Both compounds increased the cytochrome P-450 content, when calculated on a "per mg protein" basis. However, this result was not statistically significant for pheneturide when expressed as nmoles/g liver. When rates of specific reactions catalysed by the microsomal mixed function oxidase system were measured (Table 3), phenobarbitone stimulated the reactions in all cases. Although pheneturide increased *N*- and *O*-demethylation, it had no apparent effect on ring hydroxylation.

Effects on vitamin D_3. Results of measurements to determine total radioactivity and metabolite levels in plasma 24 hr after intravenous administration of [^{14}C]-vitamin D_3 are summarised in Table 4. The total radioactivity content of plasma appeared to be greater in the phenobarbitone group, a result found by other workers [28]. With the exception of radioactivity remaining at the origin (less than 2 per cent), the remainder was separated into three distinct bands, namely vitamin D_3, 25-hydroxycholecalciferol and a third band assumed to contain various dihydroxy derivatives of cholecalciferol. With phenobarbitone treated rats there was an increase in the hydroxylation of parent compound, this increase being significant in the 22 and 43 day experiments (Fig. 1). The vitamin D_3: 25-hydroxycholecalciferol ratio, which appears to give a reliable indication of effects on vitamin D_3 metabolism, was also markedly reduced. Pheneturide had no apparent effect on any of the parameters determined.

Effects on plasma folate levels. Plasma folate levels and the magnitude of their reduction in the phenobarbitone group on days 22 and 43, when compared to control values (Table 5), were similar to those reported by Spray and Burns [29]. Pheneturide again had no effect. It is of interest to note that the folate concentration decreased significantly over the duration of the study. Part of the decrease is presumably related to the decreased relative intake of dietary folate over the course of the experiment.

Effects on urinary FIGLU levels. The results of the urinary FIGLU determinations were very variable and no correlation could be made either with plasma folate levels or between animals of the same group. The exact reason could not be found, but was probably related to endogenous material in the urine (which gave high and widely different extinction readings) reacting with the crude chicken liver enzyme preparation. The histidine loading experiment did not produce more acceptable reults.

Ethyl isocyanide difference spectra. Since, when compared to phenobarbitone, pheneturide produced

Table 2. Crude microsomal protein and cytochrome P-450 levels

Group	Day 8		Day 22		Day 44	
	Protein	P-450	Protein	P-450	Protein	P-450
A	119 ± 11	0.205 ± 0.026	120 ± 4	0.210 ± 0.003	168 ± 24	0.159 ± 0.026
B	120 ± 7	0.633 ± 0.147*	103 ± 8	0.450 ± 0.024*	179 ± 15	0.243 ± 0.089*
C	92 ± 9	0.332 ± 0.045*	66 ± 7*	0.378 ± 0.036*	124 ± 3	0.236 ± 0.040*

Results are expressed as mean values ± S.E.M. of mg protein per gram wet weight of liver or nmoles cytochrome P-450 per mg crude microsomal protein for 3 or 4 rats per group.
* $P < 0.05$ or better.

Table 4. Effects on vitamin D_3 metabolism in rats

Day	Treatment	Plasma radioactivity (dpm/ml)	% Incorporation of radioactivity DHC†	25HC†	VIT D_3†	Vitamin D_3/25HC ratio
8	Control	1422 ± 23	4 ± 1	37 ± 6	59 ± 7	1.79 ± 0.57
	Phenobarbitone	2235 ± 170*	6 ± 1	44 ± 4	50 ± 4	1.15 ± 0.17
22	Control	995 ± 160	3 ± 2	22 ± 4	75 ± 5	3.68 ± 0.75
	Phenobarbitone	1372 ± 230	8 ± 1	47 ± 2*	45 ± 2*	0.96 ± 0.07*
44	Control	1682 ± 84	2 ± 0	23 ± 3	75 ± 3	3.49 ± 0.70
	Phenobarbitone	2273 ± 363	8 ± 2*	45 ± 2*	47 ± 3*	1.08 ± 0.12*
	Pheneturide	2169 ± 169	3 ± 1	20 ± 3	77 ± 3	4.16 ± 0.83

The results are expressed as mean values ± S.E.M. for 3 or 4 rats.
*$P < 0.05$ or better.
† Abbreviations: DHC, dihydroxycholecalciferol; 25HC, 25-hydroxycholecalciferol; VIT D_3, vitamin D_3.

both similar and dissimilar effects on the various parameters determined, ethyl isocyanide difference spectra were measured as a possible guide to the type of cytochrome being utilised. The polycyclic hydrocarbon 3-methylcholanthrene (3-MC), which produces a difference spectrum in dithionite reduced microsomes unlike that from phenobarbitone [30], was employed as a positive control. It was administered intraperitoneally to rats once daily for 5 days at a dose level of 20 mg/kg and produced a significant ($0.05 > P > 0.01$) increase in the levels of crude microsomal cytochrome P-450. It also produced a ratio of 455/430 nm absorption peaks significantly different from that of control rats (Table 6). The ratios for phenobarbitone and pheneturide were both similar to that of the control animals.

DISCUSSION

The oral administration of phenobarbitone and pheneturide to rats has been shown to cause hepatic microsomal induction as judged by measurement of various parameters. However, the spectrum of activity between the two anti-convulsant compounds is often different, although apparently the same form of cytochrome P-450 is inducible. Stevenson *et al.* [31] reported that following oral administration of pheneturide to rats for 4 days at 10 or 100 mg/kg, hexobarbitone oxidation was induced whereas cytochrome P-450 levels were unaffected. It can be concluded that cytochrome P-450 probably behaves catalytically differently with the two anti-convulsants. Similar differences in substrate specificities have been reported for phenobarbitone and the steroid pregnenolone-16α-carbonitrile [30].

The present study has shown that a potent enzyme inducer like phenobarbitone can significantly reduce the vitamin D_3:25-hydroxycholecalciferol ratio. This observation might not at first seem compatible with the fact that the concentration of the hydroxy metabolite is lower than normal in epileptic patients undergoing anti-convulsant therapy [2, 32]. However, it is probable that enzyme inducers produce a general increase in vitamin D_3 metabolism [7], with higher levels of metabolites in the bile [28] and possible increased oxidation of 1,25-dihydroxycholecalciferol [33]. This does not mean that the present method employed to assess effects on vitamin D_3 metabolism is invalid for inducers like phenobarbitone. The lack of published data on the other specific inducers (including pheneturide) which cause changes in vitamin D_3 metabolism and clinical effects (i.e. rickets) limits further direct validation of the method at this stage.

Although doubts have been expressed concerning the relationship between anti-convulsant therapy and decreased serum folate levels [34], it is probable that a connection does exist [5, 9]. The work presented here has demonstrated that plasma levels of folate are reduced when phenobarbitone is administered for 22 days or longer. However, although pheneturide had some effect on enzyme induction, it failed to reduce plasma folate levels. Pheneturide, which in man is apparently a more potent enzyme inducer than

Table 5. Effects on plasma folate levels in rats

Group	Plasma folate levels Day 8	Day 22	Day 44
A	40.2 ± 1.3	28.2 ± 1.0	14.6 ± 0.6
B	43.7 ± 1.7	22.5 ± 0.9*	9.9 ± 0.7*
C	49.0 ± 5.3	27.8 ± 1.8	18.3 ± 1.8

Results are expressed as mean values of folate levels (ng/ml) ± S.E.M. for 3 or 4 rats.
* $P < 0.05$ or better.

Table 6. Ethyl isocyanide difference spectra

Group	Ratio of 455/430 nm absorption peaks
A	0.327 ± 0.025
B	0.343 ± 0.040
C	0.340 ± 0.045
3-MC	0.520 ± 0.080*

The results are mean values ± S.E.M. for 3 rats measured at pH 7.4.
* $P < 0.05$ or better.

phenobarbitone [10, 35] on the basis of urinary glucaric acid excretion, significantly reduces the serum folate concentration in epileptic patients. It therefore seems possible that pheneturide behaves differently in rat and man.

The main purpose of the present study was to investigate whether changes in levels of endogenous substrates in man, which produce certain clinical effects and are presumably related to enzyme induction, could be detected in laboratory animals at an early stage. Whereas in man the clinical symptoms do not normally manifest themselves unless the therapeutic agent is administered for periods in the order of 1 year of longer, the approach in rats has been to choose a higher dose level, but to maintain dosing for a shorter period. One obvious difficulty with this approach is to try and correlate the dosing regimes in laboratory animals and man. With the two compounds selected for the present study, the average daily doses to epileptic patients are approximately 2 and 8 mg/kg for phenobarbitone and pheneturide respectively: the corresponding dose levels for rats were about 80 and 40 mg/kg. From these figures it might be argued that pheneturide did not affect the endogenous substrates under investigation due to the dose being too low. However, Latham *et al.* [10] did demonstrate increased glucaric acid excretion in man at a dose level of approximately 3 mg/kg.

Is the approach presented here for the early diagnosis of effects on endogenous substrates in man valid? Normally clinical symptoms in man associated with altered metabolism of endogenous substrates only appear after long term drug therapy. This might be due to man being subjected to many variables and continually being partly in an induced state, so that changes in endogenous substrates due to drug related induction, although possibly present at an early stage, do not manifest themselves until later. Certainly for a compound like phenobarbitone, the extrapolation from animals to man would seem valid. The results for pheneturide, for a number of reasons outlined above, would not support the present approach. However, it can be stated that the suggestion to use pheneturide instead of phenobarbitone in routine enzyme induction studies in animals [10] might be erroneous.

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