EFFECT OF PHENOBARBITONE TREATMENT ON VITAMIN D METABOLISM IN MAMMALS

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(Received 1 October 1973)

SUMMARY

1. The metabolism of radioactive cholecalciferol was studied in control and phenobarbitone-treated rats and pigs.

2. Treatment with phenobarbitone enhanced the appearance in plasma of 25-hydroxycholecalciferol (peak IV on silicic acid chromatography), and of more-polar metabolites (peak V), but not of the most-polar metabolites (peak VI). Peak IV had the chromatographic properties of authentic 25-hydroxycholecalciferol (25-HCC) and had biological activity.

3. There was no effect on the appearance of peaks V and VI in plasma after an injection of radioactive 25-HCC.

4. Treatment with phenobarbitone enhanced the excretion of metabolites of radioactive vitamin D₃ in bile. These metabolites were largely water-soluble conjugates of peaks IV, V and VI, which included glucuronides. Peak IV in bile was not identical with 25-HCC.

5. Prolonged treatment with phenobarbitone depleted the tissue radioactivity of rats given radioactive vitamin D₃.

Key words: cholecalciferol, vitamin D, phenobarbital, enzyme induction, bile, glucuronides.

An increased incidence of vitamin D deficiency has been described in patients receiving long-term therapy with combinations of the anticonvulsant drugs phenobarbitone, primidone and diphenylhydantoin (Kruse, 1968; Richens & Rowe, 1970). Phenobarbitone and primidone are known to induce hepatic microsomal enzymes (Conney, 1967) and it has been suggested that this leads to an enhanced rate of metabolism of vitamin D (Lancet, 1972). The administration of enzyme-inducing drugs to humans results in an increased rate of conversion of vitamin D₃ into more-polar compounds, including 25-hydroxycholecalciferol (Hahn, Birge, Scharp & Avioli, 1972a; Greenwood, Prunty & Silver, 1973), a biologically active metabolite known to be pro-

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duced in the liver (Ponchon & De Luca, 1969). Somewhat paradoxically, however, the serum concentrations of 25-hydroxycholecalciferol (25-HCC) are subnormal in patients receiving phenobarbitone on a long-term basis (Hahn, Hendin, Scharp & Haddad, 1972b).

We have carried out a series of experiments in rats and pigs to study the effect of phenobarbitone administration on vitamin D metabolism. The results show that phenobarbitone increases the hydroxylation of vitamin D₃ to 25-HCC, and this is accompanied by an enhanced biliary excretion of biologically inactive conjugated metabolites, the net result being a decrease in body stores of vitamin D. Some of these findings have already been reported in a preliminary form (Silver, Neale, Davies, Breckenridge & Thompson, 1972a; Silver, Quill, Neale & Thompson, 1972b; Silver, Thompson, Neale, Orme, Davies & Breckenridge, 1972c).

METHODS

Experimental design

(a) Metabolites of vitamin D₃ (cholecalciferol) were studied in the plasma of control and phenobarbitone-induced animals after intravenous injections of radioactively labelled cholecalciferol or 25-HCC. In pilot studies, in vitamin D-deficient rats, the plasma concentrations of the circulating metabolites were measured at 6, 12 and 24 h after injection. The differences between control and phenobarbitone-induced animals were slightly greater at 12 h than at 24 h. All definitive studies were subsequently performed in rats on a normal laboratory diet and at 24 h. In similar experiments on pigs vitamin D metabolism could be assessed in a different animal species, which also allowed sufficiently large quantities of plasma to be obtained for assessment of biological (anti-rachitic) activity.

(b) Bile fistulae were created in control and "induced" rats and the enterohepatic circulation was maintained by an infusion of donor bile through an intraduodenal tube. Bile was collected for 24 h after an intravenous injection of either labelled cholecalciferol or 25-HCC, assayed for radioactivity and analysed by silicic acid chromatography before and after incubation with β-glucuronidase.

(c) The tissue distribution of radioactivity was determined in control and induced rats 30 days after an intravenous injection of [¹⁴C]cholecalciferol.

Dietary and drug regimes

Inbred male and female Wistar rats (40–50 g) from vitamin D-deficient mothers were maintained after weaning on a rachitogenic vitamin D-deficient diet (calcium 0.72%, phosphorus 0.18%), and were shielded from ultraviolet light. Radiography of the tibia under ether anaesthesia confirmed rickets by wide decalcified epiphyseal cartilages, generalized decalcification and cupping of the metaphyses. In another series of experiments, male Wistar rats and young pigs fed on a normal laboratory diet were used. In each experiment half the rats were controls and half were given phenobarbitone in their drinking water (1 mg/ml) for a minimum of 4 days. Induction of hepatic microsomal enzymes was confirmed by an increased ability to tolerate the anaesthetic effects of an intraperitoneal injection of sodium pentobarbitone (35 mg/kg) (Conney, 1967). In the pig experiments, phenobarbitone (15 mg/kg) was injected intraperitoneally for 10 days, and enzyme induction was confirmed retrospectively by the enhanced ability of the microsomal fraction of the induced pigs' livers to demethylate ethylmorphine (Breckenridge, Orme, Thorgeirsson, Davies & Brooks, 1971).
Induction of vitamin D metabolism

Preparation and administration of radioactive doses and collection of samples

[1,2-3H]Cholecalciferol (595–720 μCi/mmol), [4-14C]cholecalciferol (32.3 μCi/mmol) and 25-[26,27-methyl-3H]hydroxycholecalciferol (13.1 mCi/mmol) were dissolved separately or together in 0.5 ml of ethanol and mixed with 1 ml of propylene glycol. The ethanol was evaporated under nitrogen and approximately 4 ml of rat plasma was then mixed with the propylene glycol to provide a stock solution for injecting intravenously into rats. This was stored at −20°C under nitrogen. For the pig experiments, doses were made up in propylene glycol, and mixed with 10 ml of venous blood immediately before injection into the internal jugular vein of the pig, which had been anaesthetized with intravenous pentobarbitone. The radioactive purity of the injected material exceeded 90% as assessed by silicic acid column chromatography. To ensure this each of the labelled compounds was analysed chromatographically before use and repurified if necessary upon silicic acid columns.

TABLE 1. Procedure for silicic acid column chromatography

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Volume (ml)</th>
<th>Peak</th>
<th>Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diethyl ether–hexane (1:3)</td>
<td>450</td>
<td>I</td>
<td>Cholecalciferol ester</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II</td>
<td>Precholecalciferol</td>
</tr>
<tr>
<td></td>
<td></td>
<td>III</td>
<td>Cholecalciferol(1)</td>
</tr>
<tr>
<td>Diethyl ether–hexane (1:1)</td>
<td>50</td>
<td>Wash</td>
<td>25-Hydroxycholecalciferol</td>
</tr>
<tr>
<td>Diethyl ether</td>
<td>300</td>
<td>IV</td>
<td></td>
</tr>
<tr>
<td>Diethyl ether</td>
<td>50</td>
<td>Wash</td>
<td></td>
</tr>
<tr>
<td>Methanol–ether (1:19)</td>
<td>150</td>
<td>V</td>
<td>Dihydroxycholecalciferol(2) compounds, including 1,25-, 24,25-, 25,26-dihydroxycholecalciferol</td>
</tr>
<tr>
<td>Methanol–ether (1:19)</td>
<td>50</td>
<td>Wash</td>
<td>Unidentified</td>
</tr>
<tr>
<td>Methanol–ether (1:1)</td>
<td>150</td>
<td>VI</td>
<td></td>
</tr>
<tr>
<td>Methanol–ether (1:1)</td>
<td>50</td>
<td>Wash</td>
<td></td>
</tr>
<tr>
<td>Methanol</td>
<td>100</td>
<td>Flush</td>
<td></td>
</tr>
</tbody>
</table>

(2) Holick & De Luca (1971).

At appropriate time-intervals rats were bled to death under ether anaesthesia. Blood was drawn into heparinized syringes and plasma was separated by centrifugation at 4°C. Plasma samples were subsequently stored at −20°C. Pigs were anaesthetized with intravenous pentobarbitone and exsanguinated via the axillary vessels. Plasma was separated from blood collected into acid–citrate anticoagulant and was stored at −20°C. Pig livers were perfused through the portal vein with ice-cold 0.9% sodium chloride solution before excision and then kept on ice for no longer than 4 h, during which time the microsomal fractions were prepared (De Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955).

Extraction and chromatography

Aliquots of plasma were extracted with 20 vol. of chloroform–methanol (2:1, v/v) and the phases separated by the addition of 4 vol. of distilled water (Folch, Lees & Sloane-Stanley,
The chloroform phase was collected and evaporated to dryness under nitrogen in a rotary evaporator at less than 50°C. The dried extract was taken up in 5 ml of hexane, of which 4 ml was applied to a silicic acid column. Chromatography was performed through glass columns 2 cm in diameter, containing 40 g of silicic acid (Sigma 325 mesh) that had not been heat-inactivated and, as described by Mawer & Backhouse (1969), the sample was sequentially eluted with solvents of increasing polarity (Table 1), and either collected in fractions or batch-wise. The elution patterns of cholecalciferol and 25-HCC were confirmed by using authentic radioactive markers. The columns were run at 0–4°C under nitrogen. Eluates were dried down and dissolved in 10 ml of 5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole (butyl-PBD) in toluene (6 g/l), or, if water-soluble, in scintillation fluid containing butyl 5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole (8 g), naphthalene (80 g), ethoxyethanol (400 ml) and toluene (600 ml). Radioactivity was assayed with a Beckman LS 250 liquid-scintillation counter. Quenching was corrected by means of an automatic external standardization system. Overall recovery of radioactivity applied to columns was 75–100%.

When unlabelled 25-HCC (gift of Dr J. C. Babcock, Upjohn Co., Michigan) was added to silicic acid columns for spectrophotometric analysis of material migrating as 25-[3H]hydroxycholecalciferol, 20 ml fractions were collected, of which 10 ml was dried down and assayed for radioactivity and 10 ml was evaporated under nitrogen; this was then dissolved in 3 ml of ethanol, and the extinction of the solution read at 264 nm in a Hitachi Perkin–Elmer 139 UV-vis spectrophotometer. Sephadex LH 20 column chromatography as described by Holick & de Luca (1971) was also used. Fractions were collected at room temperature from glass columns (1.5 cm diameter) containing 20 g of Sephadex LH 20, chloroform–n-hexane (65:35, v/v) being used as the eluent. This system separates peak III (cholecalciferol) and peak IV (25-HCC) from peak V (dihydroxy derivatives), which chromatographs with as many as four peaks.

Bioassay

The biological activities of peaks III and IV, obtained from the plasma of two control pigs and two phenobarbitone-induced pigs, were assessed 24 h after an intravenous injection of 20 µCi of [3H]cholecalciferol. The animals were exsanguinated 24 h later and plasma extracts were chromatographed on silicic acid columns.

Pooled peaks I–III, and pooled peaks IV of plasma from both control and induced pigs, were each first taken up in 2 ml of ethanol and then dissolved in 10 ml of olive oil. The samples were flushed with nitrogen until the ethanol had evaporated. The olive oil solutions and Medical Research Council reference standards of vitamin D3 were administered intra-oesophageally with calibrated 1 ml syringes to vitamin D-deficient rats. Each rat received 0.4 ml initially, and one-half of the rats from each group received a further 0.4 ml 24 h later. Six days after the first dose the animals were killed. A hindleg from each animal was radiographed and assessed for the healing of rickets.

Biliary excretion of vitamin D metabolites

Rats with surgically created bile fistulae were kept in restraining cages and allowed access to food and water containing 0.9% sodium chloride and 3% of glucose. The enterohepatic circulation of bile was maintained throughout the experiment by mechanically infused pooled donor bile into the duodenum, so as to match the total bile output with volume of bile infused.
Bile was collected at 4°C for 24 h after the administration of [14C]cholecalciferol or 25-[3H]HCC intravenously and 0.1 ml aliquots were assayed for radioactivity.

The effect of phenobarbitone on the nature of the metabolites in the bile was investigated by the extraction, partition and chromatography of biliary radioactivity before and after enzymic hydrolysis with β-glucuronidase. β-Glucuronidase obtained from ox liver (Ketodase, Warner and Chilcott), bacteria (Boehringer, Mannheim) and limpet (gift of Dr R. V. Brooks, St Thomas' Hospital, London) was incubated with aliquots of bile at 37°C and pH 5.0 for 18 h. The effect of the antibiotic chloramphenicol (20 μg/ml) on the incubations was investigated. The absence of endogenous biliary β-glucuronidase was confirmed by incubating bile with a p-nitrophenyl-β-glucuronide substrate; the absence of endogenous biliary inhibition of glucuronidase was confirmed by incubating β-glucuronidase with p-nitrophenylglucuronide in bile. After incubation the free p-nitrophenol was separated from its glucuronide by thin-layer chromatography on silicic acid plates (Merck HF254) developed in butan-1-ol-acetic acid-water as solvent (4:1:2, by vol.).

Bile was extracted with 20 vol. of chloroform–methanol (2:1, v/v) and the phases were separated with 0.02 mol/l KOH. The chloroform-soluble radioactivity was applied to silicic acid columns and assayed for radioactivity as for plasma, except that after transfer of the dissolved eluates to scintillation vials, the round-bottom flasks were washed with 0.5 ml of methanol and 10 ml of water-soluble scintillation fluid was added. This was necessary to ensure complete recovery of radioactivity in more-polar metabolites.

**Tissue distribution of vitamin D**

Five control and five phenobarbitone-treated rats (on a normal laboratory diet) were injected intravenously with [14C]cholecalciferol (4 μg) and killed 30 days later. The test group had phenobarbitone in their drinking water for the 30 days. The plasma was extracted as before; the liver, intra-abdominal fat and thigh muscles were excised and homogenized in 40 ml of chloroform–methanol (2:1, v/v), in a Waring blender, and after separating the phases with water, the chloroform extract was dried and assayed for radioactivity.

Statistical differences were evaluated by Student's t-test.

**RESULTS**

**Plasma radioactivity**

At 6, 12 and 24 h after an intravenous dose of 0.03 μCi of [1,2-3H]cholecalciferol (0.02 μg) a greater proportion of plasma radioactivity was present as polar metabolites of vitamin D (peaks IV–VI) in phenobarbitone-treated rachitic rats than in control animals (Fig. 1). This difference was most marked at 6 h.

A similar more detailed study was also carried out in rats on a normal laboratory diet. Plasma from nine control and nine induced rats was obtained 24 h after the intravenous injection of 0.2 μCi of [3H]cholecalciferol (0.12 μg). The concentration of total radioactivity in plasma (mean ± SE) expressed as d.p.m. ml⁻¹ 100 g⁻¹ body weight was found to be significantly greater in the phenobarbitone-treated rats (3115 ± 323) than in the controls (1894 ± 249; P < 0.01). The difference in radioactivity was largely accounted for by a highly significant increase in peak IV radioactivity in the 'induced' rats. There was a significant decrease in peak I–III activity (corresponding mainly to unmetabolized vitamin D₃), and increases in peak IV
and in peak V, but there was no difference in peak VI radioactivity between the two groups (Fig. 2). These results suggest that both in rachitic rats and rats on a normal diet, phenobarbitone enhanced the amount of peak IV and peak V radioactivity which appeared in the plasma after intravenous \(^{3}H\)cholecalciferol.

To determine whether the increase in peak V radioactivity was secondary to a direct effect of phenobarbitone on the metabolism of peak IV a parallel experiment was carried out, in which 0·6 μCi of 25-\(^{3}H\)HCC (0·002 μg) was administered instead of cholecalciferol. In contrast to the previous study, there was no significant difference between the total plasma radioactivity in eight control rats (5324±805) and six ‘induced’ rats (5576±523) at 24 h after the intravenous dose. Furthermore, the amount of radioactivity in peaks IV, V and VI was almost identical in the two groups of animals (Fig. 3). Therefore phenobarbitone administration had no obvious effect on the conversion of injected 25-HCC to more-polar metabolites appearing in plasma. Thus the excess of peak V radioactivity observed in ‘induced’ rats given labelled cholecalciferol in the previous experiment was presumably a reflection of the increased amount of peak IV available for metabolism.

**Characterization of peak IV**

The nature of the labelled metabolite eluted as peak IV from silicic acid columns was determined as follows. Two ‘induced’ and two control pigs were each given 15 μCi of [4-\(^{14}\)C]-
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FIG. 2

Pattern of radioactivity in rat plasma at 24 h after an intravenous injection of [3H]cholecalciferol. The mean ± SEM and the differences between them are shown. N.S., not significant.

FIG. 3

Pattern of radioactivity in rat plasma at 24 h after an intravenous injection of 25-[3H]HCC. N.S., not significant.

TABLE 2. Distribution of radioactivity in plasma of four control and four 'induced' pigs at 24 h after intravenous [3H]cholecalciferol (20 μg) or [14C]cholecalciferol (170 μg)

<table>
<thead>
<tr>
<th></th>
<th>Peak III</th>
<th>Peak IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control [3H]</td>
<td>52, 57</td>
<td>40, 35</td>
</tr>
<tr>
<td>Control [14C]</td>
<td>58, 56</td>
<td>28, 32</td>
</tr>
<tr>
<td>Mean</td>
<td>57</td>
<td>34</td>
</tr>
<tr>
<td>'Induced' [3H]</td>
<td>40, 39</td>
<td>47, 51</td>
</tr>
<tr>
<td>'Induced' [14C]</td>
<td>40, 45</td>
<td>48, 40</td>
</tr>
<tr>
<td>Mean</td>
<td>42</td>
<td>45</td>
</tr>
</tbody>
</table>
cholecalciferol (170 µg) and killed at 24 h. Their plasma was used for co-chromatography with a 25-[3H]HCC marker. Four other pigs, two ‘induced’ and two control, were each given 20 µCi of [3H]cholecalciferol (15 µg) and also killed at 24 h. Their plasma was chromatographed on silicic acid columns and the eluted peaks were used for radio- and bio-assay. The results (Table 2) show that the four ‘induced’ pigs had less radioactivity in peak III than the controls (mean of 42% versus 57%) and more in peak IV (44% versus 30%). The plasma of pigs given

![Silicic acid column co-chromatography](image)

Fig. 4. Silicic acid column co-chromatography of 25-[3H]HCC marker (●), with pig plasma obtained 24 h after an intravenous injection of [14C]cholecalciferol (○). Each point gives the mean value of results obtained from two pigs.

[14C]cholecalciferol was co-chromatographed with a preparation of 25-[3H]HCC on columns of both silicic acid (Fig. 4) and Sephadex LH 20 (Fig. 5). Plasma peak IV appeared to be identical with the major peak of radioactivity obtained from 25-[3H]HCC and co-chromatographed with unlabelled 25-HCC (Fig. 6). Furthermore, pooled peak III and pooled peak IV from both control and ‘induced’ pigs were each shown to be biologically active on the rat line test assay.
Biliary excretion

The cumulative excretion of radioactivity in five control and five 'induced' rats after the intravenous administration of 1.5 μg of \([{^{14}C}]\)cholecalciferol and 0.001 μg of 25-[\(^{3}H\)]HCC is shown in Fig. 7. 'Induced' rats excreted a significantly larger amount of bile than controls \((P<0.01)\), but there was no positive correlation between bile volume and the amount of radioactivity excreted (Fig. 8). Phenobarbitone-treated rats excreted significantly larger amounts of radioactivity than controls after both \([{^{14}C}]\)cholecalciferol (% of dose 24 h\(^{-1}\) 100 g\(^{-1}\), mean ± SEM, 9.7 ± 1.7:3 ± 0.5, \(P<0.01\)) and after 25-[\(^{3}H\)]HCC (7.0 ± 0.9:3 ± 0.5, \(P<0.01\)).

Nature of metabolites in the bile

Chloroform solubility. Extraction of bile with chloroform–methanol showed that 17% of the radioactivity was lipid-soluble. This increased to 28% after incubation alone, and to 46% after incubation with \(\beta\)-glucuronidase. This applied to bile from both control and 'induced' rats, and for radioactive metabolites derived from both \([{^{14}C}]\)cholecalciferol and 25-[\(^{3}H\)]HCC. Limpet and liver \(\beta\)-glucuronidase gave similar results but the differences were less when bacterial enzyme was used. In all instances the pH optimum was 5.0 and maximum hydrolysis was achieved in 24 h. Incubation with and without the antibiotic chloramphenicol did not alter the
ratio of water and lipid-soluble radioactivity and this suggested that bacteria in bile were not responsible for changes in lipid solubility, such as occurs with glucuronidase hydrolysis.

**Chromatography.** Silicic acid column chromatography of the chloroform-soluble radioactivity (Fig. 9) showed that the metabolites were mainly in peak VI before incubation. There was little radioactivity in peak III. After incubation more radioactivity appeared in peaks IV and V. Co-chromatography of the peak IV fraction, both before and after incubation with 25-[²H]HCC (Fig. 10) showed that the bile peak IV had a more heterogeneous elution pattern than the 25-HCC marker. Peak IV from bile of rats given 25-[²H]HCC was identical with that found in animals given [¹⁴C]cholecalciferol.

![Silicic acid column co-chromatography of unlabelled 25-HCC (E264;○) and peak IV from plasma of a pig, 24 h after an intravenous injection of [¹⁴C]cholecalciferol (●).](image1)

**Fig. 6.** Silicic acid column co-chromatography of unlabelled 25-HCC (E264;○) and peak IV from plasma of a pig, 24 h after an intravenous injection of [¹⁴C]cholecalciferol (●).

**Tissue distribution**

The amount of radioactivity in plasma and tissues analysed at 30 days after an intravenous injection of 4 µg of [¹⁴C]cholecalciferol is shown in Table 3. There was less radioactivity in the adipose tissue ($P = 0.02$) and kidneys ($P = 0.01$) of ‘induced’ rats than in the controls, but similar amounts in plasma, muscle and per unit weight of liver. The phenobarbitone-treated rat livers weighed $22.1 ± 1.4$ g, and the control livers $15.8 ± 0.3$ g, which accounts for the greater total radioactivity in the livers of induced animals.
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FIG. 7. Biliary excretion of radioactivity (a) after 1.5 µg of [14C]cholecalciferol and (b) after 0.01 µg of 25-[3H]HCC in control (○) and phenobarbitone-treated rats (●). Each point is the mean value from five rats; vertical bars represent the SEM.

FIG. 8. Correlation between biliary radioactivity after an intravenous injection of [14C]cholecalciferol and bile flow in five control (○) and five phenobarbitone-treated rats (●).
DISCUSSION

These studies demonstrate that the administration of phenobarbitone caused an increased conversion of radioactive vitamin D₃ into more-polar metabolites in Wistar rats and pigs on a normal diet, and also in vitamin D-deficient rachitic rats. In all animals the major labelled metabolite found in plasma, peak IV, had the same $R_p$ value as 25-HCC when chromatographed on silicic acid and on Sephadex LH 20, and was biologically active. Thus it may be concluded that phenobarbitone stimulates the conversion of vitamin D₃ into 25-HCC in experimental animals as well as in humans (Hahn et al., 1972a). The increased metabolism of vitamin D in

<table>
<thead>
<tr>
<th></th>
<th>Body wt. (d.p.m./ml)</th>
<th>Plasma (d.p.m./g)</th>
<th>Fat (d.p.m./g)</th>
<th>Muscle (d.p.m./g)</th>
<th>Kidneys (total d.p.m.)</th>
<th>Liver Total d.p.m. (d.p.m./g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control rats (n = 5)</td>
<td>336 ± 7</td>
<td>405 ± 50</td>
<td>437 ± 20</td>
<td>79 ± 6</td>
<td>4409 ± 187</td>
<td>1452 ± 115</td>
</tr>
<tr>
<td>Phenobarbitone-treated rats (n = 5)</td>
<td>373 ± 16</td>
<td>511 ± 110</td>
<td>340 ± 24</td>
<td>73 ± 9</td>
<td>2373 ± 418</td>
<td>1900 ± 52</td>
</tr>
<tr>
<td>$P$</td>
<td>N.S.</td>
<td>N.S.</td>
<td>0.02</td>
<td>N.S.</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

The means ± SEM are shown. N.S., not significant.
rats given phenobarbitone may be due in part to an increased hepatic blood flow (Ohnhaus, Thorgeirsson, Davies & Breckenridge, 1971) but it is also likely that cholecalciferol-25-hydroxylase is a microsomal enzyme which can be induced by the drug. We have tried to test this hypothesis by isolating the enzyme and measuring its activity in vitro, but the results of these experiments have been inconclusive (unpublished observations). It is unlikely that our observations are due to a non-specific effect of phenobarbitone on the physical activity of the

animals because we delayed the experiments until the sedative effect of phenobarbitone was no longer noticeable and because similar results were obtained in rachitic rats which in other respects behave quite differently from vitamin D-replete animals. Furthermore the effect of phenobarbitone is independent not only of vitamin D status but also of the size of the dose administered (in pigs, over the range 15–170 μg of cholecalciferol).

The administration of phenobarbitone also resulted in the appearance of increased amounts of peak V radioactivity (dihydroxy derivatives) in plasma after administration of labelled cholecalciferol but not after 25-HCC. This suggests that the increase in peak V was not due to a direct effect of phenobarbitone on peak IV metabolism. Peak V contains 1,25-dihydroxy-
cholecalciferol (Mawer, Lumb, Schaeffer & Stanbury, 1971), but it seems unlikely that formation of this metabolite was enhanced in 'induced' animals, since it has been shown that its synthesis by the kidney cannot be promoted by increasing the supply of precursor 25-HCC (Fraser & Kodicek, 1971). A similar increase in metabolites more polar than peak IV has also been observed in the plasma of patients taking enzyme-inducing drugs (Hahn et al., 1972a). Peak V may contain derivatives of cholecalciferol which have not been hydroxylated in the 25 position. It might be possible to study this by using doubly labelled cholecalciferol (e.g. [1-3H, 4-14C]-cholecalciferol) and measuring the 3H/14C ratio in peak V.

Rats given phenobarbitone excreted more radioactivity in the bile than control animals after the administration of either [3H]cholecalciferol or 25-[3H]HCC. Thus phenobarbitone appears to enhance the metabolism of 25-HCC as well as that of cholecalciferol even though this effect could not be demonstrated by the measurement of radioactive metabolites in plasma. These results suggest that phenobarbitone may accelerate more than one pathway for the metabolism of vitamin D and that to elucidate such changes it will be necessary to undertake a more detailed study of the distribution of metabolites between plasma and tissues.

Analysis of metabolites in bile from control and phenobarbitone-induced rats showed that they were mainly in the form of water-soluble conjugates, including glucuronides. A mean of 29% of the water-soluble radioactivity was rendered soluble in chloroform by incubating bile with β-glucuronidase derived from ox liver, bacteria or limpets. Additional studies excluded the possibility of endogenous inhibitors or activators of β-glucuronidase in bile. Bile was shown to contain little unaltered cholecalciferol, either free or conjugated, most of the radioactivity being in the form of conjugates of more-polar metabolites, separable on column chromatography into peaks IV, V and VI. Similar findings have been reported in bile after vitamin D administration by Avioli, Lee, McDonald, Lund & De Luca (1967) and Bell & Kodicek (1969). In contrast to plasma, biliary peak IV was heterogeneous with a similar chromatographic pattern both before and after incubation with β-glucuronidase. It did not co-chromatograph with 25-[3H]HCC and probably consisted mainly of other metabolites. In our studies bile from control rats was qualitatively similar to that from phenobarbitone-induced rats and this suggests that phenobarbitone enhances but does not alter the normal processes of hydroxylation and conjugation.

The effect of phenobarbitone administration on the tissue distribution of vitamin D and its metabolites was studied in rats killed 30 days after an injection of a 4 μg dose of [14C]cholecalciferol. It was found that induced animals retained significantly less radioactivity in their adipose tissue and kidneys than the controls. Since fat is the major storage site of vitamin D in the rat, accounting for 50% of total body stores (Rosenstreich, Rich & Volwiler, 1971), the decreased amount of radioactivity found in the adipose tissue of 'induced' rats probably represents a significant degree of vitamin D depletion. The significance of the decreased amount of radioactivity in the kidney of induced rats is not immediately apparent, but is in agreement with the findings of Von Herrath, Kraft, Schaefer & Koepppe (1972). Further studies would be necessary to determine whether this represents a decrease in 1,25-dihydroxycholecalciferol.

In conclusion, we have shown that phenobarbitone causes both an increased conversion of cholecalciferol into 25-HCC and a more rapid excretion of their metabolites in bile. Since this was accompanied by a decrease in adipose tissue levels of [14C]cholecalciferol measured radioactivity, and since chronic administration of phenobarbitone reduces serum 25-HCC levels (Hahn et al., 1972b), the effect on biliary excretion must be the metabolically dominant one. If this effect of phenobarbitone applies in man, then it is not surprising that vitamin D
deficiency may be precipitated by the administration of phenobarbitone or other enzyme-inducing drugs, especially as the available supply of vitamin D from sunlight and diet in this country is close to the minimal requirements in many otherwise normal subjects (Stanbury & Mawer, 1972). In the absence of a compensatory increase in intake, an accelerated rate of turnover of vitamin D must inevitably lead to a decrease in pool size in subjects on a borderline diet.

ACKNOWLEDGMENTS

This work was supported by a grant from the Medical Research Council. We are grateful to Miss Helen Quill for excellent technical assistance, and to Dr A. Breckenridge and Dr D. S. Davies for helpful discussions and suggestions.

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