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THE EFFECTS OF PHENOBARBITONE ON URINARY 6β-HYDROXYCORTISOL EXCRETION AND HEPATIC ENZYME ACTIVITY IN THE MARMOSET MONKEY (*CALLITHRIX JACCHUS*)

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Abstract—The effect of phenobarbitone administration on hepatic drug metabolising activity and urinary 6β-hydroxycortisol excretion was investigated in the marmoset monkey (*Callithrix jacchus*). Phenobarbitone produced a significant increase in hepatic microsomal cytochrome P-450 content, aminopyrine *N*-demethylase activity, 7-ethoxycoumarin *O*-deethylase activity but no change in ethoxyresorufin *O*-deethylase activity. A significant increase in the urinary excretion of 6β-hydroxycortisol was observed after two days treatment with phenobarbitone.

6β-Hydroxycortisol is a polar metabolite of cortisol in man formed primarily in the endoplasmic reticulum of hepatocytes by mixed-function oxidases [1, 2] and is excreted in urine [3]. Many agents that induce microsomal drug metabolism also stimulate the hydroxylation of steroids in both man and animals [4]. It was therefore suggested that measurement of the urinary excretion of 6β-hydroxycortisol in relation to total urinary 17-hydroxycorticosteroids may provide and index of enzyme induction of the hepatic microsomal mixed-function oxidases in man [4]. Subsequent studies have shown that urinary 6β-hydroxycortisol is a useful *in vivo* parameter for the assessment of the enzyme inducing capacity of drugs such as phenobarbitone, rifampicin, antipyrine and phenytoin [5–7]. However, the qualitative changes in the microsomal enzymes associated with increased urinary 6β-hydroxycortisol excretion have not been determined. We are therefore seeking a suitable animal model with which to investigate the biochemical changes associated with enhanced cortisol 6β-hydroxylation.

In this paper we report the effects of phenobarbitone administration on the relationship between hepatic microsomal mixed-function oxidase activity and urinary 6β-hydroxycortisol excretion in the marmoset monkey (*Callithrix jacchus*).

MATERIALS AND METHODS

Chemicals. Tritiated 6β-hydroxycortisol (S.A. 52 Ci/mmole) was a gift from the Radiochemical Centre, Amersham. Standard radioimmunoassay reagents were prepared as described previously [8]. 6β-Hydroxycortisol was synthesised according to the method of Dusza *et al.* [9]. *p*-Hydrazinobenzenesulphonic acid was obtained from Eastman Ltd. and purified by recrystallisation [10]. Ketodase (β-glucuronidase) was purchased from Warner Lambert (U.K.) Ltd. 7-Ethoxycoumarin and umbeliferone were obtained from Sigma Ltd. and ethoxyresorufin from Pierce & Wariner Ltd. All other general reagents were obtained from B.D.H. All solvents were redistilled before use. For high pressure liquid chromatography purposes, solvents were filtered using Millipore filters (Whatman). Scintillant (NE 260) was obtained from Nuclear Enterprises.

Experimental animals. Eight male marmoset monkeys (bred at I.C.I. Pharmaceutical Alderley Park) weighing between 260 and 420 g were housed in individual metabolism cages. On day zero, control 24 hr urine samples were collected for each animal. On days 1, 2 and 3, four animals were injected intraperitoneally with sodium phenobarbitone (20 mg/kg) in 0.9% saline (2ml/kg) and four animals were injected i.p. with saline alone. Complete 24-hr urine collections were made on days 1, 2 and 3. After the final urine collection, the animals were killed with carbon dioxide.

Preparation of liver microsomes. The livers were removed from the animals immediately after death and a 25% w/v homogenate in 10 mM phosphate buffer pH 7.4 containing 0.15M potassium chloride was prepared using a glass Potter–Elvehjem homogeniser with a Teflon pestle. The homogenates were centrifuged at 18,000*g* for ten min, and the microsomal fraction isolated by centrifugation of the supernatant at 250,000 *g* for 30 min. The microsomal pellet was resuspended in phosphate buffered potassium chloride and recentrifuged. The microsomes were stored frozen in liquid nitrogen as a suspension in Tris–HCl buffer pH 7.5 (50 mM) containing 0.25 M sucrose and EDTA (1 mM).

Determination of 6β-hydroxycortisol. Urinary 6β-hydroxycortisol was measured in marmoset urine diluted (1:1,000–1:20,000) in phosphate buffered saline with thiomersal (PBSM), by radioimmunoassay as described previously [8]. The specificity of

this assay for 6β-hydroxycortisol in marmoset urine was investigated by assay of urine samples with and without prior separation of 6β-hydroxycortisol by high performance liquid chromatography (h.p.l.c.)

Extraction of urine samples prior to h.p.l.c. Sodium sulphate (20%, w/v) was dissolved in a 1 ml aliquot of urine. Approximately 15,000 c.p.m [^{3}H]-6β-hydroxycortisol in 20 μl methanol was added and allowed to equilibrate for 30 min. The sample was then extracted with 4 vol. of freshly redistilled ethyl acetate and the extract was then evaporated to dryness under a stream of nitrogen. The dried extract was redissolved in 50μl ethanol. A 10μl aliquot was used for chromatography and the radioactivity in duplicate 10μl aliquots was measured by liquid scintillation spectrometry.

High performance liquid chromatography. A Varian 8500 high performance liquid chromatography isochratic pump was used in conjunction with a Pye Unicam LC3 ultraviolet detector and a M.S.E. Fisons vitatron pen recorder. A Whatman partisil column (P×S 10/25) with a pre-column (7cm; packed with HC Pellosil) were used. The eluent consisted of methylene chloride (410 ml), *n*-hexane (470 ml), ethanol (112 ml) and water (10 ml) prepared as described by Roots *et al.* [6]. The flow rate was 90 ml/hr and the ultraviolet absorbance was measured at 240 nm. After each determination the pump was switched to 'fast pump' mode until a volume of 20ml of eluent had passed through the column. Samples were injected with a 10 μl, Hamilton syringe. The fraction containing 6β-hydroxycortisol was collected over a period of 5 min. A suitable aliquot was taken in duplicate for radioimmunoassay [8]. A aliquot of the 6β-hydroxycortisol fraction was evaporated to dryness under a stream of nitrogen and the radioactive content determined. The recovery of [^{3}H]-6β-hydroxycortisol was used to determine the recovery of the endogenous 6β-hydroxycortisol which was measured by radioimmunoassay.

Determination of ethyl acetate-extractable 17-hydroxycorticosteroids in marmoset urine. 17-Hydroxycorticosteroids were determined by the method of Sanghvi [10] with some modifications. An aliquot (0.25 ml) of urine was diluted to 4ml with 2M acetate buffer (pH 5.0) and incubated overnight (16 hr) at 42° with β-glucuronidase (8,000 Fishman units). Sodium sulphate (20%, w/v) was dissolved in the incubation mixture prior to extraction with 25 ml of freshly redistilled ethyl acetate. The ethyl acetate extract was separated into two 10 ml aliquots which were evaporated to dryness under a stream of nitrogen. Complete reagent was added to one of the tubes and blank reagent to the other. The tubes were covered and heated at 100° for 60 min. Optical density was measured for each tube against its own blank at 325, 355 and 375 nm using a Pye Unicam SP8-100 ultraviolet spectrophotometer. All calculations were made using the Allen correction [11]. Cortisol was used as standard.

Metabolism of model substrates. Microsomal aminopyrine *N*-demethylase activity was measured as described by Mazel [12]. Aminopyrine (15 mM) was incubated with microsomal protein (1 mg) and NADPH (0.5 mM) in 0.1M phosphate buffer pH 7.4 (5 ml) for 7 min at 37°. Ethoxyresorufin *O*-deethylase activity was determined by the method of Burke and Meyer [13]. The incubation mixture contained ethoxyresorufin (250 nM), microsomal protein (1 mg) and NADPH (0.25 mM) in 0.1M phosphate buffer pH 7.8 (2 ml). The reaction was monitored in a cuvette for 3 min at 30°. NADPH–cytochrome *c* reductase activity was determined by the method of Mazel [12]. The incubation mixture contained microsomal protein (0.025 mg), NADPH (0.15 mM), and 0.45 mM KCN in 0.1 M phosphate buffer pH 7.6 (1 ml). 7-Ethoxycoumarin *O*-deethylation was measured by a modification of the method of Pohl *et al.* [14]. The reaction was monitored in a cuvette for three minutes at 30° by measuring the emission at 456 nm using an excitation wavelength of 394 nm using a Perkin Elmer 3000 spectrofluorimeter. The reaction mixture contained 1 mg microsomal protein and 0.25 mM NADPH in 0.1 M phosphate buffer pH 7.4 (2 ml). The reaction was started by the addition of 7-ethoxycoumarin (40 mM) in dimethylformamide (10 μl) to give a final substrate concentration of 0.2 mM. Cytochrome P-450 concentrations were estimated using a Pye Unicam SP8-200 spectrophotometer by the method of Omura and Sato [15] using a microsomal protein concentration of 2 mg/ml.

Gel electrophoresis. SDS polyacrylamide gel electrophoresis of microsomal protein was carried out as described by Dent *et al.* [16]. A 1.5 mm Slab gel was used with a 2 cm 3% w/v acrylamide upper stacking gel and a 10 cm 10% w/v separating gel. 15 μg microsomal protein was applied per sample. A constant current power supply was used at 15 mA while the samples were in the stacking gel, increasing to 30 mA for the separating gel. Proteins were visualised using Coomassie blue stain before photography.

Characterisation of 6β-hydroxycortisol. Ultraviolet spectra were recorded on a Pye Unicam SP8-100 spectrophotometer and mass spectra were determined on a LKB 9000 mass spectrometer.

RESULTS

Characterisation of 6β-hydroxycortisol in marmoset urine. The mass spectrum of 6β-hydroxycortisol isolated from marmoset urine by h.p.l.c. was identical to the spectrum obtained from a synthetic sample [9]. In addition to the molecular ion (M^+ = 378) the spectrum contains peaks at 360 (M^+-H_2O), 348 (M^+-CH_2O) and 318 (M^+-$C_2H_4O_2$) consistent with the presence of the dihydroxyacetone side-chain and the 6β-hydroxyl group [17]. The u.v. spectrum of the biological material was also identical to that of authentic synthetic material and contained λ max 240 nm for the enone system in the A-ring. The specificity of the radioimmunoassay [8] was found to be satisfactory for direct measurement of 6β-hydroxycortisol in marmoset urine. The correlation coefficient of samples measured by radioimmunoassay with and without purification was $r = 0.99$ ($n = 11$; $P < 0.001$; slope 1.04).

Excretion of steroids in marmoset urine. There was a wide variation in normal urinary excretion of 6β-hydroxycortisol (33.5 – 360 μg/24 hr) but when it was expressed as a percentage of ethyl acetate-extract-

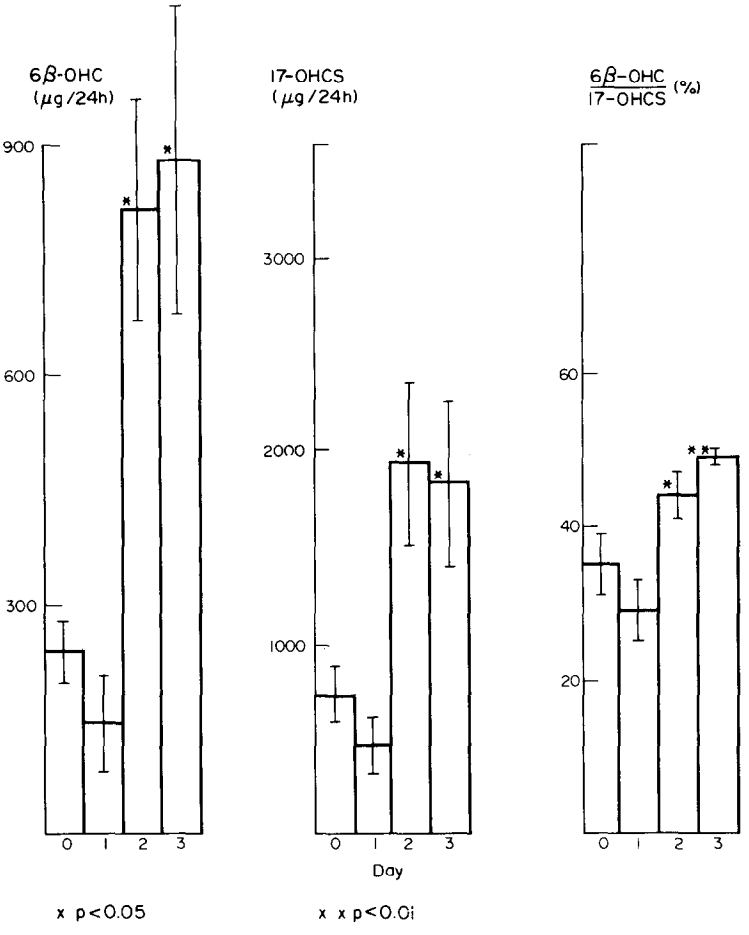

Fig. 1. The 24-hr urinary excretion of 6β-hydroxycortisol (6β-OHC), 17-hydroxycorticosteroids (17-OHCS) and the calculated ratio 6β-hydroxycortisol: 17-hydroxycorticosteroids (6β-OHCS/17-OHCS) in marmoset monkeys before (day 0) and during (days 1, 2, 3) treatment with phenobarbitone 20 mg/kg. Results are means ($n = 4$) ± S.E. Statistical significance from control day using Student's t-test *P < 0.05 and **P < 0.01.

able 17-hydroxycorticosteroids the variation was much less (17–46%). There was a good correlation ($r = 0.95$; $P < 0.001$) between 6β-hydroxycortisol and 17-hydroxycorticosteroids supporting the use of the ratio [7] for investigating cortisol metabolism.

The effect of phenobarbitone treatment on urinary 6β-hydroxycortisol, ethyl acetate-extractable 17-hydroxycorticosteroids and the calculated ratio 6β-hydroxycortisol/17-hydroxycortisteroids is shown in Fig. 1 and the corresponding data for saline control animals is given in Fig. 2. Phenobarbitone treatment produced significant changes in all three parameters

Table 1. The effect of phenobarbitone on liver weight, microsomal enzyme activity and microsomal cytochrome P-450 content in male marmosets

	Saline	Phenobarbitone
Body weight (g)	320 ± 33	308 ± 34
Liver weight (g/100g body wt.)	4.33 ± 0.79	4.44 ± 0.93
Cytochrome P-450 (nmoles/mg protein)	0.59 ± 0.01	1.39 ± 0.09*
Cytochrome *c* reductase (nmoles/min/mg protein)	287 ± 27	334 ± 8.6
Aminopyrine *N*-demethylase (nmoles/min/mg protein)	6.57 ± 0.58	22.66 ± 0.80*
7-Ethoxycoumarin *O*-deethylase (nmoles/min/mg protein)	0.76 ± 0.17	4.58 ± 0.49*
Ethoxyresorufin *O*-deethylase (nmoles/min/mg protein)	0.0572 ± 0.0127	0.073 ± 0.004

Values are given as mean ($n = 4$) ± S.E.
Statistical significance from saline-treated animals using Student's t-test.
* $P < 0.001$.

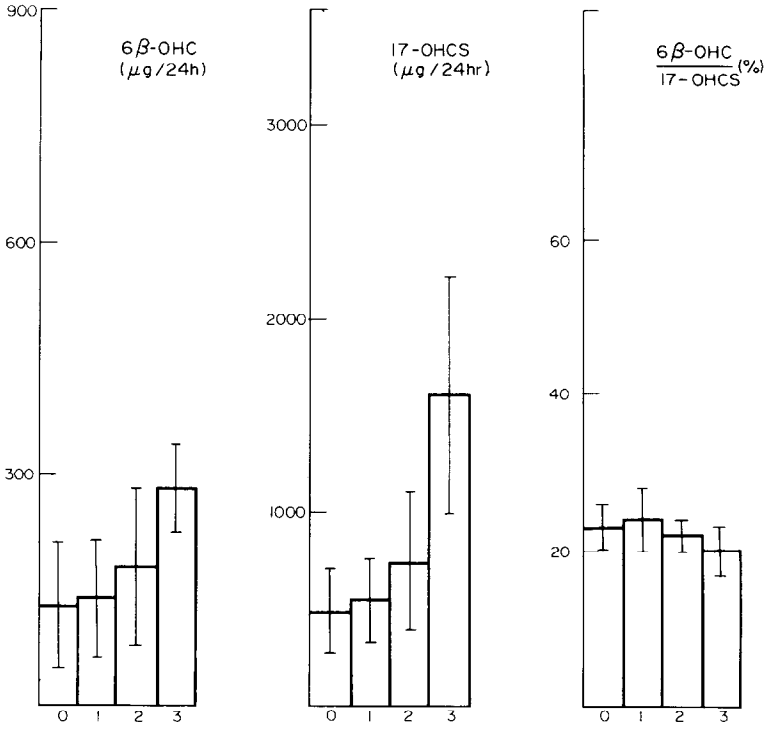

Fig. 2. The 24-hr urinary excretion of 6β-hydroxycortisol (6β-OHC), 17-hydroxycorticosteroids (17-OHCS) and the calculated ratio 6β-hydroxycortisol: 17-hydroxycorticosteroids (6β-OHC/17-OHCS) in marmoset monkeys before (day 0) and during (days 1, 2, 3) treatment with saline. Results are means ($n = 4$) ± S.E.

on days two and three. No statistically significant changes were observed for the control group.

Measurement of hepatic microsomal enzyme activity. The effect of administration of phenobarbitone on hepatic microsomal aminopyrine *N*-demethylation activity, 7-ethoxycoumarin deethylation activity, ethoxyresorufin deethylation activity, cytochrome P-450 content and cytochrome *c* reductase activity is shown in Table 1. There was a significant 2.4-fold increase in the concentration of cytochrome P-450 but only a small (20%), statistically insignificant increase in cytochrome *c* reductase. There were significant increases in aminopyrine *N*-demethylase (3.6-fold) and ethoxycoumarin *O*-deethylase (6-fold) activities but not in ethoxyresorufin *O*-deethylase activity.

SDS polyacrylamide gel electrophoresis (Fig. 3) showed induction of microsomal proteins in the 45,000–55,000 molecular weight range following phenobarbitone treatment. Three protein staining bands of mol. wt 49,000, 52,000 and 54,000, which were just visible in control microsomal protein, were markedly induced in microsomal protein from phenobarbitone-treated animals. This pattern differs from that seen in phenobarbitone-induced rat liver microsomes where one major band of mol. wt 53,000 is increased.

DISCUSSION

The marmoset monkey (*Callithrix jacchus*) was chosen for this study because it is a small animal used in long term toxicity studies [18, 19] which has high plasma corticosteroid concentrations [20], the major corticoid being cortisol. In contrast to a previous report [21], we have found that 6β-hydroxycortisol is a major urinary metabolite of cortisol in the marmoset monkey. The excreted steroid was characterised by radioimmunoassay, mass spectrometry and ultraviolet spectroscopy after purification by high pressure liquid chromatography. The specificity of the radioimmunoassay was satisfactory for direct measurement of 6β-hydroxycortisol in diluted urine.

The mean daily urinary excretion of 6β-hydroxycortisol was approximately 600 μg/kg which is considerably greater than found for man (4 μg/kg [8]) or the monkey *Cebus albifrons* (40 μg/kg [22]). There was a large inter-animal variation in the absolute excretion of 6β-hydroxycortisol but the proportion of cortisol that is 6β-hydroxylated remains fairly constant as indicated by the ratio 6β-hydroxycortisol:17-hydroxycorticosteroids (Figs. 1 and 2). The calculated ratio is thought to be a better index than absolute levels of 6β-hydroxycortisol in studies dealing with cortisol metabolism [7] and accordingly we found a good correlation between 6β-hydroxycortisol and 17-hydroxycorticosteroids in marmoset urine. Ethyl acetate, rather than chloroform, was necessary for extraction of 17-hydroxycorticosteroids from hydrolysed marmoset urine which suggests they are more polar than human 17-hydroxycorticosteroids.

Administration of phenobarbitone to marmosets produced significant increases in the urinary excre-

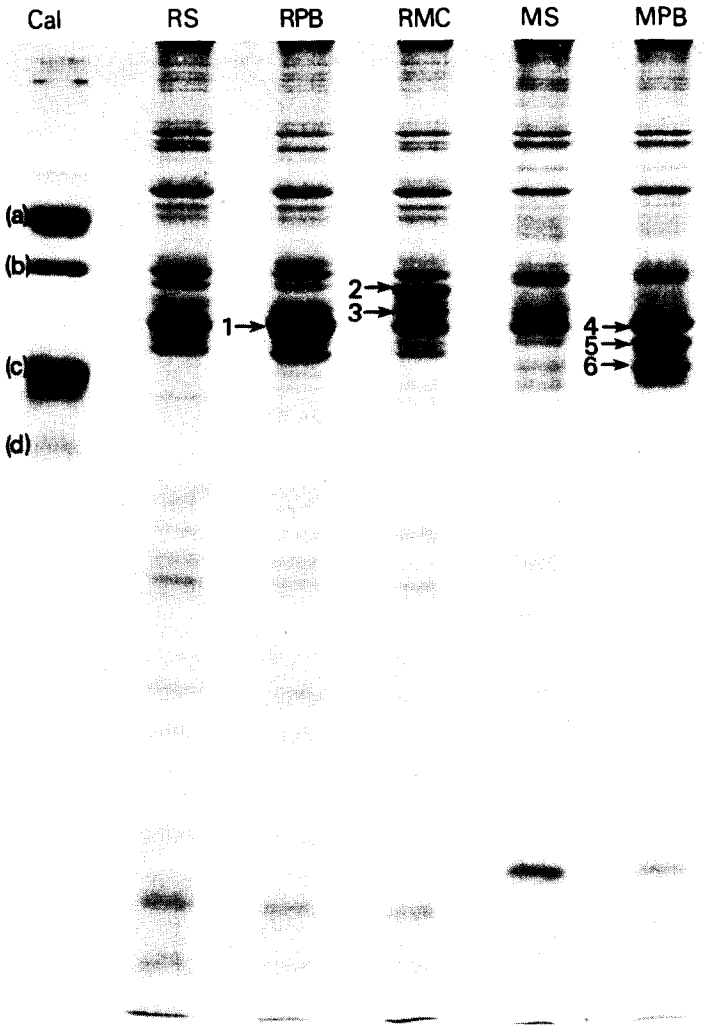

Fig. 3. Electrophoretogram of liver microsomes from animals treated with inducing agents. The induced protein bands are labelled with arrows. Abbreviations: Cal: calibration proteins (a) bovine serum albumin, mol. wt 68000 (b) catalase, mol. wt 60000 (c) hen egg lb, mol. wt 45000 (d) aldolase, mol. wt 39500; RS: rat saline control; RPB: rat phenobarbitone, band 1—mol. wt 53000; RMC: rat 3-methyalbumincholanthrene, band 2—mol. wt 58000, band 3—mol. wt 56000; MS: marmoset saline control; MPB: marmoset phenobarbitone, band 4—mol. wt 54000, band 5—mol. wt 52000, band 6—mol. wt 49000.

tion of 6β-hydroxycortisol and in the calculated ratio 6β-hydroxycortisol:17-hydroxycorticosteroids indicating that the proportion of cortisol that had been 6β-hydroxylated was increased. There may also be an increase in the overall rate of cortisol metabolism as there was a significant increase in urinary 17-hydroxycorticosteroids on days 2 and 3. However, part of this increase may be due to stress as three of the four control animals showed an increase in 17-hydroxycorticosteroids, but only on day 3 (there was no effect on the calculated ratio).

The 20 mg/kg dose of phenobarbitone used for enzyme induction purposes was found to be the maximum dose tolerated by the marmoset. Nevertheless, enzyme induction is clearly evident from the significant increase in cytochrome P-450 content and in the microsomal activities of aminopyrine *N*-demethylase and ethoxycoumarin *O*-deethylase both of which are mediated by P-450 enzyme systems. There was no significant increase in ethoxyresofurin *O*-deethylase activity but this is thought to be a P-448 dependent process. There was only a slight increase in cytochrome *c* reductase activity but this does not always increase in proportion to cytochrome P-450 [23, 24]. It is interesting to note that phenobarbitone induction was not accompanied by an increase in liver mass as has been observed in other species (rat, guinea pig, monkey) [23, 24, 25], possibly indicating that liver hypertrophy and enzyme induction are distinct processes [26]. There was however a clear increase in three distinct microsomal proteins in the 45,000–55,000 mol. wt range as shown by SDS–polyacrylamide gel electrophoresis (Fig. 3). Three protein staining bands of mol. wt 49,000, 52,000 and 54,000 were markedly increased. It is not known whether all these proteins are cytochrome P-

450 haemoproteins. However they do migrate in the same molecular weight region as the major protein induced by phenobarbitone in rat liver microsomes which is a haemoprotein.

The present work shows that the marmoset (*Callithrix jacchus*) is a useful animal model for investigating the relationship between hepatic microsomal enzyme induction and excretion of 6β-hydroxycortisol. Phenobarbitone produced significantly increased microsomal P-450-dependent drug metabolising activity at the same time as increasing urinary 6β-hydroxycortisol excretion, as has been observed in man [5]. However, phenobarbitone is thought to be a relatively non-specific inducing agent and in the present work has been shown to induce several microsomal proteins (Fig. 3). Further work is necessary to determine which of these proteins are involved in cortisol 6β-hydroxylation.

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