Biochemical Pharmacology, Vol. 20, pp. 2561–2570. Pergamon Press, 1971. Printed in Great Britain

CHANGES IN LIVER BLOOD FLOW DURING ENZYME INDUCTION

E. E. OHNHAUS,* S. S. THORGEIRSSON, D. S. DAVIES and A. BRECKENRIDGE

Department of Clinical Pharmacology, Royal Postgraduate Medical School, London, U.K.

(*Received* 11 *March* 1971; *accepted* 25 *March* 1971)

Abstract—There is a difference between *in vitro* and *in vivo* measurements of the extent of enzyme induction. To explain this disparity we have measured changes in liver blood flow produced by three inducing agents—phenobarbitone (30 mg/kg for 4 days), antipyrine (30 mg/kg for 4 days) and 3,4-benzpyrene (40 mg/kg as a single injection). Flow was measured by a heat exchange method in conscious rats. Liver blood flow increased by 33–175 per cent above control values in rats treated with phenobarbitone and antipyrine but not in those given benzpyrene. The increase in liver blood flow was first seen at 24 hr and remained above control values for 2–8 days after treatment. In similarly operated rats phenobarbitone shortened the sleeping time to 5–10 per cent of control values with a similar time course to the changes in liver blood flow. The maximal velocity of *N*-demethylation of ethylmorphine increased from 4·9 to 17·2 nm/mg protein/min, the maximal increase occurring 2–3 days after starting phenobarbitone. It is suggested that the increase in liver blood flow may be due to increases in liver weight and enzyme activity.

IN STUDIES on the effects of inducing agents in rats we have observed that pretreatment with phenobarbitone which causes a 12- to 15-fold decrease in the plasma half-life of drugs produces only a 3- to 4-fold increase in liver enzyme activity measured *in vitro*. These observations suggest that factors other than specific enzyme activity may be important in controlling rates of drug oxidation *in vivo*. A change in liver blood flow produced by an inducing agent could contribute to the disparity. This paper describes studies of the effects of three inducing agents in liver blood flow in the rat. The inducers studied were phenobarbitone, antipyrine and 3,4-benzpyrene.

METHODS

1. *Measurement of liver blood flow*

Liver blood flow was measured utilizing the principle of internal calorimetry.[1,2] For long term studies the thermocouple as described by Grayson had to be redesigned.[3]

Male rats (R.P.M.S. strain) weighing 200–400 g were used throughout these studies. Nineteen rats were operated under ether anaesthesia and the heaters of the redesigned thermocouple were implanted in the liver substance usually in the middle of the right lobe.

Measurements of liver blood flow were started 3 days after operation. The daily measurements were carried out on the conscious animal in a restraining cage. Stresses which may influence the liver blood flow were minimized by periods of short training

* Present address: E. E. Ohnhaus, Department of Experimental Therapeutics, Sandoz A.G. Basel, Switzerland.

in the restraining cage after operation and before starting daily measurements. Measurements were made for 20–30 min at the same time each day for 5–10 days to obtain resting baseline conductivity. The following inducing agents were then given by intraperitoneal injection to individual rats:

(a) Phenobarbitone 30 mg/kg daily for 4 days.
(b) Antipyrine 30 mg/kg daily for 4 days.
(c) 3,4-benzpyrene 40 mg/kg, one injection.[4]

During the period of injections liver blood flow measurements were made before and after every injection to observe an acute effect of the inducing drug. After stopping injections daily measurements were carried out until the increased conductivity returned to baseline and remained so for at least 4 days. If no increased conductivity occurred, measurements were made for at least 6 days.

The rats were killed with ether at the end of the experiment and measurements of conductivity were made in the dead liver. The position of the implanted flow probe was checked, the liver tissue around the thermocouple was sectioned, stained with haematoxylin–eosin and examined microscopically. Results are expressed as conductivity increment (δk) obtained by measuring conductivity in the liver of the living animal minus conductivity of the dead liver ($11{\cdot}8 \times 10^{-4}$ c.g.s.). Details of the calculation are given elsewhere.[3] The mean values of the baseline control conductivity measurements were compared with the mean values after drug injection using an unpaired *t*-test. If the variances of the two samples were found to be significantly different as shown by an *F*-test, the statistical analysis was then performed using an extension to the *t*-test.[6]

2. *Measurements of pentobarbitone sleeping time*

Pentobarbitone sleeping time was measured in two different groups of rats. Twenty male rats were operated under ether anaesthesia and the thermocouple was implanted as described above. Ten days after the operation sleeping time was measured and afterwards injections of phenobarbitone 30 mg/kg were given for 4 days. Further sleeping times were measured daily during and after the period of phenobarbitone administration until the sleeping time returned to the original value. Sleeping time before treatment was expressed as 100 per cent in every animal and the change during and after phenobarbitone calculated.

In nine unoperated rats, measurements of pentobarbitone sleeping time were made before and after phenobarbitone administration and the results were compared with the operated group.

3. *Activity of mixed function oxidase in liver microsomes*

The microsomal enzyme activity was estimated in normal rats and in rats 10 days after operation. Following phenobarbitone (30 mg/kg/day for 8 days) enzyme activity was measured on the first, second, third, fourth and eighth day of the administration. In another group of rats the enzyme activity was estimated on the third day after stopping phenobarbitone.

Preparation of enzymes. The animals were killed by cervical dislocation. Livers were removed, chilled on ice, and homogenized with 4 vol. of ice-cold 1·15% KCl in 0·02 M tris buffer (pH 7·4) in a glass homogenizer with a motor driven teflon pestle. The homogenate was centrifuged at 9000 *g* for 20 min in MSE-Superspeed 50 refrigerated

ultracentrifuge. The supernatant fraction was decanted and centrifuged at 78,000 *g* for 60 min. The microsomal pellet was suspended in ice-cold tris–KCl buffer by manual homogenization and centrifuged again at 150,000 *g* for 35 min. The microsomal pellet was resuspended in tris–KCl buffer to give approximately 4·5 mg protein per ml. All *g* values were calculated for the centre of the centrifuge tubes. Protein concentration was determined as described by Lowry *et al.*[7] Crystalline bovine albumin was used as a standard.

Assay of enzyme. The activity of microsomal *N*-demethylase was measured using ethylmorphine as substrate. The incubations were carried out at 37° in a medium containing 50 mM tris–KCl buffer (pH 7·4), 5 mM $MgCl_2$, 0·33 mM NADP, 8 mM sodium isocitrate and 0·36 units of isocitrate dehydrogenase in a final volume of 3 ml. Microsomal protein concentration was 1·5 mg/ml. Eight different concentrations of ethylmorphine were used for each incubation ranging from 0·25 to 2·0 mM. The mixture was incubated for 15 min in a Gallenkamp shaking incubator. Under these conditions ethylmorphine demethylation was linear with respect to time and protein concentration.

The reaction was stopped by the addition of 1 ml of 1N perchloric acid. The mixture was centrifuged for 15 min at 3000 rev/min and 3-ml aliquots of the supernatant were assayed for formaldehyde according to the method of Nash,[8] except that the reagent consisted of 4 M ammonium acetate, 0·04 M acetyl acetone and 0·1 M acetic acid. The maximal velocity (V_{max}) and apparent Michaelis constant (K_m) were obtained by a least squares method as described by Davies *et al.*[9]

Cytochrome c reductase activity was measured by the method of Phillips and Langdon[10] and NADPH cytochrome P-450 reductase activity was assayed by the method described by Davies *et al.*[9] Cytochrome P-450 content was measured by the method of Omura and Sato,[1] using molar extinction coefficient of 91 $cm^{-1}mM^{-1}$ between 450 and 490 nm.

RESULTS

(1) *Liver blood flow*

From the 19 operated rats, seven were excluded from the study. In three rats excess connective tissue was found round the thermocouple as manifest by a serial decrease in conductivity during the baseline measurements and confirmed histologically. In another four rats for technical reasons dead liver readings could not be obtained and therefore conductivity could not be calculated.

In the remaining 12 animals measurements of baseline conductivity and conductivity after administration of the enzyme inducing agents were made. The results are shown in Table 1, while Fig. 1 shows an example of the measurements of blood flow before and after phenobarbitone administration in rat 12.

An increased conductivity was found in every animal given phenobarbitone 30 mg/kg for 4 days. This was usually first seen 24 hr after the first dose of phenobarbitone. There was no change in liver blood flow immediately after individual injections of phenobarbitone. The changes in liver blood flow observed lasted from 2 to 8 days following the 4 days of phenobarbitone administration. The changes shown in Table 1 are expressed as a percentage increase in conductivity calculated from the mean baseline and the mean increased values. The percentage increase in conductivity ranged from 33 to 175 per cent, and was significant in all 12 animals.

TABLE 1

Rat no.	Baseline conductivity Mean ($\delta k \times 10^{-4}$) ± S.E.	Inducing drug	Conductivity after induction Mean ($\delta k \times 10^{-4}$) ± S.E.	Increase (%)	Duration of the increased conductivity in days	*t*-test
Rat 1	2·0 ± 0·13	Phenobarb.	3·9 ± 0·05	95·0	8	$P < 0·001$
Rat 3	4·7 ± 0·20	Phenobarb.	7·5 ± 0·19	60·0	6	$P < 0·001$
Rat 6	2·7 ± 0·15	Phenobarb.	5·4 ± 0·37	100·0	2	$P < 0·001$
Rat 8	4·3 ± 0·18	Phenobarb.	6·5 ± 0·19	51·0	2	$P < 0·001$
Rat 10	1·1 ± 0·08	Phenobarb.	1·8 ± 0·17	64·0	2	$P < 0·001$
Rat 12	1·5 ± 0·11	Phenobarb.	2·3 ± 0·09	53·0	3	$P < 0·001$
Rat 14	3·0 ± 0·12	Phenobarb.	4·0 ± 0·17	33·0	5	$P < 0·001$
Rat 15	0·8 ± 0·22	Phenobarb.	2·2 ± 0·27	175·0	2	$P < 0·001$
	0·9 ± 0·11	Antipyrine	2·0 ± 0·14	122·0	4	$P < 0·001$
Rat 16	0·9 ± 0·12	Phenobarb.	2·0 ± 0·11	122·0	5	$P < 0·001$
Rat 17	2·1 ± 0·14	Benzpyrene	2·3 ± 0·10	0·0		$0·4 < P < 0·3$
	2·3 ± 0·10	Phenobarb.	3·2 ± 0·14	39·0	2	$P < 0·001$
Rat 18	1·0 ± 0·10	Benzpyrine	1·0 ± 0·06	0·0		$0·7 < P < 0·6$
	1·0 ± 0·06	Antipyrine	2·1 ± 0·18	110·0	2	$P < 0·001$
Rat 19	0·6 ± 0·11	Benzpyrene	0·9 ± 0·12	0·0		$0·2 < P < 0·1$
	0·8 ± 0·08	Antipyrine	1·6 ± 0·12	100·0	2	$P < 0·001$

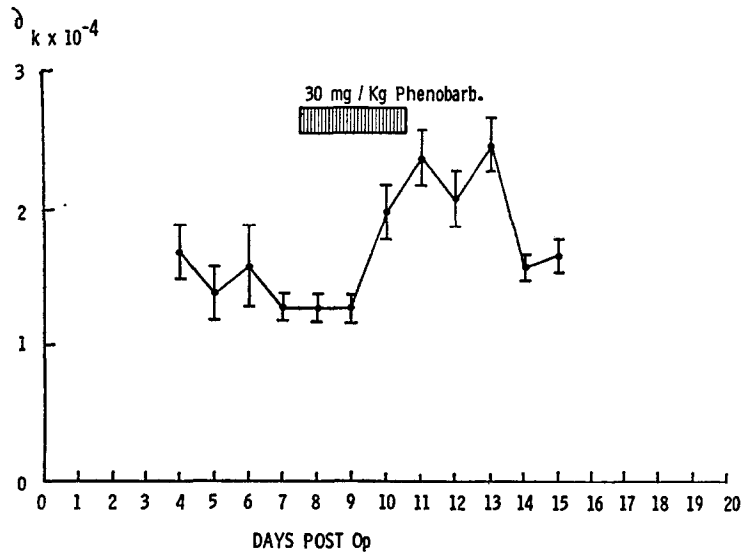

FIG. 1. Liver blood flow before and after phenobarbitone administration in rat 12. Vertical axis conductivity increment (δk) $\pm$ S.E., horizontal axis days after operation. Increase of conductivity can be seen after three daily injections of phenobarbitone. This increase remains for 3 days and returns to the baseline level.

Similar results were obtained after antipyrine 30 mg/kg daily for 4 days. In one study phenobarbitone and antipyrine produced similar changes in the same animal when studied on two occasions.

No changes in conductivity were found after giving 3,4-benzpyrene 40 mg/kg as one single injection, while the same three animals responded to phenobarbitone or antipyrine (Table 1).

(2) *Sleeping time*

Operation produced a significant increase in the sleeping time. On day 0 in the operated group of rats the sleeping time was 91·6 $\pm$ S.E. 9·2 min and in the non-operated group 43·8 $\pm$ S.E. 3·3 min. After starting phenobarbitone administration the percentage fall in sleeping time was the same in both groups. Therefore the results of both groups were pooled and are shown in Fig. 2. Sleeping time was decreased to 39 per cent of the control value on day 1 and to 5·7 per cent on day 3 of phenobarbitone administration. There was no significant change on days 4 and 5 but the sleeping times slowly returned to control values on days 6–9.

(3) *Mixed function oxidase activity*

Changes in liver weight following phenobarbitone are shown in Table 2. There was no difference in liver weight between normal rats and operated rats 10 days after operation. The maximal increase in liver weight of +27 per cent was reached on the 8th day of phenobarbitone administration. There was no change in liver weight, however, after a single dose of 3,4-benzpyrene.

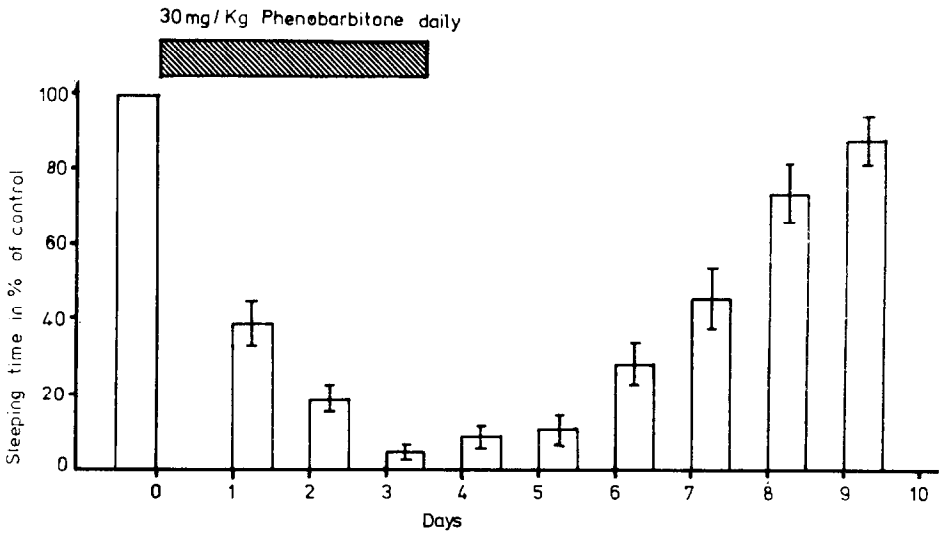

FIG. 2. Pentobarbitone sleeping time of the operated and non-operated rats expressed as percentage of the control sleeping time. Vertical axis—sleeping time in per cent of control values; horizontal axis—time in days.

Table 3 shows that operated rats had a significantly ($P < 0.05$) lower liver microsomal protein content, cytochrome P-450 content, cytochrome c reductase activity, cytochrome P-450 reductase activity and maximal velocity of *N*-demethylation of ethylmorphine. However pretreatment with phenobarbitone increased all these parameters in the operated rats.

There was an increase in microsomal protein from 16·88 to 26·43 mg/g liver, and the highest level of microsomal protein 30·87 mg/g liver was found 3 days after stopping phenobarbitone. P-450 content was increased during phenobarbitone administration from 0·635 to 1·684 nmole/mg reaching a plateau after 2 days. Cytochrome c reductase increased during phenobarbitone administration from 118 to 231·35 nmole/mg/min, the highest activity was found after 8 days of phenobarbitone administration. P-450 reductase activity increased from 7·96 to 28·72 nmole/mg/min

TABLE 2. CHANGES IN LIVER WEIGHTS

Control	No. of rats	g liver/100 g body wt.	Change (%)
(10 days post operation)	4	4·76 ± 0·31	100
1st day PB*	4	4·74 ± 0·13	99·6
2nd day PB	3	5·70 ± 0·11	119·70
3rd day PB	4	5·61 ± 0·86	117·86
4th day PB	4	5·16 ± 0·34	108·4
8th day PB	3	6·05 ± 0·35	127·10
3rd day after stopping PB	3	5·23 ± 0·38	109·87

* PB = Phenobarbitone.

TABLE 3

	Microsomal protein (mg/g liver ± S.D.)	Cytochrome P-450 content (nmole/mg microsomal protein ± S.D.)	Cytochrome c reductase (nmole/mg/min reduced ± S.D.)	Cytochrome P-450 reductase (nmole/mg/min reduced ± S.D.)	*N*-demethylase (Ethylmorphine)	
					K_m (mM ± S.E.)	V_{max} (HCHO formed/ mg/min ± S.E.)
Control	21·00 ± 2·0	0·977 ± 0·060	158·0 ± 24·0	9·50 ± 0·72	0·32 ± 0·07	10·72 ± 1·32
Operated (10 days after op.) $n = 4$	16·88 ± 1·7	0·635 ± 0·100	118·0 ± 16·9	7·96 ± 0·33	0·26 ± 0·04	4·9 ± 0·18
1st day of PB treatment $n = 4$	21·30 ± 2·1	1·330 ± 0·090	198·61 ± 8·5	13·00 ± 1·15	0·30 ± 0·03	10·98 ± 0·26
2nd day of PB treatment $n = 3$	25·70 ± 2·5	1·684 ± 0·130	177·7 ± 3·0	21·10 ± 1·10	0·45 ± 0·097	15·7 ± 1·0
3rd day of PB treatment $n = 4$	22·30 ± 2·5	1·612 ± 0·126	174·7 ± 6·5	25·70 ± 2·9	0·60 ± 0·07	13·85 ± 0·60
4th day of PB treatment $n = 4$	23·23 ± 2·3	1·500 ± 0·090	197·5 ± 10·2	28·72 ± 0·5	0·93 ± 0·097	17·2 ± 0·73
8th day of PB treatment $n = 3$	26·43 ± 2·6	1·587 ± 0·050	231·3 ± 8·0	21·92 ± 1·5	0·44 ± 0·04	16·22 ± 0·45
3rd day after stopping PB treatment $n = 3$	30·87 ± 3·0	1·148 ± 0·103	209·7 ± 4·0	15·95 ± 0·98	0·44 ± 0·067	11·6 ± 0·03

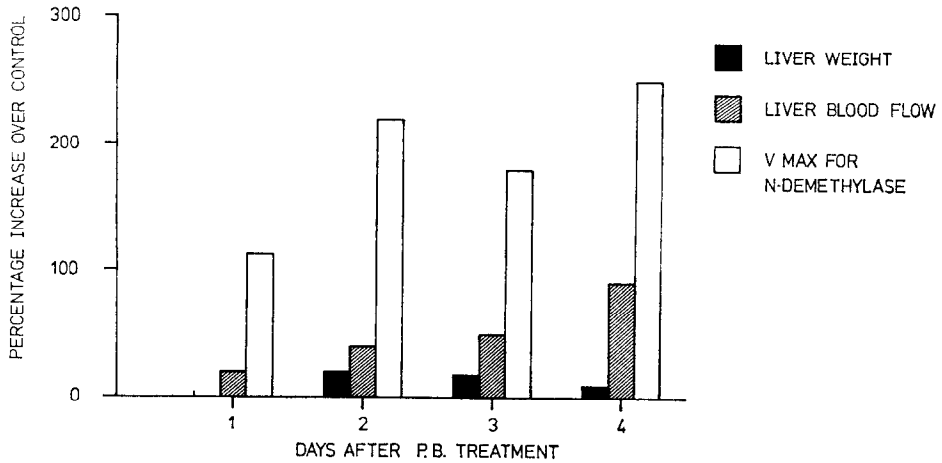

FIG. 3. Time course of changes in liver weight, blood flow and enzyme activity in phenobarbitone treated rats.

and reached a plateau after 3 days of phenobarbitone administration. V_{max} for *N*-demethylase increased from 4·9 to 17·2 nmole HCHO formed per mg per min, and reached peak values after 2–3 days of phenobarbitone. The K_m for ethylmorphine metabolism increased from 0·26 to 0·93 mM during the first 4 days of phenobarbitone administration but fell to 0·44 mM after 8 days of phenobarbitone.

(4) *Time course of the changes observed*

Figure 3 shows the changes in liver weight, liver blood flow and maximal velocity (V_{max}) of ethylmorphine *N*-demethylation during the 4 days of phenobarbitone administration. The greatest change was observed in V_{max} (an increase of 260 per cent), with lesser changes in liver blood flow (an increase of 91 per cent) and liver weight (an increase of 19·7 per cent). Liver blood flow and V_{max} were significantly increased 24 hr after phenobarbitone; increases in liver weight did not occur for 48 hr.

DISCUSSION

The principle of internal calorimetry in which changes in thermal conductivity are measured can be used to monitor liver blood flow. Grayson and Johnson[12] found that the thermal conductivity increment is a linear function of blood flow. The advantage of this method is that it can be used in small animals for long term experiments. In recent studies we have shown that the resting daily values of liver blood flow were constant in individual rats.[3] This has been confirmed in the current experiments in which significant increases in blood flow occurred after chronic administration of an inducing agent. Phenobarbitone or antipyrine administered for 4 days caused a significant increase in conductivity, whereas a single administration of benzpyrene did not. The baseline level and the percentage increase in conductivity and the duration of this increase varied in individual rats. Baseline conductivity ranged from a δk value of 0·8–4·7 $\times$ 10^{-4} c.g.s. This variability probably reflects the relation of the thermocouple to nearby blood vessels. A thermocouple sited near a bigger vessel gives a

higher δk than one sited in an area of capillary vessels.[12] Table 1 shows that the administration of phenobarbitone caused an increase in thermal conductivity irrespective of the baseline value. The variations in the increase of conductivity and in duration of this increase are not related to the baseline values but may reflect differences in the extent of induction in each rat. Rats do not have an anatomical intrahepatic sphincter mechanism, so intrahepatic autoregulation and changes in intrahepatic distribution are unlikely.[13,14] Therefore, the increase in liver blood flow is not a manifestation of intrahepatic autoregulation or distribution.

Acute effects of the inducing drugs were not found and there is no evidence that prolonged administration of these drugs affect the cardiovascular system in a way which could increase liver blood flow. Therefore it is likely that changes in liver blood flow originate from changes in the splanchnic area. The main receptors for the regulation of the capacity and resistance vessels in the splanchnic area are situated in the central mesenteric vessels and the extrahepatic part of the portal vein. Changes in blood flow are regulated by reflex arcs[15] or by myogenic reactions.[16,17]

Changes in pentobarbitone sleeping times are a valuable method of studying agents which may stimulate microsomal enzymes. In our studies daily injections of phenobarbitone produced a maximal fall in sleeping time after 3 days administration, and this level was maintained for 2 days after stopping phenobarbitone. These results from operated rats correspond closely to those obtained by Conney *et al.*[18] in normal rats.

Pretreatment with phenobarbitone beside changing liver blood flow and sleeping time caused an increase in liver weight, microsomal protein concentration, and a number of components of the microsomal mixed function oxidase system viz. cytochrome c reductase, cytochrome P-450 content, cytochrome P-450 reductase and ethylmorphine oxidation (V_{max}). As shown in Fig. 3, there is a progressive increase in liver blood flow over the 4 days of phenobarbitone administration. The first increase in liver weight was observed on the second day and no further increase occurred thereafter. The time course of the changes in V_{max} was similar to liver blood flow.

The increase in liver weight caused by phenobarbitone in these and other studies[18] may result in a change in the distribution of blood flow in the splanchnic area. Since the liver weight reached its maximum value at a time when the liver blood flow was still increasing, it would appear unlikely that the changes in liver weight are entirely responsible for changes in liver blood flow.

A better correlation was found between the rate of increase of blood flow and the rate of change of liver enzyme activity. An increased metabolic rate may cause an increased liver blood flow. Grayson and Kinnear[19] attributed changes in liver blood flow following glucose and glucose and insulin administration to an increase in rate of metabolism. An increased liver blood flow was also found in patients given hypertonic glucose during cholangiography.[20]

Our results do not permit us to define clearly the mechanism whereby liver blood flow increases following treatment with phenobarbitone, but it is most likely to be the result of an interplay of circulatory and metabolic factors. Nevertheless the increased liver blood flow is likely to contribute to the differences observed between *in vitro* and *in vivo* changes in rates of drug oxidation following treatment with phenobarbitone.

Acknowledgements—We acknowledge the helpful advice of Professor C. T. Dollery and the technical assistance of Miss Wendy Green and Larry Davies.

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