Triacylglycerol metabolism in the phenobarbital-treated rat

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1. Various aspects of triacylglycerol metabolism were compared in rats given phenobarbital at a dose of 100 mg/kg body wt. per day by intraperitoneal injection; controls were injected with an equal volume of 0.15 M-NaCl by the same route. Animals were killed after 5 days of treatment. 2. Rats injected with phenobarbital demonstrated increased liver weight, and increased microsomal protein per g of liver. Other evidence of microsomal enzyme induction was provided by increased activity of aminopyrine *N*-demethylase and cytochrome *P*-450 content. Increased hepatic activity of γ -glutamyltransferase (EC 2.3.2.2) occurred in male rats, but not in females, and was not accompanied by any detectable change in the activity of this enzyme in serum. 3. Phenobarbital treatment increased the hepatic content of triacylglycerol after 5 days in starved male and female rats, as well as in non-starved male rats; non-starved females were not tested in this regard. At 5 days after withdrawal of the drug, there was no difference in hepatic triacylglycerol content or in hepatic functions of microsomal enzyme induction between the treated and control rats. 4. After 5 days, phenobarbital increased the synthesis *in vitro* of glycerolipids in cell-free liver fractions fortified with optimal concentrations of substrates and co-substrates when results were expressed per whole liver. The drug caused a significant increment in the activity of hepatic diacylglycerol acyltransferase (EC 2.3.1.20), but did not affect the activity per liver of phosphatidate phosphohydrolase (EC 3.1.3.4) in cytosolic or washed microsomal fractions. A remarkable sex-dependent difference was observed for this latter enzyme. In female rats, the activity of the microsomal enzyme per liver was 10-fold greater than that of the cytosolic enzyme, whereas in males, the activities of phosphohydrolases per liver from both subcellular fractions were similar. 5. The phenobarbital-mediated increase in hepatic triacylglycerol content could not be explained by a decrease in the hepatic triacylglycerol secretion rate as measured by the Triton WR1339 technique. Since the hepatic triacylglycerol showed significant correlation with microsomal enzyme induction functions, with hepatic glycerolipid synthesis *in vitro* and with diacylglycerol acyltransferase activity, it is likely to be due to enhanced triacylglycerol synthesis consequent on hepatic microsomal enzyme induction. 6. In contrast with rabbits and guinea pigs, rats injected with phenobarbital showed a decrease in serum triacylglycerol concentration in the starved state; this decrease persisted for up to 5 days after drug administration stopped, and did not occur in non-starved animals. It seems to be independent of the microsomal enzyme-inducing properties of the drug, and may be due to the action of phenobarbital at an extrahepatic site.

The effect of drugs on lipid metabolism is a topic of growing interest (Sturton & Brindley, 1977; Brindley, 1978), in which phenobarbital has attrac-

ted attention. In an early study with rats, the saponifiable fatty acid content of liver increased 53% after 7 days of phenobarbital treatment without change in the apparent rate of glycerolipid synthesis (Karvinen *et al.*, 1965). However, in phenobarbital-treated mice, liver triacylglycerol showed a significant increase and serum triacylglycerol a sig-

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nificant decrease in the female, whereas the male showed only marginal changes in these parameters (Salvador *et al.*, 1970).

We have demonstrated that rabbits and guinea pigs treated with phenobarbital manifest increased hepatic content and serum concentration of triacylglycerol, which correlated with functions of microsomal enzyme induction (Goldberg *et al.*, 1980b,c). Phenobarbital enhanced the ability of cell-free fractions of rabbit and guinea pig liver to synthesize glycerolipids *in vitro*, and increased activity per whole liver of cytosolic and microsomal phosphatidate phosphohydrolase. In a preliminary study, we found that phenobarbital significantly decreased serum-triacylglycerol concentration in starved male rats, whereas hepatic triacylglycerol was concomitantly increased (Goldberg *et al.*, 1980a). The present paper reports studies in male and female rats under starving and fed conditions aimed at pursuing these initial observations. Their enzymic basis was explored by assessing the possible effects of phenobarbital on critical enzymes in the triacylglycerol biosynthetic pathway, and on glycerolipid formation *in vitro* in cell-free preparations of rat liver. The relationship between hepatic glycerolipid synthesis and microsomal enzyme induction was also examined, and a major sex-dependent difference in the distribution of cytosolic and microsomal phosphatidate phosphohydrolase in rat liver was demonstrated.

Materials and methods

General

We followed the procedures described previously (Goldberg *et al.*, 1980b), which outlined instrumentation and reagent sources, additional items in the present study being the following: Triton WR1339 (Ruger Chemical Co., Irvington, NJ, U.S.A.); bovine serum ^{125}I -labelled albumin (New England Nuclear; sp. radioactivity 1.45 mCi/mg of protein); [$1\text{-}^{14}\text{C}$]palmitic acid (Nuclear-Chicago, sp. radioactivity 50.2 Ci/mol), which was converted into the potassium salt before use.

Animals

Male and female Wistar rats weighing approx. 100 g (range 95–105 g) were purchased from Canadian Biobreeding Lab., Ottawa, Ont., Canada, and fed laboratory chow *ad lib*. They were weighed daily and a chart of food intake was maintained until they were killed. At 3 days after purchase, by which time the rats had gained approx. 10 g each, phenobarbital dissolved in 0.15 M-NaCl was injected intraperitoneally at a dose of 100 mg/day per kg body wt. at 09:00 h for 5 days. Controls were injected with 0.15 M-NaCl alone for the same period. Some rats were killed at 09:00 h on the day after the

last injection (day 6 of the experiment) after withdrawal of food for 18 h. Others were allowed to feed up to the time of killing at 09:00 h on day 6. A third group was starved as described above before determination of the hepatic triacylglycerol secretion rate by the Triton WR1339 technique at 09:00 h on day 6, one day after the last dose of drug or saline. In brief, this necessitated injection into the tail vein of Triton WR1339 (40 mg/kg body wt., dissolved in 0.15 M-NaCl) and bovine serum ^{125}I -labelled albumin (0.5 μCi /rat). Blood was withdrawn by orbital puncture at 0, 10 and 90 min for determination of radioactivity, Triton WR1339 and triacylglycerol concentrations. The hepatic triacylglycerol secretion rate was then calculated from the formula of Kim & Kalkhoff (1975).

Preparation of serum and cell-free fractions from liver

The previously described procedures were employed to prepare serum and homogenates, post-mitochondrial supernatant, as well as washed microsomal and cytosolic fractions of rat liver (Goldberg *et al.*, 1980b). These procedures were intended to achieve quantitative recovery of the various sub-cellular fractions. Phenobarbital does not affect the recovery of endoplasmic reticulum (Cooper & Feuer, 1972). Specific assays were conducted in duplicate under optimal and linear conditions (Goldberg *et al.*, 1980b) and were as follows.

Esterification assays with *sn*-[^{14}C]glycerol 3-phosphate. Glycerolipid synthesis from *sn*-[^{14}C]glycerol 3-phosphate and fatty acids was determined in postmitochondrial and microsomal fractions with palmitate as the exogenous fatty acid (Roncari & Murthy, 1975). In contrast with rabbits (Goldberg *et al.*, 1980b), albumin did not increase glycerolipid synthesis in rat liver microsomal fractions and was consequently not used. Incubations were stopped after 5 min at 25°C. In all other respects, the assays were performed as previously described.

Phosphatidate phosphohydrolase (EC 3.1.3.4). These assays with membrane-bound 1,2-diacyl-*sn*-[^{14}C]glycerol 3-phosphate ([^{14}C]phosphatidate) were carried out in microsomal (7 min incubation) and cytosolic (15 min incubation) fractions at 37°C by a modification of previously described methods (Brindley & Bowley, 1975a; Heusden & Bosche, 1978; Roncari *et al.*, 1979). Full details have been given previously (Goldberg *et al.*, 1980b).

Diacylglycerol acyltransferase (EC 2.3.1.20). Activity in washed liver microsomal fractions was assayed by a modification of the method of Mangiapane *et al.* (1973). Assays were performed in a final volume of 0.2 ml containing 25 mM-Tris buffer (pH 7.4), 5 mM-glutathione, 1 mM-ATP, 0.16 mM-coenzyme A, 20 mM-MgCl₂, 3 mg of bovine serum

albumin (fatty acid-poor), 200 μ g of microsomal protein, 4.2 mM *sn*-1,2-dioleoylglycerol, 2.0 mM-potassium palmitate and potassium [1- 14 C]-palmitate (0.1 μ Ci per tube). The mixture was incubated at 37°C for 30 min. The reaction was terminated by adding 1.5 ml of propan-2-ol/heptane/water (40:10:1, by vol.). After 5 min, 1 ml of heptane and 0.5 ml of water were added, and the mixture was vortex-mixed for 1 min. The heptane layer was removed carefully and washed twice with 2 ml of a solution containing 0.5 M-NaOH, ethanol and water in the ratio by volume of 1:5:5 to remove the labelled non-esterified fatty acids. A blank was performed containing all the constituents except dioleoylglycerol, which was added after terminating the reaction. The heptane phase was transferred to scintillation vials containing 10 ml of Bray's mixture and the radioactivity was measured. Results were expressed as nmol of triacylglycerol formed.

Aminopyrine N-demethylase. Activity of this enzyme was measured in postmitochondrial supernatant (Nash, 1953).

γ -Glutamyltransferase (EC 2.3.2.2). Activity was measured in serum by the method of Szasz (1976), and in liver homogenates and microsomal fraction by a modification of the method of Orlowski & Meister (1965).

Triacylglycerol. Concentrations of triacylglycerol in serum and liver homogenates were determined by the enzymic method of Bucolo & David (1973) after extraction and saponification (Goldberg *et al.*, 1980b).

Cytochrome P-450. This was determined in washed microsomal fractions (Omura & Sato, 1964).

Protein. The method of Lowry *et al.* (1951), as modified by Miller (1959), was used with bovine serum albumin as standard.

Triton WR1339. This was measured by the method of Cornforth *et al.* (1951) as modified by Schurr *et al.* (1972).

Results

Studies in starving male rats

General. Mean food consumption for the 6 days of the experiment in rats given phenobarbital (71 ± 0.6 g) was similar to that in the control group (73 ± 0.6 g). Mean weight gain in the test group over the 6-day observation period (23 ± 10 g) was not statistically different from that of the controls (20 ± 0.9 g). Phenobarbital did, however, bring about a significant increase in liver weight, from 5.1 ± 0.2 g in the controls to 6.9 ± 0.2 g in the treated animals ($t = 5.77$; $P < 0.001$). The protein content of all fractions per liver was significantly increased in the rats given phenobarbital (Table 1), but only the microsomal fractions showed an increment per g of liver (63%) in the phenobarbital-treated rats.

Functions of microsomal enzyme induction. Phenobarbital increased microsomal cytochrome P-450 and aminopyrine N-demethylase about 3-fold per mg of protein, and even more markedly per g of liver or per whole liver (Table 2). Activity of γ -glutamyltransferase increased in liver homogenates of treated rats, but in microsomal fraction increases only occurred per g of liver and per whole liver (Table 2). Activity of this enzyme in serum was at the limit of detection in control and treated rats and did not seem to be increased by phenobarbital.

Serum and liver triacylglycerol. Significant increases in liver triacylglycerol occurred in starved male rats injected with phenobarbital (Table 3). Paradoxically, the serum triacylglycerol concentration in these rats (40.2 ± 2.2 mg/dl) was lower than in the control animals (70.7 ± 4.6 mg/dl) on the sixth day of the study ($t = 5.96$; $P < 0.001$). The time course of serum triacylglycerol concentration was followed in male rats injected with phenobarbital or saline for 5 days, the animals being killed after an overnight starvation period on the eleventh day. Serum triacylglycerol concentration after starvation fell in both groups during the 5-day injection period,

Table 1. *Effect of phenobarbital on protein content of starved male rat liver*

Protein content of liver fractions measured in control ($n = 22$) and treated ($n = 22$) rats after 5 days of phenobarbital sodium treatment at a dose of 100 mg/day per kg body wt. Protein data, by the method of Lowry *et al.* (1951), are reported as means $\pm$ S.E.M. and are analysed for statistical significance by Student's *t* test (control versus treated group for each parameter): * $t = 3.21$; $P < 0.005$. All other differences in protein content per g of liver in other fractions are not statistically significant.

Fraction	Whole homogenate		Postmitochondrial supernatant		Microsomal fraction		Cytosol	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated
g of protein/liver	1.03 ± 0.04	1.39 ± 0.05	0.36 ± 0.02	0.48 ± 0.03	0.05 ± 0.006	0.11 ± 0.01	0.07 ± 0.006	0.11 ± 0.01
	$(t = 6.13; P < 0.001)$		$(t = 3.32; P < 0.005)$		$(t = 5.10; P < 0.001)$		$(t = 3.06; P < 0.005)$	
mg of protein/g of liver	203 ± 4	195 ± 9	71 ± 5	71 ± 6	$10 \pm 1^*$	16 ± 2	15 ± 1	16 ± 1

Table 2. *Effect of phenobarbital on functions of microsomal enzyme induction in starved male rat liver*

Phenobarbital sodium was injected intraperitoneally into test animals ($n = 22$) at a dose rate of 100 mg/day per kg body wt. for 5 days and the animals were killed after starvation overnight. Control rats ($n = 22$) were injected with 0.15 M-NaCl for the same period. Results are reported as means $\pm$ s.e.m. The Student's t test was used to assess significance of differences between test and control rats (values for t and P are not given where differences were not significant). Cytochrome P -450 content was measured in washed microsomal fractions and aminopyrine N -demethylase activity in postmitochondrial supernatant.

	Control	Test	t	P
Cytochrome P -450				
(nmol/liver)	32.4 ± 7.0	262 ± 44.2	4.28	<0.001
(nmol/g of liver)	8.56 ± 1.80	36.50 ± 5.07	3.87	<0.001
(nmol/mg of protein)	0.66 ± 0.09	1.91 ± 0.21	5.21	<0.001
Aminopyrine N -demethylase				
(nmol/min per liver)	129 ± 7	514 ± 45	8.45	<0.001
(nmol/min per g of liver)	25 ± 2	77 ± 6	9.21	<0.001
(nmol/min per mg of protein)	0.36 ± 0.02	1.07 ± 0.04	16.12	<0.001
γ -Glutamyltransferase (whole homogenate)				
(mol/min per liver)	1.48 ± 0.11	3.63 ± 0.33	6.40	<0.001
(mmol/min per g of liver)	305 ± 27	534 ± 46	4.26	<0.001
(mmol/min per mg of protein)	1.52 ± 0.13	2.62 ± 0.22	4.21	<0.001
γ -Glutamyltransferase (microsomal fraction)				
(mmol/min per liver)	150 ± 17	382 ± 66	3.55	<0.001
(mmol/min per g of liver)	30 ± 4	56 ± 10	2.50	<0.02
(mmol/min per mg of protein)	3.12 ± 0.30	3.07 ± 0.29	—	—

Table 3. *Effect of phenobarbital on functions of glycerolipid metabolism in starved male rat liver*

The protocol as described in Table 2. Triacylglycerol data refer to whole homogenate. The esterification assay with sn -[14 C]glycerol 3-phosphate is reported as the sum of d.p.s. incorporated into phosphatidate and di- and triacylglycerol in the presence of optimal concentrations of palmitate and cofactors and converted into nmol of glycerolipid formed/min. Incubation was for 5 min at 25°C for postmitochondrial supernatant and microsomal fractions.

	Control	Test	t	P
Hepatic triacylglycerol				
(mg/liver)	34.7 ± 3.4	72.6 ± 7.6	5.96	<0.001
(mg/g of liver)	6.96 ± 0.73	10.40 ± 1.06	2.71	<0.01
(μ g/mg of protein)	36.7 ± 3.8	51.4 ± 4.4	2.51	<0.01
Esterification assay (postmitochondrial supernatant)				
(nmol/min per liver)	713 ± 60	987 ± 88	2.89	<0.01
(nmol/min per g of liver)	143 ± 14	145 ± 13	—	—
(nmol/min per mg of protein)	2.04 ± 0.17	2.07 ± 0.15	—	—
Esterification assay (microsomal fraction)				
(nmol/min per liver)	176 ± 17	281 ± 39	2.46	<0.02
(nmol/min per g of liver)	35.6 ± 3.8	42.8 ± 6.3	—	—
(nmol/min per mg of protein)	4.11 ± 0.36	2.63 ± 0.22	3.56	<0.001

but this decrease was more marked in the phenobarbital-treated rats (Fig. 1). After this period, the values remained at a plateau, failing to rise to pre-treatment values, although the livers on the eleventh day revealed no differences in hepatic functions of microsomal enzyme induction or triacylglycerol content between the test and control animals.

The triacylglycerol secretion rates were 49.3 nmol/min ($n = 7$) in the control rats and 51.8 ($n = 7$) in the phenobarbital-treated rats. These rates are somewhat lower than in female Sprague-Dawley rats weighing 200–250 g (Kim & Kalkhoff, 1975).

Glycerolipid synthesis in cell-free fractions. Phosphatidate was the major glycerolipid (>90%) synthesized *in vitro* in microsomal and postmitochondrial liver fractions in our experiments (Goldberg *et al.*, 1980b,c) and those of others utilizing comparable conditions (Roncari & Murthy, 1975; Brindley & Bowley, 1975b; Whiting *et al.*, 1977). Significantly increased synthesis of glycerolipids from postmitochondrial and microsomal fractions fortified with optimal concentrations of substrates and co-substrates occurred per liver of phenobarbital-treated rats (Table 3), but glycerolipid synthesis per mg of protein in the post-

Fig. 1. Effect of phenobarbital on serum triacylglycerol concentration of male rats

Six animals in each group were given phenobarbital (100mg/day per kg body wt.) or 0.15M-NaCl (controls) by intraperitoneal injection for the first 5 days of the experiment. Blood was drawn under light anaesthesia by retro-orbital puncture at 09:00h on the days indicated after withdrawal of food 18h previously (■, phenobarbital; ●, saline), or in the non-starved state (□, phenobarbital). Triacylglycerol concentration was measured in the serum by the method of Bucolo & David (1973). Data are means $\pm$ S.E.M. The starved phenobarbital-treated group differed significantly from the other two groups from day 5 onwards, but differences between the starved control and non-starved phenobarbital-treated groups were not statistically significant (Student's *t* test). At no point was there a significant difference in serum triacylglycerol concentration between phenobarbital-treated and control non-starved rats; therefore the data for the latter group are not presented.

mitochondrial supernatant was unchanged, and it was significantly decreased in the microsomal fraction of treated rats.

Activities of critical enzymes of glycerolipid synthesis. Total phosphatidate phosphohydrolase activities per liver of the microsomal and cytosolic fractions were unaltered by phenobarbital (Fig. 2), and the phosphohydrolase activities in these two fractions were almost identical. By contrast, diacylglycerol acyltransferase activity per liver of phenobarbital-treated rats was 260% that of the controls (Fig. 2), and its activity remained significantly increased per g of liver and per mg of protein. Activity of this enzyme was 10-fold that of the microsomal or cytosolic phosphatidate phos-

phohydrolase in the controls, a difference that was even more marked in phenobarbital-treated rats.

Studies with phenobarbital in non-starved male rats

Liver weight and microsomal protein content per g of liver were significantly increased in the phenobarbital-treated rats. Phenobarbital, in the described regimen, increased cytochrome *P*-450 content of microsomal fractions 3-fold, aminopyrine *N*-demethylase activity of postmitochondrial supernatants 2-fold and γ -glutamyltransferase activity of liver homogenates by the same amount (Table 4). Hepatic triacylglycerol content showed a 2-fold increment in phenobarbital treated rats, but the serum triacylglycerol concentrations in test and control animals, taken at the same time as in the fasted male rats, were comparable. In a group of male rats, non-starved-rat serum triacylglycerol determinations were made at 11:00h during 5 days of daily injections with phenobarbital or with saline, and for a further 5 days after stopping the injections. No differences occurred between the test and control groups, and the decrease in serum triacylglycerol concentrations seen in starved male rats (Fig. 1) did not take place. In contrast with the increased synthesis per liver *in vitro* of glycerolipids from *sn*-[14 C]glycerol 3-phosphate and fatty acids that occurred in the starved phenobarbital-treated rats relative to starved control rats, there was no significant difference between the two groups of non-starved male rats (Table 4). However, in contrast with the controls, where starvation caused a trend towards lower glycerolipid synthesis *in vitro* by rat liver postmitochondrial supernatant and microsomal fractions, which was not statistically significant, the starved phenobarbital-treated rats demonstrated 54% more glycerolipid synthesis *in vitro* per liver by the postmitochondrial supernatant ($P < 0.05$) and 138% more glycerolipid synthesis per liver by the microsomal fractions ($P < 0.01$) than non-starved phenobarbital-treated rats. At present, we cannot offer an explanation for this unexpected effect of phenobarbital. Activities of phosphatidate phosphohydrolase and diacylglycerol acyltransferase were not measured in these animals.

Studies with starved female rats

Phenobarbital led to a significant increase in liver weight (approx. 32%) and in microsomal protein per g of liver (approx. 72%). Microsomal cytochrome *P*-450 was higher in male than in female rats (Table 5), and was increased 2-fold by phenobarbital. In contrast with males, γ -glutamyltransferase activity of liver homogenate and microsomal fractions per liver showed no increase in phenobarbital-treated female rats. This treatment caused a significant

Fig. 2. Effect of phenobarbital on hepatic enzymes of glycerolipid synthesis in starved-rat liver

Rats given phenobarbital at a dose of 100mg/day per kg body wt. (■) or 0.15 M-NaCl (□) for 5 days by intraperitoneal injection were killed at 09:00h on the sixth day after starvation for 18h. Hepatic microsomal and cytosolic phosphatidate phosphohydrolase activities were measured in 14 male rats in each of the two groups (a), in six female control rats and five female phenobarbital-treated rats (b) and diacylglycerol acyltransferase activity was measured only in the male rats (c). Note that there are separate scales for the male phosphohydrolases, the female microsomal phosphohydrolase and the female cytosolic phosphohydrolase. Activity of the microsomal phosphohydrolase was thus much higher than that of cytosolic phosphohydrolase ($P < 0.001$ for both test and control group) in females (b) and not in males (a). The differences in phosphohydrolase activity between test and control rats were in no instance significant, but the difference between diacylglycerol acyltransferase activity in phenobarbital-treated and control rats (c) was highly significant ($P < 0.001$). Data are means $\pm$ S.E.M. by Student's t test.

Table 4. Effect of phenobarbital on selected functions of microsomal enzyme induction and glycerolipid metabolism in non-starved male rat liver

Drug regimen, data expression and organization of the Table are as for Tables 2 and 3.

	Control (n = 10)	Test (n = 9)	t	P
Cytochrome P-450 (nmol/mg of protein)	0.46 $\pm$ 0.09	1.53 $\pm$ 0.11	7.68	<0.001
Aminopyrine N-demethylase (nmol/min per mg of protein)	0.41 $\pm$ 0.04	0.82 $\pm$ 0.04	6.59	<0.001
γ -Glutamyltransferase (mol/min per liver)* (whole homogenate)	1.50 $\pm$ 0.33	3.07 $\pm$ 0.62	2.38	<0.05
Hepatic triacylglycerol (μ g/mg of protein)	25.3 $\pm$ 2.5	49.6 $\pm$ 4.4	4.86	<0.001
Serum triacylglycerol (mg/dl)	67.0 $\pm$ 3.5	69.9 $\pm$ 3.8	—	—
Esterification assay (nmol/min per liver)				
Postmitochondrial supernatant	858 $\pm$ 123	642 $\pm$ 91	—	—
Microsomal fraction	219 $\pm$ 41	118 $\pm$ 58	—	—

* Serum activity barely measurable.

Table 5. *Effect of phenobarbital on selected functions of microsomal enzyme induction and glycerolipid metabolism in starved female rat liver*

Drug regimen, data expression and organization of the Table are as for Tables 2 and 3.

	Control (n = 6)	Test (n = 5)	t	P
Liver weight (g)	4.62 ± 0.33	6.08 ± 0.28	3.27	<0.01
Microsomal protein (mg/g of liver)	12.1 ± 0.7	20.8 ± 1.0	7.24	<0.001
Cytochrome P-450 (nmol/mg of protein)	1.26 ± 0.05	2.60 ± 0.39	3.39	<0.01
γ-Glutamyltransferase (mol/min per liver)*				
Whole homogenate	1.35 ± 0.30	1.23 ± 0.18	—	—
Microsomal fraction	0.23 ± 0.04	0.30 ± 0.03	—	—
Hepatic triacylglycerol (μg/mg of protein)	56.4 ± 3.0	75.2 ± 6.9	2.64	<0.05
Serum triacylglycerol (mg/dl)	61.9 ± 6.0	44.2 ± 4.2	2.33	<0.05
Esterification assay (nmol/min per liver) (microsomal fraction)	163 ± 19	241 ± 30	2.79	<0.025

* Serum activity barely measurable.

Table 6. *Correlation of hepatic triacylglycerol content with functions of glycerolipid synthesis and microsomal enzyme induction*

Data from test and control male starved rats were pooled and the correlation coefficient (*r*) was calculated. The hepatic triacylglycerol content was always expressed in the same fashion as the other variable with which its correlation was tested (per liver or per mg of protein, as appropriate). Abbreviation used: NS, not significant.

	r	P
Functions of glycerolipid synthesis		
Esterification assay (per liver)		
Postmitochondrial supernatant	0.467	<0.01
Microsomal fraction	0.883	<0.001
Phosphatidate phosphohydrolase (per liver)		
Microsomal	0.182	NS
Cytosolic	-0.052	NS
Diacylglycerol acyltransferase (per liver)	0.866	<0.001
Functions of microsomal enzyme induction		
Cytochrome P-450 (per mg of protein)	0.654	<0.001
Aminopyrine N-demethylase (per mg of protein)	0.712	<0.001
γ-Glutamyltransferase (per liver)		
Whole homogenate	0.869	<0.001
Microsomal fraction	0.589	<0.01

increase in hepatic triacylglycerol and a decrease in serum triacylglycerol in starved female rats (Table 5). Synthesis of glycerolipids *in vitro* from *sn*-[¹⁴C]glycerol 3-phosphate and fatty acids was measured only in the microsomal fraction of these animals, and was significantly increased per liver by phenobarbital. The activities of microsomal and cytosolic phosphatidate phosphohydrolases were increased approx. 50% per liver in phenobarbital-treated female rats, but due to the wide variance, the differences were not statistically significant (Fig. 2). Whereas the activities of these two phosphohydrolases were comparable in starving male rats, the microsomal activity per liver was considerably greater than that of the cytosolic activity in the female rats. Moreover, the activity of cytosolic phosphatidate phosphohydrolase in the female was an order of magnitude less than that of male rat

liver. Diacylglycerol acyltransferase activity was not measured in female animals.

Correlation of enzyme induction functions with indices of glycerolipid metabolism

Hepatic triacylglycerol content correlated significantly with microsomal enzyme induction functions and with glycerolipid synthesis *in vitro* when data for control and phenobarbital-treated starved male rats were pooled, but did not correlate with microsomal or cytosolic phosphatidate phosphohydrolase activity (Table 6). Similar results were obtained on analysing the data from non-starved male rats and starved female rats. A close correlation between hepatic triacylglycerol content and diacylglycerol acyltransferase activity was found in starved male rats, the only group in which this enzyme was measured (Table 6).

Discussion

Starved male rats treated with phenobarbital showed increments in liver weight, microsomal protein, aminopyrine *N*-demethylase, cytochrome *P*-450 and γ -glutamyltransferase, all of which provide evidence of microsomal enzyme induction. Accompanying these changes, hepatic triacylglycerol content was increased. In starved male rabbits, a similar but more intense response to phenobarbital by hepatic triacylglycerol content and indices of microsomal enzyme induction were seen (Goldberg *et al.*, 1980b). Although the dosage regimens were different in the two species, preliminary experiments showed that they cause maximal or near-maximal induction of hepatic microsomal enzymes (Roomi & Goldberg, 1981). The concurrence of increments in functions indicating microsomal enzyme induction with increased hepatic triacylglycerol content was also observed in non-starved male and starved female rats. Moreover, when starved and non-starved male rats were examined 5 days after the last injection of phenobarbital, the indices of microsomal enzyme induction and hepatic triacylglycerol content had returned to control values. Statistically, there were excellent correlations between hepatic triacylglycerol and microsomal enzyme induction functions (Table 6).

In starved male and female rats, but not in non-starved males, phenobarbital after 5 days enhanced synthesis per liver of glycerolipids from *sn*-[^{14}C]glycerol 3-phosphate and fatty acids by cell-free fractions; non-starved female rats were not studied. Phenobarbital may modulate glycerolipid synthesis in rat liver only in the starved state, and this enhanced synthesis may cause accumulation of hepatic triacylglycerol in starved rats. The mechanism whereby hepatic triacylglycerol is increased in non-starved phenobarbital-treated rats remains to be elucidated.

Previous studies have shown that *sn*-glycerophosphate acyltransferase (EC 2.3.1.15) is not increased in rat liver after 5 days of phenobarbital treatment (Higgins & Barnett, 1972; Joly *et al.*, 1973). In an attempt to determine the locus in the synthetic pathway wherein phenobarbital acts, we measured the activity of phosphatidate phosphohydrolases, because they may be rate-controlling for triacylglycerol synthesis in hepatocytes (Lamb & Fallon, 1974a; Christie *et al.*, 1976). Cytosolic and microsomal phosphohydrolases exist, but their relative importance is not as yet known. The cytosolic phosphohydrolase increases after subtotal hepatectomy (Mangiapanne *et al.*, 1973), ethanol administration (Pritchard *et al.*, 1977; Savolainen, 1977), in diabetic rats (Whiting *et al.*, 1977; Murthy & Shipp, 1979) and in rats injected with various hormones (Glenny & Brindley, 1978; Savolainen & Hassinen, 1978). These situations are associated

with increased hepatic triacylglycerol synthesis, and may be glucocorticoid-dependent (Sturton *et al.*, 1978; Brindley *et al.*, 1979; Knox *et al.*, 1979). The enzyme is inhibited by drugs that decrease neutral lipid synthesis (Brindley & Bowley, 1975b; Mitchell *et al.*, 1976).

The microsomal phosphohydrolase activity is also augmented in many conditions associated with increased hepatic triacylglycerol synthesis, such as a high-carbohydrate diet, sorbitol, glycerol and ethanol (Lamb & Fallon, 1974b; Sturton *et al.*, 1978; Lamb *et al.*, 1979). In our studies, phenobarbital did not affect phosphatidate phosphohydrolase activities per liver or per g of liver, but decreased activity of the microsomal enzyme per mg of protein in rats of both sexes because of the increased protein content of this fraction (results not shown). These results are different from those with this enzyme system in phenobarbital-treated rabbits and guinea pigs, wherein increased activities per liver were recorded (Goldberg *et al.*, 1980b,c). The specific activity of microsomal phosphatidate phosphohydrolase in livers from starved male rats was similar to that previously determined for rabbit liver (Goldberg *et al.*, 1980b), and by other authors for rat liver (Lamb & Fallon, 1974a; Lamb & Banks, 1979). On the other hand, the activity of cytosolic phosphohydrolase per mg of protein, which, in the male rat, approximated that of the microsomal phosphohydrolase, was more than 20-fold higher than that of cytosolic phosphohydrolase in male rabbit liver. Our data are close to those of others who have measured its activity in male rat liver (Glenny *et al.*, 1978; Glenny & Brindley, 1978; Murthy & Shipp, 1979), although variations in pH and addition of phosphatidylcholine can greatly enhance activity with emulsified substrates (Sturton & Brindley, 1978). The specific activity of microsomal phosphatidate phosphohydrolase in livers from female control rats was higher than that from males; but the specific activity of the cytosolic enzyme was lower by an order of magnitude in females (results not given). Thus, whereas the ratio of cytosolic to microsomal phosphohydrolase in male rat liver approximated unity, as in the experiments of Sturton *et al.* (1978), the corresponding ratio for female rats was much lower.

Since, in contrast with the situation in rabbits, phenobarbital did not influence microsomal or cytosolic phosphohydrolase in rats, we sought other mechanisms whereby it might lead to increases in glycerolipid synthesis and triacylglycerol content in this species. Thus, we determined the activity of the final enzyme in triacylglycerol synthesis, diacylglycerol acyltransferase, and demonstrated that it was significantly increased in livers from starved male rats injected with phenobarbital. It might appear that this enzyme, which had a specific

activity 4–5-fold higher than either phosphohydrolase, could not be rate-limiting for triacylglycerol synthesis in rat liver. However, without knowing how these enzymes function in intact liver cells, their K_m values and the relative concentrations of their natural substrates, the possibility that increased diacylglycerol acyltransferase activity contributes in part to enhanced triacylglycerol synthesis and content in livers of phenobarbital-treated rats cannot be excluded. This enzyme might also expand hepatic triacylglycerol pools by re-esterifying diacylglycerol derived by partial lipolysis.

The decrease by phenobarbital of serum triacylglycerol concentrations in starved male and female rats contrasts with their increase in phenobarbital-treated rabbits and guinea pigs (Goldberg *et al.*, 1980b,c). It is not clear why this decrease in serum triacylglycerol concentration occurred only in starved animals. The possibility that phenobarbital might block secretion of triacylglycerol-rich lipoproteins (very-low-density lipoproteins) from the liver was not validated experimentally, although the Triton WR1339 technique may underestimate this process (Lipkin *et al.*, 1978; Palmer *et al.*, 1978). Modulation of circulating lipoprotein-triacylglycerol hydrolysis may be another mechanism of phenobarbital action, which requires further investigation.

The phenobarbital-mediated changes in liver triacylglycerol metabolism and in serum triacylglycerol concentration seem to be independent. The hepatic effects, but not the decrease in serum triacylglycerol concentration, were reversed within 5 days of withdrawing the drug. At that time, we could not detect phenobarbital or its metabolites in blood by using highly sensitive immunoassay and chromatographic procedures (Soldin & Hill, 1976). Furthermore, the present studies indicate that, although the increased hepatic triacylglycerol content in the rat may be due to enhanced synthesis consequent on hepatic microsomal enzyme induction, the decrease in serum triacylglycerol concentration is independent of such induction and may be due to action of the drug at an extrahepatic location.

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