Effects of phenobarbital upon triacylglycerol metabolism in the rabbit

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1. The association between hepatic microsomal enzyme induction and triacylglycerol metabolism was examined in fasting male rabbits (2 kg body wt.) injected intraperitoneally with 50 mg of phenobarbital per kg for 10 days. 2. Occurrence of enzyme induction was established by a significant increase in hepatic aminopyrine *N*-demethylase activity and cytochrome *P*-450 content, as well as a doubling of microsomal protein per g of liver and a 54% increase in liver weight. Parallel increments in hepatic γ -glutamyltransferase (EC 2.3.2.2) activity occurred; these were more pronounced in the whole homogenate than in the microsomes, which only accounted for 12.5% of the total enzyme activity in the controls and 17.0% in the animals given phenobarbital. Increased activity of γ -glutamyltransferase activity was also observed in the blood serum of the test animals. 3. The rabbits given phenobarbital manifested increased hepatic triacylglycerol content and the triacylglycerol concentration of blood serum was also elevated. These changes were accompanied by a significantly enhanced ability of cell-free fractions of liver from the test animals (postmitochondrial supernatant and microsomal fractions) to synthesize glycerolipids *in vitro* from *sn*-[14 C]glycerol 3-phosphate and fatty acids, when expressed per whole liver. Relative to the protein content of the fraction, glycerolipid synthesis *in vitro* was significantly decreased in the microsomes, presumably consequent upon the dramatic increase in their total protein content, whereas no change occurred in the postmitochondrial supernatant, possibly due to the protective effect of cytosolic factors present in this fraction and known to enhance glycerolipid synthesis. 4. Microsomal phosphatidate phosphohydrolase accounted for 85% of the total liver activity of this enzyme and its specific activity was 20-fold higher than that of the cytosolic phosphatidate phosphohydrolase (EC 3.1.3.4), when each was measured under optimal conditions. A significant increase in the activity of both enzymes per whole liver occurred in the rabbits given phenobarbital. A closer correlation between hepatic triacylglycerol content and microsomal phosphatidate phosphohydrolase, as well as the above observations, suggest that this, rather than the cytosolic enzyme, may be rate-limiting for triacylglycerol synthesis in rabbit liver. 5. Significant correlations were observed between the various factors of hepatic microsomal-enzyme induction (aminopyrine *N*-demethylase and γ -glutamyltransferase activity as well as cytochrome *P*-450 content) and hepatic triacylglycerol content, suggesting that microsomal enzyme induction may promote hepatic triacylglycerol synthesis and consequently hypertriglyceridaemia in the rabbit.

The enzymes that catalyse triacylglycerol synthesis are, with the possible exception of cytosolic

Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

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phosphatidate phosphohydrolase (EC 3.1.3.4), localized in smooth endoplasmic reticulum (Hübscher, 1970). This intracellular structure in the liver is also the site where the synthesis of various enzymes can be induced by a variety of exogenous and endogenous compounds (Conney, 1967; Lu *et al.*, 1976).

Previous investigations in human subjects have

indicated a possible correlation between hepatic microsomal enzyme induction and serum triacylglycerol concentration (Martin *et al.*, 1975, 1976*a,b*). In these studies, activity of serum γ -glutamyltransferase (EC 2.3.2.2) was employed as an index of hepatic microsomal enzyme induction (Rosalki, 1975; Goldberg, 1979). Support for this relationship has come from authors who have confirmed the association in humans between serum γ -glutamyltransferase activity and triacylglycerol concentration (Cucuianu *et al.*, 1976), whereas other workers reported parallel increases in serum triacylglycerol concentration, serum γ -glutamyltransferase activity and urinary excretion of D-glucaric acid (another index of hepatic microsomal enzyme induction) in human subjects after starting phenobarbital therapy (Annoni *et al.*, 1976). In order to elucidate the biochemical basis for these findings, we have determined, as presented in this paper, the activity of critical enzymes involved in triacylglycerol synthesis and have quantified the overall formation of glycerolipids in cell-free systems. These were obtained from livers of control rabbits and animals in whom microsomal enzyme synthesis had been induced with phenobarbital.

Materials and methods

General

All assays were performed in duplicate. Results were rejected and repeated if duplicates differed by >10%. Centrifugations were performed with the International Equipment centrifuge, model B-20A, for speeds up to 15000g (rotor no. IEC 870) and with the Beckman model L5-65 ultracentrifuge for higher speeds (rotor no. 60i).

Radioactivity was measured with the Nuclear Chicago Mark II liquid-scintillation spectrometer, counting efficiency being >90%. All buffers were prepared at room temperature.

The following reagents were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.: ATP (disodium salt), CoA (lithium salt), *sn*-glycerol 3-phosphate, *rac*-glycerol phosphate, potassium palmitate, di- and tri-palmitoylglycerol, Triton X-100, D-glucose 6-phosphate (disodium salt), NADP⁺, glucose 6-phosphate dehydrogenase (Cat. no. G8875, approx. 600 units per ml), L- γ -glutamyl-*p*-nitroanilide, glycylglycine and nicotinamide. *sn*-[¹⁴C]Glycerol 3-phosphate (sp. radioactivity 165 Ci per mol) was from New England Nuclear Corporation, Boston, MA, U.S.A., aminopyrine from Aldrich Chemical Co., Milwaukee, WI, U.S.A., bovine serum albumin (fraction V, fatty acid free, water content <2.0%) from Miles Laboratories, Elkhart, IN, U.S.A., dithioerythritol from Pierce Chemical Co., Rockford, IL, U.S.A., phenobarbital

sodium from Allen and Hanbury, Toronto, Canada, and 2-mercaptoethanol from Eastman Kodak, Rochester, NY, U.S.A. Silica gel H was purchased from E. Merck, Darmstadt, W. Germany, and spread on glass plates to a thickness of 0.25 mm. All other chemicals were of the highest reagent grade.

Animals

Male New Zealand albino rabbits, weighing 2 kg, were obtained from Reiman's, Kitchener, Ontario. They were given laboratory chow (Ralston Purina Co., St. Louis, MO, U.S.A.) *ad libitum*. The animals were weighed daily and a chart of their food intake was also maintained. Phenobarbital was dissolved in 0.15 M-NaCl and injected intraperitoneally at a dose of 50 mg/kg for 10 days. Injections were performed at 09.00h. The control animals were injected with 0.15 M-NaCl alone. Food, but not water, was withdrawn for 18 h before killing.

Preparation of serum and cell-free fractions from liver

Animals were briefly anaesthetized with chloroform and blood was withdrawn by cardiac puncture, this allowed to clot and the serum removed by centrifugation to determine triacylglycerol concentration and γ -glutamyltransferase activity. All further preparative procedures were performed at 2–4°C. Livers were weighed and homogenized in 3 vol. of soln. A (0.2 M-sucrose, 0.05 M-Hepes, pH 7.0, 1 mM-EDTA and 2 mM-dithioerythritol). A measured portion of the homogenate was removed for assay of triacylglycerol concentration and γ -glutamyltransferase activity and the remainder was centrifuged at 700g for 10 min. The supernatant was transferred to a clean tube, the sediment rehomogenized in a volume of soln. A equal to the original, and the washings, after centrifugation at 700g for 10 min, were added to the first supernatant. The pellet was discarded, the combined supernatant centrifuged at 15000g for 30 min, and the post-mitochondrial supernatant removed; the sediment was washed with an equal volume of soln. A and the washings, after recentrifugation at the original speed, were added to the postmitochondrial supernatant. A measured portion of the latter was taken for assay of aminopyrine *N*-demethylase activity and *in vitro* synthesis of glycerolipids from *sn*-[¹⁴C]glycerol 3-phosphate.

The mitochondrial pellet was discarded and the remainder of the postmitochondrial supernatant was centrifuged at 105000g for 60 min. The cytosol and microsomal pellet were separated and the latter rehomogenized in a measured volume of soln. A, the washings being transferred to the cytosol after resedimentation at the original speed. The assays performed on the microsome fraction (after its dispersion in a measured volume of soln. A) included

cytochrome *P*-450 content, *in vitro* glycerolipid synthesis and the activities of γ -glutamyltransferase and phosphatidate phosphohydrolase. The latter was also assayed in the cytosol. Protein concentration was determined in all fractions and results of the assays for each animal were calculated per mg of protein, per g of liver and per whole liver.

Esterification assays with sn -[^{14}C]glycerol 3-phosphate

All activities were assayed under optimal and linear reaction-rate conditions. Glycerolipid synthesis from sn -[^{14}C]glycerol 3-phosphate and fatty acids was determined in postmitochondrial and microsomal fractions by a modification of a reported procedure (Roncari & Murthy, 1975).

The incubation mixture for the postmitochondrial supernatant contained: 6 mM- sn -[^{14}C]glycerol 3-phosphate, 0.08 mM-potassium palmitate, 3.3 mM-ATP, 0.08 mM-CoA, 2 mM-MgCl₂, 0.1 M-potassium phosphate buffer (pH 7.0), 0.05 M-KCl, 0.01 M-2-mercaptoethanol, 0.8–1.0 mg of postmitochondrial supernatant protein and de-ionized redistilled water, to a total volume of 0.1 ml. Incubations were carried out at 25°C for 5 min. Since the fasted-rabbit liver was rich in endogenous fatty acids, 75% of maximal esterification activity under our conditions occurred without exogenous palmitate.

Albumin was found to increase the incorporation of sn -[^{14}C]glycerol 3-phosphate into glycerolipids in the presence of microsomal fractions by about 50% (it decreased incorporation in the presence of postmitochondrial supernatant by about 30%). Consequently, it was added as a 400 μM -palmitate–145 μg albumin complex [bovine serum albumin delipidated by the method of Goodman (1957) and dissolved (10%, w/v) in 0.1 M-potassium phosphate buffer, pH 7.4, 1.0 M-NaCl, 0.1 M-KCl and 0.1 M-MgSO₄] to an assay mixture modified for microsomes. Each reaction tube contained 40 nmol of palmitate and 145 μg of albumin. The incubation mixture also contained 12 mM- sn -[^{14}C]glycerol 3-phosphate (final sp. activity 0.83 $\mu\text{Ci}/\mu\text{mol}$ prepared by diluting the stock isotope solution with cold sn -glycerol 3-phosphate), 5 mM-ATP, 0.2 mM-CoA, 2.5 mM-MgCl₂, 0.1 M-Tris/HCl buffer (pH 7.4), 0.3–0.4 mg of microsomal protein and deionized redistilled water, to a total volume of 0.1 ml. Incubations were carried out at 37°C for 10 min.

For each assay, the reactions were stopped by the addition of 0.7 ml of ice-cold water followed by 3 ml of chloroform/methanol (1:2, v/v); the methanol contained 0.01 M-*rac*-glycerol phosphate. Lipid extraction was carried out by the method of Bligh & Dyer (1959). Glycerolipids were separated on a t.l.c. plate coated with silica gel H and developed with light petroleum (b.p. 40–60°C)/diethyl ether/acetic acid (190:55:5, by vol.), as

previously reported (Roncari & Hollenberg, 1967). 50 μg of authentic di- and tri-palmitoylglycerol were added as carriers for newly synthesized glycerolipids. After visualization with iodine, the areas containing the various lipid fractions were scraped and radioactivity was counted by using Bray's mixture. Appropriate corrections were made for quenching of radioactivity and self-absorption, with both internal and external standardization. A blank was also performed containing all constituents, but substituting water for the tissue fraction. Lyso-phosphatidate extracted with butan-1-ol according to Monroy *et al.* (1972), on completion of the esterification assay, was found to be a minor constituent (<5%) in all samples tested and was not routinely measured in our final assay.

Phosphatidate phosphohydrolase assay

This assay with membrane-bound 1,2-diacyl ([^{14}C]palmitoyl)- sn -glycerol 3-phosphate (abbreviated [^{14}C]phosphatidate) was carried out in microsomal and cytosolic fractions by a modification of previously described methods (Brindley & Bowley, 1975a; Heusden & Bosche, 1978; Roncari *et al.*, 1979). The substrate, [^{14}C]phosphatidate, was synthesized from sn -[^{14}C]glycerol 3-phosphate with rat liver microsomes. The reaction was carried out in a total volume of 10 ml containing the same concentrations of reagents as in the above esterification assay. The mixture was incubated at 37°C for 20 min and the reaction was stopped by cooling on ice for 10 min, with constant stirring. It was layered carefully on top of 2 vol. of 0.3 M-sucrose and centrifuged at 105 000 *g* for 60 min. The pellet was resuspended in 0.1 M-potassium phosphate buffer, pH 7.0, centrifuged at 105 000 *g* for 60 min, and the pellet resuspended again in the same buffer. Protein concentration was then determined, and the specific radioactivity of the [^{14}C]phosphatidate was measured in a portion of the labelled microsomes by performing chloroform/methanol extraction, t.l.c. separation, scintillation counting and P_i determination by using the method of Fiske & Subbarow (1925). This was found to be 2.94 $\mu\text{Ci}/\mu\text{mol}$.

In microsomal fractions, the assay was carried out in a total volume of 0.1 ml containing membrane-bound 3.4 mM-[^{14}C]phosphatidate corresponding to 500 μg of microsomal protein, which had been previously heated to 85°C for 15 min, 0.1 M-potassium phosphate buffer, pH 7.0, 1 μl of Triton X-100, 300 μg of microsomal protein from the sample to be analysed and deionized redistilled water to make up the volume. The reaction mixture was incubated at 37°C for 7 min and the reaction was stopped by the addition of 0.7 ml of ice-cold water followed by 3 ml of chloroform/methanol (1:2, v/v); the methanol contained 0.01 M-*rac*-glycerol phosphate. Extraction of glycerolipids and

their separation by t.l.c. followed the procedures used in the esterification assay. After visualization with iodine, the area corresponding to diacylglycerol was scraped, the radioactivity was counted in a Nuclear Chicago liquid-scintillation counter by using Bray's mixture, and the results converted into nmol of diacylglycerol formed in the assay by referring to the specific radioactivity of the [^{14}C]phosphatidate substrate.

A similar assay was carried out with the cytosolic fractions. These were brought to 40%-satn. with $(\text{NH}_4)_2\text{SO}_4$, stirred for 30 min and centrifuged at 20000g for 10 min. The pellet was suspended in 0.1 M-potassium phosphate buffer, pH 7.0. The assay was performed in a total volume of 250 μl containing 250 mM-potassium phosphate buffer, pH 6.8, 2 mM- MgCl_2 , 1.5 mg of fatty acid-free bovine serum albumin, [^{14}C]phosphatidate, corresponding to 500 μg of microsomal protein preheated to 85°C for 15 min, 300 μg of cytosolic protein and distilled water to make up the volume. The reaction mixture was incubated at 37°C for 15 min. The rest of the procedure was identical to that described for the microsomal phosphatidate phosphohydrolase assay.

Some aspects of the assay for phosphatidate phosphohydrolase activity are controversial. It has been reported that the microsomal enzyme differs from its cytosolic counterpart in requiring Mg^{2+} , emulsified (as opposed to membrane-bound) substrate and a 5-fold higher substrate concentration (Lamb & Fallon, 1974a). Measurement of diacylglycerol formation (as utilized in our assays) has been recommended in place of determining phosphate release, since the latter overestimates phosphatidate phosphohydrolase activity (Sturton & Brindley, 1978). Savolainen (1977), in measuring cytoplasmic phosphatidate phosphohydrolase, employed both emulsified and membrane-bound substrate and found them to be equally effective. The equipotence of both substrate forms with soluble phosphatidate phosphohydrolase has been confirmed (Brindley *et al.*, 1979). Our conditions for the assay of cytosolic and microsomal phosphatidate phosphohydrolase activity were optimal with regard to substrate concentration, ionic conditions and the time of incubation. For the cytosolic phosphatidate phosphohydrolase, we used a lower pH and Mg^{2+} ; the latter were not required for the microsomal enzyme in the presence of Triton X-100. Under these conditions, EDTA in concentrations up to 10 mM had no effect upon the microsomal phosphohydrolase whether or not Mg^{2+} was added, whereas the cytosolic phosphohydrolase was inhibited by EDTA when in excess of the Mg^{2+} concentration. Omission of Mg^{2+} from the cytosolic assay yielded < 50% of the activity obtained with an optimal concentration of MgCl_2 (2 mM). The endogenous Mg^{2+} concentration of microsomes was such

that the final concentration in the assay for the microsomal phosphohydrolase was < 0.2 mM.

Other enzyme assays

Aminopyrine N-demethylase. The reaction was carried out in a total volume of 5 ml containing the following: 0.03 M-potassium phosphate buffer, pH 7.4, 0.2 mM-NADP $^+$, 8 mM-glucose 6-phosphate, 10 mM-nicotinamide, 5 mM- MgCl_2 , 1 mM-aminopyrine, glucose 6-phosphate dehydrogenase (3 units/assay), 20–25 mg of postmitochondrial supernatant protein and distilled water as required. The mixture was incubated, with shaking, at 37°C. After 30 min, the reaction was stopped by addition of 4 ml of 10% (w/v) ZnCl_2 , mixed well and then 2 ml of satd.-Ba(OH) $_2$ was added, mixed well and the mixture was centrifuged at 1000g for 10 min. A 5 ml portion was removed from the top layer and transferred to a test tube. 2 ml of the reagent, described by Nash (1953), was added, mixed well, and the contents placed in a water bath at 60°C for 30 min, cooled to room temperature and A_{415} was measured. Results were converted into nmol of formaldehyde produced in each assay by construction of a standard curve for formaldehyde with each batch.

Serum γ -glutamyltransferase activity. This was determined according to the method of Szasz (1976).

Hepatic γ -glutamyltransferase activity. This was measured in liver homogenates and microsome preparations by a modification of the method of Orlowski & Meister (1965). 0.5 ml of enzyme source was incubated with 1 ml of substrate mixture (4 mM- γ -glutamyl-*p*-nitroanilide, 11 mM- MgCl_2 and 40 mM-glycylglycine in 185 mM-Tris buffer (pH 8.25) at 37°C. 1 ml of ice-cold 25% trichloroacetic acid was added after 10 min, mixed, centrifuged and A_{405} of the supernatant was read. ϵ of free *p*-nitroaniline in the above buffer/trichloroacetic acid mixture at 405 nm was experimentally determined to be $1.74 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$.

Triacylglycerol determination

The serum triacylglycerol concentration was determined by the method of Bucolo & David (1973). Triacylglycerol was hydrolysed to glycerol and fatty acid by saponification with ethanolic KOH (approx. 0.7 mol/litre) followed by enzymatic determination of liberated glycerol with a Boehringer Mannheim triglyceride kit, cat. no. 125032. Blanks for free glycerol were performed and the results deducted from the serum triacylglycerol concentration. Results were expressed as mg of triacylglycerol/dl of serum. After lipid extraction by the method of Folch *et al.* (1957), the triacylglycerol content of liver homogenates was quantified by the same procedure.

Other assays

Cytochrome *P*-450 was determined in the microsomal fraction (protein concentration of 2 mg/ml) according to the method of Omura & Sato (1964). ϵ of 91 litre·mol⁻¹·cm⁻¹ was used for the optical density difference (450–480 nm) of the spectrum in dithionite and carbon monoxide.

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

Statistics

Student's *t* test was used to assess the significance of differences between the test and the control animals. Unless otherwise stated, all results in this paper are reported as means $\pm$ S.E.M. Fisher's transformed correlation coefficient (Snedecor & Cochran, 1967) was used to assess the degree of association between various biochemical procedures. Results were obtained from the HP 9810A programmable calculator (Hewlett Packard, Loveland, CO, U.S.A.).

Results

General

Preliminary experiments on the time sequence of changes in aminopyrine *N*-demethylase activity and cytochrome *P*-450 content showed a rise after phenobarbital was given, reaching a plateau between day 7 and day 10 and remained constant until day 21, beyond which no further observations were made. We therefore chose 10 days of phenobarbital administration for our definitive protocol. Food consumption in the group given phenobarbital (61.8 $\pm$ 6.8 g per day) was similar to that in the control group (56.2 $\pm$ 7.4 g per day) and although the weight gain in the test group over the 10-day observation period (182 $\pm$ 52.9 g) was double that of the controls (96 $\pm$ 31.2 g), this difference was not statistically significant (*t* = 1.34). The weight of the livers of the rabbits given phenobarbital showed a mean increase of 54% above that of the controls (*t* = 4.97; *P* < 0.001) at the time of killing. Thus the protein content of all liver fractions was significantly increased in the rabbits given phenobarbital when expressed per whole liver (Table 1), but when expressed per g of liver, only the microsomal fraction showed an increment in the test animals; microsomal protein per g of liver averaged 223% of the value for the control animals (*t* = 3.88; *P* < 0.01). Phenobarbital administration does not influence recovery of hepatic subcellular fractions (Cooper & Feuer, 1972).

Parameters of microsomal enzyme induction

Aminopyrine *N*-demethylase activity and cytochrome *P*-450 content increased almost 5- and

Table 1. Effect of phenobarbital on protein content of rabbit liver

Fraction	Whole homogenate		Postmitochondrial supernatant		Microsomes		Cytosol	
	Control	Test	Control	Test	Control	Test	Control	Test
g of protein/liver	11.4 $\pm$ 0.8	17.9 $\pm$ 0.8	6.8 $\pm$ 0.5	11.3 $\pm$ 0.6	0.43 $\pm$ 0.03	1.40 $\pm$ 0.15	1.68 $\pm$ 0.17	3.12 $\pm$ 0.38
	<i>t</i> = 5.88; <i>P</i> < 0.001		<i>t</i> = 4.89; <i>P</i> < 0.001		<i>t</i> = 6.04; <i>P</i> < 0.001		<i>t</i> = 3.35; <i>P</i> < 0.005	
mg of protein/g of liver	187 $\pm$ 3	190 $\pm$ 6	111 $\pm$ 3	121 $\pm$ 7	6.9 $\pm$ 0.2	15.4 $\pm$ 2.1*	27.5 $\pm$ 2.2	32.1 $\pm$ 2.6

Protein content of liver fractions measured in control (*n* = 9) and test (*n* = 10) rabbits after 10 days of phenobarbital sodium at a dose of 50 mg day⁻¹·kg⁻¹. Results by the method of Lowry *et al.* (1951) are reported as means $\pm$ S.E.M. analysed for statistical significance by Student's *t* test (control versus test for each factor): **t* = 3.88; *P* < 0.005. All other differences in protein content per g of liver in other fractions are not statistically significant.

3-fold respectively per mg of protein after 10 days of phenobarbital administration, and even more dramatically when expressed per g of liver or per whole liver (Table 2). Increased activity of γ -glutamyltransferase was also observed in liver homogenates and microsomes. All these increments in the γ -glutamyltransferase activity of the test animals were statistically significant, apart from the microsomal activity per mg of protein (Table 2). A significant increase in the serum γ -glutamyltrans-

ferase activity accompanied these changes; the mean for the control group was $4.00 \pm 0.49 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{litre}^{-1}$, and for the test group was $9.88 \pm 0.72 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{litre}^{-1}$ ($t = 6.67$; $P < 0.001$).

Serum and liver triacylglycerol content

Significant increases in liver triacylglycerol content occurred in the rabbits given phenobarbital (Table 3). These increments were nearly as dramatic

Table 2. *Effect of phenobarbital on factors of microsomal enzyme induction in rabbit liver*

Phenobarbital sodium was injected intraperitoneally into test animals ($n = 10$) at a dose of $50 \text{ mg day}^{-1} \text{ kg}^{-1}$ for 10 days and the animals killed after an overnight fast. Control animals ($n = 9$) were injected with 0.15 M-NaCl for the same period. Results are reported as means $\pm$ S.E.M.. Student's t test was used to assess significance of differences between test and control animals (values for t and P are not given where differences are not significant). Cytochrome P -450 content was measured in washed microsomes and aminopyrine N -demethylase activity in postmitochondrial supernatant.

	Control	Test	t	P
Cytochrome P -450				
$\mu\text{mol/whole liver}$	0.53 ± 0.05	4.54 ± 0.58	6.52	<0.001
nmol/g of liver	8.65 ± 0.67	51.80 ± 8.59	4.74	<0.001
$\text{nmol/mg of protein}$	1.25 ± 0.08	3.26 ± 0.13	12.5	<0.001
Aminopyrine N -demethylase				
$\text{nmol/min per whole liver}$	26 ± 3	381 ± 39	8.52	<0.001
$\text{nmol min}^{-1} \text{ g}^{-1}$ of liver	0.42 ± 0.04	4.20 ± 0.56	6.41	<0.001
$\text{pmol min}^{-1} \text{ mg}^{-1}$ of protein	60 ± 5	275 ± 8	21.50	<0.001
γ -Glutamyltransferase (whole homogenate)				
$\text{mol/min per whole liver}$	88 ± 10	180 ± 11	6.12	<0.001
$\text{mol min}^{-1} \text{ g}^{-1}$ of liver	1.44 ± 0.12	1.94 ± 0.14	2.63	<0.02
$\text{mmol min}^{-1} \text{ mg}^{-1}$ of protein	7.74 ± 0.58	10.20 ± 0.74	2.50	<0.025
γ -Glutamyltransferase (microsomes)				
$\text{mol/min per whole liver}$	10.6 ± 1.2	31.1 ± 4.5	4.17	<0.001
$\text{mol min}^{-1} \text{ g}^{-1}$ of liver	171 ± 14	342 ± 55	2.85	<0.02
$\text{mmol min}^{-1} \text{ mg}^{-1}$ of protein	25.1 ± 2.3	25.0 ± 4.8	—	—

Table 3. *Effect of phenobarbital on factors of glycerolipid metabolism in rabbit liver*

Protocol as described in Table 2. Triacylglycerol results refer to whole homogenate. Esterification assay with sn - $[^{14}\text{C}]$ glycerol 3-phosphate reported as the sum of d.p.s. incorporated into phosphatidate, di- and tri-acyl glycerol in the presence of optimal concentrations of plamitate and cofactors and converted into nmol of glycerolipid formed per min. For the postmitochondrial supernatant, albumin was not included and the reaction was stopped after 5 min at 25°C . For the microsomes, albumin was present and the reaction was stopped after 10 min at 37°C . Full details are given in the text.

	Control	Test	t	P
Hepatic triacylglycerol				
g/whole liver	0.59 ± 0.01	1.70 ± 0.10	8.07	<0.001
mg/g of liver	9.4 ± 1.1	18.2 ± 1.2	5.40	<0.001
$\mu\text{g/mg of protein}$	49.7 ± 5.1	93.7 ± 4.0	6.84	<0.001
Esterification assay (postmitochondrial supernatant)				
$\text{mmol/min per whole liver}$	0.69 ± 0.16	1.36 ± 0.22	2.40	<0.05
$\text{nmol min}^{-1} \text{ g}^{-1}$ of liver	12.1 ± 3.0	15.4 ± 3.0	—	—
$\text{pmol min}^{-1} \text{ mg}^{-1}$ of protein	88 ± 22	97 ± 13	—	—
Esterification assay (microsomes)				
$\text{nmol/min per whole liver}$	327 ± 55	544 ± 65	2.57	<0.02
$\text{nmol min}^{-1} \text{ g}^{-1}$ of liver	5.39 ± 0.83	6.04 ± 0.80	—	—
$\text{nmol min}^{-1} \text{ mg}^{-1}$ of protein	0.78 ± 0.11	0.43 ± 0.06	2.68	<0.02

when the data were expressed per mg of protein (mean test value 189% of the controls) as per g of liver (mean test value 193% of the controls) and per whole liver (mean test value 288% of the controls). In line with these changes, serum triacylglycerol concentration was significantly increased in the test animals. The mean value rose from $137 \pm 11 \text{ mg} \cdot \text{dl}^{-1}$ in the control group to $191 \pm 19 \text{ mg} \cdot \text{dl}^{-1}$ in the test group ($t = 2.37$; $P < 0.05$).

Glycerolipid synthesis in cell-free fractions

Under conditions of zero-order kinetics, diacyl-*sn*-glycerol 3-phosphate is the major glycerolipid (>90%, the remainder being diacyl- and triacylglycerol) synthesized *in vitro* in microsomal and postmitochondrial (microsomes + cytosol) liver fractions fortified with optimal concentrations of substrates and cosubstrates (Roncari & Murthy, 1975; Brindley & Bowley, 1975b; Whiting *et al.*, 1977; Soler-Argilaga *et al.*, 1978). When incubation is prolonged, increasing quantities of diacyl- and triacylglycerol are formed. Because we wanted to conduct our assays under linear reaction-rate conditions, we stopped the reactions at a time when the

triacylglycerol precursor, diacyl-*sn*-glycerol 3-phosphate, was still the major product *in vitro*.

As illustrated in Table 3, the incorporation of *sn*-[^{14}C]glycerol 3-phosphate into glycerolipids was significantly higher in postmitochondrial supernatant and microsomal preparations obtained from rabbits given phenobarbital, when expressed per whole liver. When the results were expressed per mg of protein, there was no significant difference for the postmitochondrial supernatant, although a significant decrease in *sn*-[^{14}C]glycerol 3-phosphate incorporation occurred in microsomes from the test animals.

Microsomal and cytosolic phosphatidate phosphohydrolase activity

Of the total phosphatidate phosphohydrolase activity in control rabbit liver attributable to these two fractions, 85% was accounted for by the microsomal enzyme (Fig. 1). Its specific activity was 20-fold higher than that of the cytosolic phosphohydrolase. When expressed per whole liver, a significant increase in phosphatidate phosphohydro-

Fig. 1. Effect of phenobarbital on microsomal and cytosolic phosphatidate phosphohydrolase activities of rabbit liver. ■, Rabbits ($n = 10$) given phenobarbital at a dose of $50 \text{ mg} \cdot \text{day}^{-1} \cdot \text{kg}^{-1}$ for 10 days by intraperitoneal injection and □, rabbits ($n = 9$) given, for the same period, the vehicle alone. Data are activities expressed as rate of diacylglycerol formed per whole liver, per g of liver and per mg of protein and are presented as means $\pm$ S.E.M. Significant differences are indicated by the appropriate value for P based on the Student's t test. N.S., Differences not statistically significant. Note that scales for microsomal and cytosolic enzymes differ only when expressed per mg of protein.

Table 4. *Correlation of hepatic triacylglycerol content with factors of glycerolipid synthesis and microsomal enzyme induction*

Results from test and control animals were pooled and the correlation coefficient (r) was calculated. The hepatic triacylglycerol content was always expressed in the same way as the other variable with which its correlation was tested (per whole liver, per g of liver or per mg of protein, as appropriate).

Factors of glycerolipid synthesis	r	P
Phosphatidate phosphohydrolase		
Microsomal (per whole liver)	0.927	<0.001
Microsomal (per g of liver)	0.715	<0.001
Cytosolic (per whole liver)	0.690	<0.01; >0.001
Cytosolic (per g of liver)	0.390	<0.1; >0.05
<i>sn</i> -[^{14}C]Glycerol 3-phosphate incorporation into glycerolipids		
Postmitochondrial supernatant (per whole liver)	0.456	<0.05; >0.01
Microsomal (per whole liver)	0.300	<0.1; >0.05
Factors of microsomal enzyme induction		
Aminopyrine <i>N</i> -demethylase (per mg of protein)	0.816	<0.001
Cytochrome <i>P</i> -450 (per mg of protein)	0.868	<0.001
γ -Glutamyltransferase		
Hepatic (per whole liver)	0.804	<0.001
Hepatic (per g of liver)	0.413	<0.1; >0.05

lase activity occurred in both microsomal and cytosolic fractions from rabbits given phenobarbital (Fig. 1). The specific activity of phosphohydrolase was decreased significantly in microsomes, although it was not altered significantly in cytosol.

Correlation of triacylglycerol metabolism and enzyme induction

The hepatic triacylglycerol content was correlated with various factors of triacylglycerol synthesis and with various indices of microsomal enzyme induction (Table 4). An excellent correlation was obtained between triacylglycerol content and microsomal phosphatidate phosphohydrolase activity, but a less close correlation with cytosolic phosphohydrolase activity. A poor correlation was found with *sn*-[^{14}C]glycerol 3-phosphate incorporation into glycerolipids by microsomal fractions; however, in the case of the postmitochondrial supernatant, which contains all the known compounds required for glycerolipid synthesis, a statistically significant correlation was found between the incorporation of *sn*-[^{14}C]glycerol 3-phosphate into glycerolipids and hepatic triacylglycerol content.

The correlation between serum triacylglycerol concentration and serum γ -glutamyltransferase activity gave a value of $r=0.374$, which was not statistically significant in the present series ($0.1 < P < 0.05$).

Discussion

Phenobarbital in the dose schedule used in these experiments induced dramatic increases in liver mass, hepatic microsomal content and hepatic

content of the classic microsomal constituents, aminopyrine *N*-demethylase and cytochrome *P*-450. We observed concurrent, significant increments in the contents of triacylglycerol and γ -glutamyltransferase in liver and serum. A good correlation was obtained between hepatic triacylglycerol content and γ -glutamyltransferase activity (Table 4). Likewise, an excellent correlation existed between hepatic triacylglycerol content and other factors of microsomal enzyme induction (aminopyrine *N*-demethylase and cytochrome *P*-450). These observations, therefore, are consistent with the hypothesis that hepatic microsomal enzyme induction may lead to increased triacylglycerol synthesis and elevated serum triacylglycerol concentrations.

The increased hepatic triacylglycerol content cannot be a consequence solely of impaired hepatic triacylglycerol secretion in view of the concomitant increase in serum triacylglycerol concentration. Decreased triacylglycerol hydrolysis in liver and concomitant impairment of lipoprotein-triacylglycerol clearance from plasma might also explain our findings, but increased hepatic synthesis of triacylglycerol is the most plausible explanation. The capability of the whole organ to synthesize triacylglycerol as judged by phosphatidate phosphohydrolase activity and incorporation of *sn*-[^{14}C]glycerol 3-phosphate into glycerolipids *in vitro* was increased, even though these increments were not apparent when expressed per unit of microsomal protein. The observed changes, therefore, seem to be part of the overall increase in microsomal protein induced by phenobarbital and not a selective response to the drug, unlike the case with the increased cytochrome *P*-450 content and amino-

pyrine *N*-demethylase activity. Final proof that the changes in triacylglycerol and γ -glutamyltransferase are a direct consequence of microsomal enzyme induction requires the simultaneous administration of phenobarbital and an appropriate agent, such as SKF 525A (Rogers & Fouts, 1964), to block enzyme induction.

We elected to measure phosphatidate phosphohydrolase activity because evidence indicates that it is rate-limiting for triacylglycerol synthesis in rat liver (Hübscher *et al.*, 1967; Fallon *et al.*, 1977; Brindley, 1978). The relative importance of the microsomal as compared with the cytosolic phosphatidate phosphohydrolase remains to be established. Most previous studies involved the cytosolic enzyme (Mangiapanne *et al.*, 1973; Brindley & Bowley, 1975b; Mitchell *et al.*, 1976; Glennly & Brindley, 1978; Murthy & Shipp, 1979), but other investigations have revealed that the microsomal phosphohydrolase responds to influences that alter the rate of triacylglycerol synthesis. For example, fructose, sorbitol, glycerol and ethanol increase the activity of not only the cytosolic, but also the microsomal, phosphatidate phosphohydrolase (Lamb & Fallon, 1974b; Lamb *et al.*, 1979; Wood & Lamb, 1979; Sturton *et al.*, 1978).

In our studies, phenobarbital brought about a significant increase in both microsomal and cytosolic phosphohydrolase activities, when expressed per whole liver. The activity of the microsomal, but not the cytosolic enzyme, when expressed per g of liver, correlated significantly with hepatic triacylglycerol content expressed in the same way. The parallelism between microsomal phosphatidate phosphohydrolase activity and glycerolipid synthesis in microsomes would suggest that this phosphohydrolase, which has the same intracellular location (smooth endoplasmic reticulum) as the other enzymes of the triacylglycerol biosynthetic pathway (Hübscher, 1970; Roncari *et al.*, 1979), is the significant phosphohydrolase *in vivo*. However, the possible regulatory influence of glycerophosphate acyltransferase (EC 2.3.1.15) and diacylglycerol acyltransferase (EC 2.3.1.20) in triacylglycerol synthesis deserves further investigation. Nevertheless, assuming that the rabbit is similar to the rat regarding the rate-controlling role of phosphatidate phosphohydrolase activity in hepatic triacylglycerol synthesis, the microsomal enzyme would be quantitatively most important because its specific activity was 20-fold higher than that of the cytosolic phosphatidate phosphohydrolase and total microsomal phosphohydrolase activity in whole rabbit liver was 5-fold greater than total phosphohydrolase activity in cytosol.

The specific activity of cytosolic phosphatidate phosphohydrolase was not altered significantly by phenobarbital, nor was overall glycerolipid syn-

thesis per mg of protein in the postmitochondrial supernatant containing both microsomes and cytosol. Thus the presence of cytosol prevented the decrease in specific activity of overall glycerolipid synthesis observed with microsomes derived from rabbits given phenobarbital. The difference between microsomes and the postmitochondrial supernatant in these respects is not surprising in view of the fact that the former, but not the latter, manifested increased protein content per g of liver in the rabbits given phenobarbital (Table 1). A significant correlation between cytosolic phosphatidate phosphohydrolase and glycerolipid synthesis from *sn*-[^{14}C]glycerol 3-phosphate by the post-mitochondrial supernatant *in vitro* was observed when both factors were expressed per g of liver ($r=0.651$; $P<0.005$) and per whole liver ($r=0.630$; $P<0.005$). Furthermore, a characterized low-molecular-weight cytosolic protein (mol.wt. about 10000) that enhances neutral acylglycerol synthesis (Roncari & Mack, 1975) may partially explain why the specific activity of overall glycerolipid synthesis is not decreased in cell-free fractions containing not only microsomes, but also cytosol derived from animals given phenobarbital.

Another cytosolic constituent, Z protein, has also been shown to promote glycerolipid synthesis (O'Doherty & Kuksis, 1975; Mishkin & Roncari, 1976). Thus the following three cytosolic proteins may account for the unaltered specific activity of overall glycerolipid synthesis in postmitochondrial fractions derived from rabbits given phenobarbital: 'soluble' phosphatidate phosphohydrolase, the cytosolic protein (mol.wt. about 10000) that enhances neutral acylglycerol synthesis and Z protein. Other less well-characterized soluble proteins enhance glycerolipid synthesis by washed liver microsomes (Manley *et al.*, 1974; Jamdar, 1979). Despite the fact that the specific activity of microsomal phosphatidate phosphohydrolase is decreased in test animals, the specific activity of overall glycerolipid synthesis would not be depressed, at least partly because of these cytosolic constituents. Phenobarbital led to an increment in the total quantity of proteins per liver not only in microsomes, but also in cytosol. An increment in the quantity of one or more of the three cytosolic proteins that have been described would account, at least in part, for the increase in glycerolipid synthesis and triacylglycerol content in whole liver of rabbits given phenobarbital. This increased hepatic glycerolipid synthesis expressed per whole liver may in turn be responsible, at least partly, for the observed elevation of serum triacylglycerol. The possible role of glucocorticoids in modulating the observed changes deserves to be mentioned. These hormones have been shown by Brindley and his group to stimulate phosphatidate phosphohydrolase activity and hepa-

tic triacylglycerol synthesis and can account for increases in these functions ascribed to various experimental manipulations (Glenny & Brindley, 1978; Brindley *et al.*, 1979; Lehtonen *et al.*, 1979).

The results of phenobarbital administration upon hepatic and serum γ -glutamyltransferase are consistent with these effects being mediated by enzyme induction. However, in the rat, the specific activity of γ -glutamyltransferase in parenchymal cells is only one-seventh that of Kupffer cells and one-twentieth that of biliary tract cells (Selden *et al.*, 1978). Analysis of its subcellular distribution in rat liver showed γ -glutamyltransferase to be present equally in the cytosol and a membrane-bound pool having the characteristics of plasma membrane (Malvoisin *et al.*, 1978). A similar distribution has been reported in guinea-pig liver (Huseby, 1979) and in rabbit liver (Ratanasavanh *et al.*, 1979). Our data (Table 2) suggest that microsomes account for only 12.5% of the total γ -glutamyltransferase activity in control rabbits and 17% in rabbits given phenobarbital. Increased synthesis of the enzyme in other cell components, as well as in microsomes, has to be postulated to explain the magnitude of the increment in γ -glutamyltransferase activity in both liver and serum.

In contrast to our findings in the rabbit, phenobarbital does not cause hypertriglyceridaemia or increased hepatic triacylglycerol synthesis in the rat or mouse (Karvinen *et al.*, 1965; Salvador *et al.*, 1970). The present results, therefore, demonstrate that the rabbit is a good model for the study of the relationship between microsomal enzyme induction and hypertriglyceridaemia and suggest that enhanced hepatic triacylglycerol synthesis is the likely mechanism underlying this relationship.

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