Phenobarbital-induced alterations in phosphatidylcholine and triglyceride synthesis in hepatic endoplasmic reticulum

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ABSTRACT Biosynthetic pathways of phosphatidylcholine and triglyceride were studied in proliferating hepatic endoplasmic reticulum of rats pretreated with phenobarbital. Phosphatidylcholine accounted for the major increment in membrane phospholipid.

In vitro measurements of hepatic microsomal enzymes which catalyze phosphatidylcholine biosynthesis revealed a significant increase in specific activity of the enzyme governing phosphatidylcholine synthesis by sequential methylation of phosphatidylethanolamine. The specific activity of phosphorylcholine-glyceride transferase, which catalyzes phosphatidylcholine synthesis from D-1,2-diglyceride and CDP-choline, was not altered. Specific activity of diglyceride acyltransferase, which catalyzes triglyceride biosynthesis, was increased to a degree comparable to the increase in specific activity found in the phenobarbital-induced drug-metabolizing enzyme which oxidatively demethylates aminopyrine.

In vivo incorporation of methyl-^{3}H from L-methionine-methyl-^{3}H into microsomal phosphatidylcholine was significantly increased, resulting in an increased methyl-^{3}H to choline-1,2-^{14}C incorporation ratio of more than three times that found in control animals. A comparable increase in this incorporation ratio was noted in serum phospholipids.

The in vitro enzyme studies, in agreement with in vivo incorporation data, indicate that the increase in phosphatidylcholine content of phenobarbital-induced proliferating endoplasmic reticulum is related to increased activity of the pathway of phosphatidylcholine biosynthesis involving the sequential methylation of phosphatidylethanolamine.

SUPPLEMENTARY KEY WORDS   phosphatidylcholine · S-adenosyl-L-methionine: phosphatidylethanolamine methyltransferase · phosphorylcholine-glyceride transferase · triglyceride · diglyceride-acyltransferase · choline · L-methionine · CDP-choline · palmitoyl CoA · D-1,2-diglyceride

ENZYMES in liver responsible for metabolism of drugs are located primarily in the membranes of the smooth endoplasmic reticulum of hepatic parenchymal cells. Many drugs, the most extensively studied of which is phenobarbital (1), induce a rapid proliferation of these membranes in association with induction of many enzymes including drug metabolizing enzymes (2, 3) and enzymes governing conjugative transformations, notably glucuronyl transferase (4, 5). The proliferation of these membranes is characterized by an increase in both phospholipid and protein content; no associated increase in serum levels of phospholipid has been demonstrated (6).

An important consideration in the understanding of hepatic lipid metabolism is the relation of the specific hepatic complex lipid biosynthetic pathways to altered rates of lipid production. Complex lipids produced in increased quantities may remain in the liver as pathologic accumulations or as membrane structural elements, or may be transported out of the liver as moieties of lipoproteins.

The present studies were undertaken in order to characterize the alterations in complex lipid biosynthetic pathways associated with drug-induced proliferation of hepatic endoplasmic reticulum.

The present report describes the effect of phenobarbital administration on the specific activity of the microsomal enzymes CDP-choline:1,2-diglyceride cholinephosphotransferase (EC 2.7.8.2) (phosphorylcholine-

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glyceride transferase), which catalyzes the biosynthesis of phosphatidylcholine (3-*sn*-phosphatidylcholine) from D-1,2-diglyceride (1,2-diacyl-*sn*-glycerol) and CDP-choline (cytidine diphosphate choline); *S*-adenosyl-L-methionine : phosphatidylethanolamine methyltransferase (SAME:PE methyltransferase), which catalyzes the biosynthesis of phosphatidylcholine by the sequential methylation of phosphatidylethanolamine utilizing *S*-adenosyl-L-methionine as the methyl donor; and acyl CoA:1,2-diglyceride *O*-acyltransferase (EC 2.3.1.20) (diglyceride-acyltransferase), which catalyzes the biosynthesis of triglyceride (triacylglycerol) from D-1,2-diglyceride and acyl coenzyme A. Alterations in the specific activity of the latter enzyme, which utilizes the substrate D-1,2-diglyceride common to the pathways for phosphatidylcholine biosynthesis, have previously (7) been noted to be associated with alterations in phosphatidylcholine biosynthesis. Also described are phenobarbital-induced alterations in the in vivo incorporation of radioactively labeled choline and the methyl group of L-methionine into the phosphatidylcholine of hepatic endoplasmic reticulum and serum.

METHODS

Materials

Choline-1,2-^{14}C bromide (1.03 mCi/mmole) and L-methionine-methyl-^{3}H (106.5 mCi/mmole) were obtained from Tracerlab, Waltham, Mass. Palmitic acid-1-^{14}C (10 mCi/mmole) and *S*-adenosyl-L-methionine-methyl-^{14}C (55 mCi/mmole) were obtained from New England Nuclear Corp., Boston, Mass. The purity of the palmitic acid-1-^{14}C was checked by thin-layer chromatography. The purity of L-methionine-methyl-^{3}H was checked by paper chromatography. *Clostridium perfringens* Type A toxin, *S*-adenosyl-L-methionine, and cytidine 5'-monophosphate were obtained from Sigma Chemical Co., St. Louis, Mo. Coenzyme A was obtained from C. F. Boehringer and Soehne, Mannheim, Germany. Reduced triphosphopyridine nucleotide was obtained from P–L Laboratories, Milwaukee, Wisc. Silica Gel G and Silica Gel H were obtained from E. Merck A. G., Darmstadt, Germany. Reference lipids were obtained from Applied Science Laboratories Inc., State College, Pa.

Cytidine diphosphate choline-1,2-^{14}C and palmitoyl-1-^{14}C CoA were chemically synthesized as previously described (7). Diglyceride was prepared from egg lecithin as previously described (7). All organic solvents were freshly distilled using glass stills.

Animals

Weanling male rats of the Osborne–Mendel strain were selected from similar size litters (5–7 animals per litter).

All animals weighed 40 ± 5 g and were raised on standard lab chow. The animals were housed in individual cages with controlled temperature and humidity. When 60 days old and weighing 140–170 g, animals with similar and normal growth curves were selected and paired for weight (within 2 g). One rat of each pair received intraperitoneal injections of phenobarbital in saline, 70 mg/kg. Paired controls received injections of an equal volume of saline. Injections were given once daily in the morning for three consecutive days and on the third day a fourth injection was given in the evening, 12 hr prior to the killing of the animals.

Dietary Management

In order to assure equal food intake and to negate the possible effect of altered feeding habits due to the sedative effects of phenobarbital, food was removed from the cages of all rats after the morning injections and withheld during periods of phenobarbital sedation. Food was returned to both phenobarbital-injected animals and paired controls in the evening during the periods of maximal food ingestion. Following the phenobarbital injection on the evening prior to killing, food was withheld from both groups. All animals had free access to water at all times. Weight gain was identical in experimental and paired control animals (5 g per day).

Collection and Handling of Samples

On the morning of the fourth day all animals were injected intraperitoneally with choline-1,2-^{14}C chloride, 30 µCi (30.9 µmoles), and L-methionine-methyl-^{3}H, 100 µCi (0.94 µmoles). At the proper time interval after injection of labeled substrates the animals were anesthetized with ether and exsanguinated via aortic puncture. The livers were then immediately excised, weighed, and placed in ice-cold 0.25 M sucrose containing 0.001 M EDTA. Livers were homogenized and microsomes were isolated by differential centrifugation as previously described (7). In one experiment two groups of paired phenobarbital-treated and control animals were killed at 30, 60, and 120 min following injection of labeled substrates. Incorporation of labeled substrates into microsomal phosphatidylcholine (per mg of microsomal protein and per total microsomal protein) was determined. Thus, time courses of relative rates of incorporation of the two substrates were established. In a second group of experiments seven pairs of experimental and control animals were killed at 90 min following injection of labeled substrates, a time interval previously determined to reflect established, maximum incorporation of both labeled substrates. In this experiment all animals were killed within a 90-min period to avoid diurnal variations in lipid metabolism (8).

Analytical Procedures

Microsomal protein was determined on fresh microsomal preparations by the method of Lowry, Rosebrough, Farr, and Randall (9). Microsomal and serum total phospholipids were isolated by ethanol extraction using the method previously described for phospholipid isolation from enzyme assays (7). For this extraction procedure small aliquots (0.25 ml) of microsomal preparations (2.5–30 mg of protein per ml) and 0.25 ml serum were utilized. Four separate 3.0-ml portions of 95% ethanol were utilized for extraction. The protein precipitate was macerated with 80–100 mesh glass beads prior to each extraction. The completeness of extraction was confirmed by the absence of significant radioactivity remaining in the protein precipitate following the last extraction of microsomal and serum samples previously labeled in vivo with choline-1,2-^{14}C and linoleic acid-1-^{14}C. Additionally, extraction of the protein precipitate with chloroform–methanol 2:1 (v/v) by the method of Folch, Lees, and Sloane Stanley (10) yielded no additional lipid phosphorus. Liver triglyceride levels were measured by the method of Stern and Shapiro (11). Serum triglyceride was determined by the method of Kessler and Lederer (12). Histologic sections stained with Oil Red O were prepared from a representative portion of liver from both phenobarbital-treated and control animals.

For determination of radioactivity of individual phospholipids, aliquots of the total phospholipid extracts were chromatographed on Silica Gel G thin-layer plates as previously described (7), and the areas containing lyso-phosphatidylcholine, sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine were scraped off and counted in a Packard Tri-Carb scintillation counter (13) using toluene–Cab-O-Sil solvent with 2,5-diphenyloxazole and 2,2-p-phenylenebis (5-phenyloxazole) as scintillators. 97% of total phospholipid counts were always found in the phosphatidylcholine fraction. The remaining counts were present largely in the lysophosphatidylcholine fraction.

Microsomal and serum total phospholipid phosphorus was determined by the method of Bartlett (14). Following the isolation of microsomal and serum phosphatidylcholine by thin-layer chromatography on washed Silica Gel H, phosphatidylcholine phosphorus was determined by the method of Parker and Peterson (15).

Enzyme Assays

Phosphorylcholine-glyceride transferase was assayed as previously described (7) using enzyme-saturating concentrations of both CDP-choline-1,2-^{14}C and D-1,2-diglyceride. Diglyceride acyltransferase activity was assayed as previously described (7) using palmitoyl-1-^{14}C CoA and D-1,2-diglyceride as substrates in enzyme-saturating concentrations.

SAME:PE methyltransferase activity was assayed by a modification of the method of Bremer and Greenberg (16). The complete reaction mixture contained the following components in a total volume of 1.0 ml: Tris–HCl, pH 9.0, 50 μmoles, S-adenosyl-L-methionine-methyl-^{14}C (specific activity 6.37 μCi/mole), 0.068 μmole, and 1.0 mg of microsomal protein. After a 20-min incubation at 37°C the reaction was terminated by the addition of 3 ml of cold 95% ethanol. The phosphatidylcholine was extracted from the reaction mixture and was isolated by thin-layer chromatography by the method used for phosphatidylcholine isolation from enzyme assays as previously described (7). As shown in Fig. 1, activity of SAME:PE methyltransferase was linear over a 40-min incubation period. When incubated at 37°C for 20 min, enzyme activity was proportional to the concentration of microsomal protein over a rather wide range (Fig. 2).

Oxidative demethylation of aminopyrine was measured by the method of LaDu, Gaudette, Trousof, and Brodie (17) as modified by Orrenius (18), in which the production of formaldehyde is measured (19, 20). Exogenous TPNH was used in saturating amounts in place of a TPNH-generating system. When assaying phosphorylcholine-glyceride transferase, SAME:PE methyltransferase, and diglyceride acyltransferase, aliquots of each enzyme incubation mixture were removed at zero time in order to determine preexisting radioactivity in phosphatidylcholine and triglyceride. Enzyme specific activities of phosphorylcholine-glyceride transferase and SAME:PE methyltransferase are thus calculated on the basis of counts per min in phosphatidylcholine at 10 min, minus counts per mg of microsomal protein. In control incubations to which no radioactive substrate was added, the content of labeled phosphatidylcholine did not change significantly during the incubation period. No significant radioactivity from the previously injected choline-1,2-^{14}C or methionine-methyl-^{3}H was found in triglyceride or diglyceride at zero time so that specific activity of diglyceride acyltransferase is expressed as counts per min per mg of protein.

RESULTS

Effect of Phenobarbital Administration on Microsomal Protein and Phospholipid Levels

The effects of the 72-hr period of phenobarbital administration on hepatic weight, hepatic microsomal protein, and phospholipid levels are shown in Table 1. Liver weight, total liver microsomal protein, and total liver microsomal phospholipid were significantly elevated in the phenobarbital-treated animals. The incorporation of controlled feeding periods for both phenobarbital-injected and control animals (see Methods) and the similar rates of weight gain in both groups make it un-
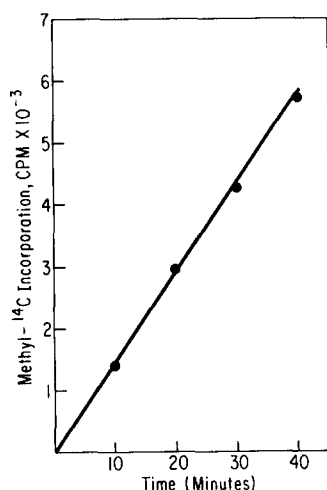
Fig. 1. Effect of variation in length of incubation on incorporation of methyl-^{14}C of S-adenosyl-L-methionine-methyl-^{14}C into phosphatidylcholine. The incubation mixture is as described under "Enzyme Assays" except for variations in the duration of incubation as indicated. The incubation was at 37°C. 1 mg of microsomal protein was added.
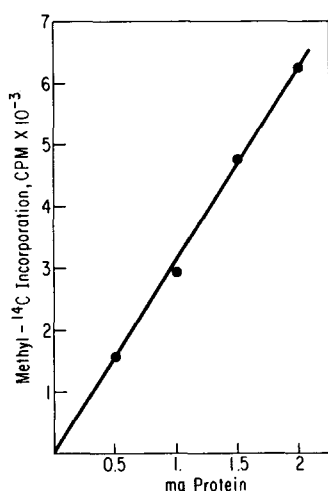

Fig. 2. Dependence of methyl-^{14}C from S-adenosyl-L-methionine-methyl-^{14}C incorporation into phosphatidylcholine on added microsomal protein. The incorporation mixture is as described under "Enzyme Assays" except for the variation of microsomal protein as indicated. The incubation was at 37°C for 20 min.

likely that differing caloric intake is responsible for the changes in microsomal phosphatidylcholine and protein levels.

Total microsomal phospholipid is elevated to a greater degree than microsomal protein. As indicated in Table 1, the increase in microsomal phosphatidylcholine content accounts for the major portion of the total phospholipid increase, resulting in a significant increase in the microsomal phosphatidylcholine to protein ratio.

Enzyme Activities

The effect of administration of phenobarbital over a 72-hr period on the enzyme catalyzing the oxidative de-

TABLE 1 EFFECT OF 72-HR PRETREATMENT WITH PHENOBARBITAL

	Control*	PB*	Fold In-crease
Liver weight (g)†	4.9	6.9‡	1.41
Total microsomal protein (mg)	97.8	152.1‡	1.56
Total microsomal phospholipid P $(\mu moles)$	28.3	58.0§	2.05
Total microsomal phosphatidyl-choline P $(\mu moles)$	11.4	30.8§	2.70
Microsomal phospholipid P $(\mu moles)$/Microsomal protein (mg)	0.289	0.381‖	1.32
Microsomal phosphatidylcholine P $(\mu moles)$/Microsomal protein (mg)	0.116	0.202‡	1.74

* Both control and phenobarbital-treated (PB) groups consisted of 7 paired animals. Mean values are shown.

† All animals were killed 72 hr after the first phenobarbital treatment.

‡ Significantly different from control values ($P < 0.01$).

§ Significantly different from control values ($P < 0.001$).

‖ Significantly different from control values ($P < 0.02$).

methylation of aminopyrine is seen in Table 2. In agreement with previous investigators (21), phenobarbital resulted in a greater than threefold increase in the specific activity of the aminopyrine demethylating enzyme.

A significant increase in the specific activity of SAME: PE methyltransferase was seen in phenobarbital-treated animals when compared with the controls. This increased enzyme specific activity corresponds in magnitude to the increased in vivo incorporation of methyl-^{3}H from L-methionine-methyl-^{3}H to be noted below. Previous work by Bremer and Greenberg (16) demonstrated that the addition of phosphatidylethanolamine to the incubation mixture caused no increased incorporation of methyl groups into phosphatidylcholine. Addition of mono- and dimethylated phosphatidylethanolamine did, however, increase the rate of incorporation. In the present study, microsomal concentrations of these individual methyl acceptors were not determined, so that an increased synthesis of only one of these methyl acceptors cannot be distinguished from an increase in the activity of the entire pathway. The specific activity of phosphorylcholine-glyceride transferase in control and phenobarbital-treated animals was not different. There was an increase in the specific activity of diglyceride acyltransferase comparable in magnitude to that noted in the enzyme catalyzing aminopyrine demethylation.

In Vivo Incorporation of Choline-1,2-^{14}C and the Methyl-^{3}H of L-Methionine

The time course of incorporation of intraperitoneally administered choline-1,2-^{14}C and L-methionine-methyl-^{3}H revealed that between 60 and 120 min incorporation

TABLE 2 SPECIFIC ACTIVITY OF HEPATIC MICROSOMAL ENZYMES AFTER 72-HR PRETREATMENT WITH PHENOBARBITAL

Enzyme	Control	Phenobarbital
Demethylation of amino-pyrine		
Specific activity*	3.9	12.2§
Total activity*	381.4	1,855.6‡
SAME:PE methyltransferase		
Specific activity†	3602	4930‖
Total activity†	352.2	749.8§
Phosphorylcholine-glyceride transferase		
Specific activity‡	12.65	12.55**
Total activity‡	123.7	190.8¶
Diglyceride acyltransferase		
Specific activity‡	7.2	22.0§
Total activity‡	70.4	334.6§

Both control and phenobarbital-treated groups consisted of 7 paired animals. All values represent means.

* Specific activity expressed as nmoles formaldehyde per min per mg of protein; total activity expressed as nmoles formaldehyde per min per total mg of microsomal protein.

† Specific activity expressed as cpm of labeled substrate incorporated per 20 min per mg of protein; total activity expressed as cpm $\times$ 10^{-3} of labeled substrate incorporated per 20 min per total mg of microsomal protein.

‡ Specific activity expressed as nmoles of labeled substrate incorporated per min per mg of protein; total activity expressed as nmoles of labeled substrate incorporated per min per total mg of microsomal protein.

§ Significantly different from control values ($P < 0.001$).

‖ Significantly different from control values ($P < 0.01$).

¶ Significantly different from control values ($P < 0.02$).

**Not significantly different from control values.

of the substrates was at maximum in both phenobarbital-pretreated animals and paired controls. This time course of incorporation is in agreement with previous studies by Hallinan, Duffy, Waddington, and Munroe (22), and Bjørnstad and Bremer (23).

In the second group of experiments, animals were killed 90 min after the injection of choline-1,2-^{14}C and L-methionine-methyl-^{3}H. Methyl-^{3}H incorporation into microsomal phosphatidylcholine per mg of microsomal protein was increased in the phenobarbital-treated rats when compared with the paired control animals, the mean values being 2,970 cpm/mg of protein and 2,234 cpm/mg of protein, respectively ($P < 0.05$). When expressed in terms of total liver microsomal protein there is a 2.05-fold increase in the incorporation of methyl-^{3}H into the phosphatidylcholine fraction from the phenobarbital-treated animals when compared with control animals ($P < 0.01$). Choline-1,2-^{14}C incorporation into microsomal phosphatidylcholine was slightly decreased in the phenobarbital-treated animals when compared with controls, the mean values being 2,320 cpm/mg of protein and 5,670 cpm/mg of protein, respectively ($P < 0.05$). The ratio of methyl-^{3}H to choline-1,2-^{14}C incorporation into microsomal phosphatidylcholine per mg

of microsomal protein was 0.39 in control animals and 1.28 in the phenobarbital-pretreated animals, a more than threefold increase ($P < 0.001$).

Serum Phospholipid

In vivo incorporation of choline-1,2-^{14}C and methyl-^{3}H from L-methionine-methyl-^{3}H into the serum phospholipid differed considerably from animal to animal when expressed in terms of counts per ml of serum, and no significant differences between the phenobarbital-treated and control animals were noted. However, when the relative incorporation of methyl-^{3}H from L-methionine-methyl-^{3}H and choline-1,2-^{14}C into the serum phospholipid of each animal was determined, a remarkably constant ratio was found. Of significance was a constant increase in the ^{3}H/^{14}C ratio observed for the phenobarbital-treated animals when compared with the controls. The mean ratio for control animals was 0.68 (SD $\pm$ 0.2) while that for the phenobarbital-treated animals was 1.81 (SD $\pm$ 0.30). This change in the ^{3}H/^{14}C ratio corresponds in both direction and magnitude to the change in the ^{3}H/^{14}C ratio noted for the incorporation of the labeled substrates into hepatic microsomal phosphatidylcholine. There was no significant difference noted in total phospholipid phosphorus per ml of serum between phenobarbital-treated and paired controls.

Histologic sections of liver stained with Oil Red O and determination of total hepatic triglyceride revealed no increase in the level of hepatic triglyceride following phenobarbital administration. There was no significant difference in serum triglyceride level noted between the two types of animals.

DISCUSSION

The present studies indicate that administration of phenobarbital to rats has a significant effect on hepatic biosynthetic pathways of certain complex lipids. This effect occurs in association with proliferation of membranes known to be the site of enzymes which catalyze a number of TPNH-dependent hydroxylation reactions, among them the oxidative demethylation of various drugs (21). It is likewise well established that these membranes contain enzymes which govern de novo biosynthesis of complex lipids (24) and are active in the synthesis of the components of lipoproteins. The lipid portion of these membranes, which are lipoprotein in nature, consists in large part of phospholipid. Accumulation of hepatic phospholipid, in contrast to triglyceride, has not been noted to any significant extent in conditions associated with altered rates of hepatic lipoprotein re-

lease (25). The induced proliferation of the endoplasmic reticulum by phenobarbital is characterized, however, by an increase in both phospholipid and protein content.

The present study demonstrates that there is a 1.56-fold increase in microsomal protein following 72 hr of phenobarbital administration (Table 1). Arias, Doyle, and Schimke (26) have also obtained evidence of a phenobarbital-induced increase in microsomal protein comparable to that indicated in the present study. Their studies in which a nonreutilizable isotope was used showed that following phenobarbital administration there is no change in turnover rate of the membrane protein. Additionally it has been demonstrated that the increase in microsomal protein which occurs following pheno-barbital administration is associated with increased amino acid incorporation into microsomal preparations (27). These data indicate that the noted increase in microsomal protein following phenobarbital administration is due primarily to increased protein synthesis and suggest that altered degradation of the membrane protein is not a significant factor.

Of interest in the consideration of complex lipid synthesis is the concomitant increase in phosphatidylcholine content of the endoplasmic reticulum following pheno-barbital pretreatment. Although other phospholipids increase to a minor extent, the major increase in microsomal phospholipid is due to phosphatidylcholine. Orrenius (3) has also presented additional evidence of the preferential increase in phosphatidylcholine, with only minor elevation of phosphatidylethanolamine, phosphatidylinositol, and sphingomyelin, following the administration of phenobarbital to rats.

As indicated in Table 1, there is a 1.74-fold increase in the ratio of microsomal phosphatidylcholine to protein following phenobarbital administration. Previous investigations by Orrenius, Ericsson, and Ernster (21) have indicated that a twofold increase in ^{32}P incorporation into phospholipids occurs as early as 3 hr after the initial phenobarbital injection, well before a demonstrable increase in either oxidative demethylation activity or microsomal protein content. At 12 hr they noted that the incorporation into mitochondrial phospholipid was only slightly increased. They state that one could not exclude that the increase, at least in part, resulted from contamination of the mitochondrial fraction with microsomes. These differing rates and magnitudes of increased synthesis of phospholipids and protein do not by themselves establish that the newly synthesized enzymes merely require new supporting membranes. Though drug-metabolizing enzymes are rapidly induced in response to administration of specific substrates, it is well recognized that activities of enzyme systems apparently unrelated to the inducing substrate are increased as well (28). This appears to be the case with certain enzymes involved in porphyrin and bilirubin metabolism (5, 29, 30). Such nonspecific induction may account in part for the noted increase in phospholipid and protein of the endoplasmic reticulum. It is also possible that the primarily-induced enzyme systems require increased amounts of specific substances as cofactors for activity. A notable example of this type of requirement is that of rat liver D-β-hydroxybutyrate dehydrogenase which has a specific requirement for phosphatidylcholine (31). Though the drug-metabolizing enzymes are localized to the endoplasmic reticulum, no such dependence on phosphatidylcholine has yet been demonstrated.

Following the intraperitoneal administration of the two labeled precursors, each being incorporated into hepatic phosphatidylcholine by different biosynthetic enzyme pathways, significant differences in the incorporation rates are seen in rats pretreated with phenobarbital when compared with controls. The increased in vivo incorporation of methyl-^{3}H from L-methionine-methyl-^{3}H suggests a phenobarbital-induced increase in the activity of the phosphatidylcholine synthetic pathway involving the sequential methylation of phosphatidylethanolamine. There is a moderate decrease, however, in the rate of incorporation of choline-1,2-^{14}C in phosphatidylcholine under the conditions used.

In vitro assays of the enzymes responsible for synthesis of phosphatidylcholine by the two pathways are in agreement with the in vivo incorporation studies. As shown in Table 2, there is a significant increase in the specific activity of SAME:PE methyltransferase while specific activity of phosphorylcholine-glyceride transferase remains unchanged. Thus, data from both in vivo incorporation studies and measurements of specific activity of the involved enzymes are consistent with a conclusion that the increased content of phosphatidylcholine noted in the proliferated endoplasmic reticulum is in part the result of an induced increase in the activity of SAME:PE methyltransferase.

In agreement with this finding, Orrenius (3) indicates that there is an increase in the specific activity of ^{32}P in microsomal phosphatidylcholine in phenobarbital-pre-treated rats. The phosphorus of lecithin derives from ATP and thus this increased incorporation of ^{32}P in the phosphatidylcholine might be due to several mechanisms. These include an increased activity of enzymes involved in synthesis of the phosphorus-containing precursors of phosphatidylcholine, namely phosphorylcholine, CDP-choline, phosphorylethanolamine, CDP-ethanolamine and phosphatidylethanolamine, as well as an increased specific activity of the enzymes which synthesize phosphatidylcholine directly, i.e., phosphatidylcholine-glyceride transferase and SAME:PE methyltransferase. The present study reveals no change in phosphatidyl-

choline-glyceride transferase but an increase in the activity of SAME:PE methyltransferase, indicating that this increase in the rate of de novo lecithin synthesis is at least one of the mechanisms by which increased phosphorus specific activity would be noted.

In contrast to these findings, Holtzman and Gillette (32) reported that there was no significant increase in ^{32}P incorporation into microsomal total phospholipid following phenobarbital administration to fasted female rats. In fasted male rats they noted a transient 41% increase in incorporation at 18 hr. They concluded that the major cause for phospholipid increase following phenobarbital induction of endoplasmic reticulum was decreased degradation of the membrane phospholipids. Though in the present study choline and the methyl group of methionine were used as precursors of phosphatidylcholine, Orrenius (3), as previously noted, indicates that there is an increase in the incorporation of ^{32}P into phosphatidylcholine in phenobarbital-pretreated rats. The cause for the discrepancy between the present work and that of Holtzman and Gillette is not apparent; however, differences in experimental design may explain this in part, in that the experimental animals used by Holtzman and Gillette (32) were fasted throughout the entire 72-hr period. Both phenobarbital-treated and paired control animals used in the present study ate food at predetermined and regular intervals and gained weight equally.

In the present study incorporation ratios of methyl-^{3}H to choline-1,2-^{14}C in phosphatidylcholine of the endoplasmic reticulum are significantly increased in the phenobarbital-treated animals, being more than three times that found in the paired control animals. A comparable increase in the methyl-^{3}H to choline-1,2-^{14}C incorporation ratio was noted in serum phospholipid. As reported by Glaumann and Dallner (33), when microsomal fractions are subjected to washing procedures utilizing Tris buffer, KCl, and distilled water, there is removal of up to 45% of the protein without significant loss of the phospholipid or triglyceride content of the microsomes. Treatment with deoxycholate (0.25%), which is known to solubilize a part of the microsomal membrane, however, results in the removal of 30–40% of both phospholipid and triglyceride from the membranes. These more easily removed proteins and lipoproteins are presumed to be in part the contents of microsomal vesicles and destined for export. In the present report, data referring to in vivo incorporation of precursors into phosphatidylcholine therefore reflects to some extent microsomal vesicle contents as well as membranes. The similarity of the in vivo incorporation ratios of methyl-^{3}H to choline-1,2-^{14}C noted in microsomes and serum, therefore, likely reflects the microsomal synthetic site of exported serum phosphatidylcholine. These findings indicate that following the administration of phenobarbital the enzymatic biosynthesis of phosphatidylcholine destined for incorporation into newly formed endoplasmic reticulum membranes and serum lipoproteins occurs to a significant extent by the sequential methylation of phosphatidylethanolamine.

As seen in Table 2, phosphorylcholine-glyceride transferase specific activity was not altered by phenobarbital pretreatment. The enzymes phosphorylcholine-glyceride transferase, diglyceride acyltransferase, and phosphorylethanolamine-glyceride transferase utilize D-1,2-diglyceride as a common substrate. SAME:PE methyltransferase, utilizing phosphatidylethanolamine as substrate, is indirectly dependent on the D-1,2-diglyceride pool as well. As previously indicated (7), an increase in the specific activity of one of these enzymes without alteration of the other might be expected to divert increasing amounts of the common substrate D-1,2-diglyceride along the induced pathway, resulting in decreased product formation from the other. The increase in the specific activity of SAME:PE methyltransferase and diglyceride acyltransferase noted in the present experiments might therefore be expected to result in a decrease in de novo phosphatidylcholine synthesis by phosphorylcholine-glyceride transferase despite the absence of significant alteration in the specific activity of the latter enzyme. The results of the in vitro incorporation studies, which indicated a moderate decrease in choline-1,2-^{14}C incorporation into phosphatidylcholine, would be consistent with this hypothesis.

In the face of increased diglyceride acyltransferase specific activity, no significant alteration in either hepatic or serum triglyceride levels was noted. A possible explanation might be a concomitant increased release of triglyceride from the liver and increased degradation and reutilization of its fatty acid extrahepatically or within the liver. The enzyme specific activity and hepatic and serum triglyceride levels, however, by no means confirm such a possibility.

The in vitro enzyme assays are designed only to evaluate the direction and magnitude of changes in enzyme specific activity. They are not intended to indicate maximal synthetic rates which might be found in vivo. These enzyme studies, in conjunction with the data from the in vivo incorporation studies, do, however, suggest that phenobarbital pretreatment clearly alters biosynthesis of some complex lipids and that in association with proliferation of the endoplasmic reticulum, there is an increase in phosphatidylcholine synthesis by the sequential methylation of phosphatidylethanolamine.

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