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# Pyrimidine Nucleotide Synthesis and Degradation in Rat Liver after Repeated Phenobarbital Administration

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SUMMARY

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The enzyme systems associated with the synthesis and degradation of pyrimidine nucleotides in rat liver after the repeated administration of phenobarbital were studied. The incorporation of [2-$^{14}$C]orotic acid into uridine components of the free nucleotide pool remains unchanged, whereas incorporation into cytidine components is decreased. The cytidine triphosphate synthetase, UTPase, and CTPase activities of cytosol are increased. The activities of 5′-nucleotidases of the cytosol and microsomal fraction as well as UTPase and CTPase of the microsomal fraction are decreased. The activities of nucleoside, nucleoside monophosphate, and diphosphate kinases of uridine and cytidine components and the activity of cytosol uridine phosphorylase are not affected. Deamination of cytidine in the presence of the whole homogenate, the microsomal fraction, and the cytosol is not altered. Finally, the ATPase activity of the cytosol is not affected, while that of the microsomal fraction is decreased.

## INTRODUCTION

Study of biochemical changes accompanying the induction of drug-metabolizing microsomal enzymes (1, 2) and hypertrophic changes in the liver following administration of phenobarbital (3–5) showed that the compound affected the synthesis of proteins (6, 7), nucleic acids (8–10), and phospholipids (11). Although some enzyme systems are activated, other enzyme activities are decreased, both in the cytosol and in the microsomal fraction of liver cells (12–15). Different enzyme systems are activated only directly after initial exposure to the drug and become less active during the course of repeated administration. This diminishing response is apparent even during the synthesis of liver RNA. A single administration of phenobarbital stimulates the incorporation of labeled orotic acid into cytoplasmic ribosomal RNA (8, 9, 16, 17), while repeated administration of the drug decreases the utilization of labeled precursor for rRNA synthesis (16, 17). In young rats administration of phenobarbital increases the RNA: DNA ratio in hepatocyte nuclei as well as in whole liver (18), indicating that the drug affects RNA synthesis and liver growth in young, rapidly growing animals to a different extent (19) than in adults, where liver proliferation, as well as over-all growth, proceeds more slowly (20).

The structural similarity between barbituric acid derivatives and the pyrimidine bases suggested that the barbiturates might interfere with the metabolism of pyrimidine nucleotides as well as with nucleic acids. The presence of barbiturates in the culture

medium depresses the penetration of orotic acid across the bacterial membrane (21, 22). In liver tissue slices some inhibitory effects of barbiturates on the synthesis of pyrimidine nucleotides *de novo* were described (23), and inhibition of dihydroorotate dehydrogenase was observed in the presence of barbituric acid even in human cell tissue cultures (24, 25). Inhibition of growth, protein synthesis (26), and nucleic acid synthesis was observed in a number of mammalian cell cultures exposed to barbiturates (27, 28).

The earlier observation that the administration of phenobarbital decreases the utilization of labeled orotic acid for the synthesis of cytidine nucleotides of rRNA as well as tRNA (16) led us to study the effect of this compound on enzyme systems involved in the synthesis and degradation of pyrimidine nucleotides in rat liver.

## METHODS

*Animals.* Male rats (Wistar, Lysolaje strain, weighing 150 g at the beginning of the experiment) were given phenobarbital (sodium salt, Merck) in drinking water (1 g/liter). The animals were fasted for 12 hr prior to death. Each experimental group included five rats.

*Chemicals.* [2-$^{14}$C]Orotic acid (specific activity, 47 mCi/mmole), [2-$^{14}$C]uridine (specific activity, 53 mCi/mmole), [U-$^{14}$C]uridine 5′-phosphate (specific activity, 300 mCi/mmole), and [U-$^{14}$C]cytidine (specific activity, 222 mCi/mmole) were obtained from the Institute for Research, Production and Uses of Radioisotopes in Prague. Labeled cytidine 5′-triphosphate was prepared enzymatically by the kinase reaction from [U-$^{14}$C]cytidine.

The rats were decapitated, and their livers were excised, weighed, and homogenized with 6 volumes of ice-cold buffered sucrose medium (29). The microsomal fraction and cytosol were isolated as described earlier (30). The microsomal fraction was suspended in buffered sucrose medium, centrifuged at 150,000 × *g* for 30 min, and resuspended in the same buffer for estimation of enzyme activities.

*Determination of total amounts and specific activities of pyrimidine nucleotide components of free nucleotide pool.* The isolation and hydrolysis of the free nucleotides were described earlier (16). Aliquots of the hydrolysate were chromatographed using Whatman No. 3 paper. Uracil and cytosine were separated in system C (see below). Following their elution with water and subsequent evaporation, both substances were rechromatographed in system B (see below). To calculate the concentrations of the pyrimidine bases, aliquots of the eluates were titrated with hydrochloric acid to 0.01 M and measured in an ultraviolet spectrophotometer. To estimate the amount of uracil, a micromolar extinction coefficient of 8.2 was used at 259 nm, and for cytosine a value of 10.2 was used at 274 nm. The radioactivity of the neutral aliquot was estimated using a dioxane scintillator (4 g of 2,5-diphenyloxazole, 100 mg of *p*-bis[2-(5-phenyl-(oxazolyl)]benzene, and 125 g of naphthalene per liter of dioxane). The specific activity is expressed as counts per minute per micromole of pyrimidine base.

*Enzyme assays.* The protein concentration of the cytosol, unless stated otherwise, was 15–20 mg/ml, and that of the microsomal fraction was 10–15 mg/ml. Reaction mixtures were incubated at 37° in a total volume of 0.5 ml. The reaction was terminated (see below), and the precipitated protein was separated by centrifugation. Aliquots of the supernatant fractions were applied to Whatman No. 3 chromatographic paper. The absorbing zones were detected under ultraviolet light. The following solvent systems were employed for paper chromatography: A, isobutyric acid–water–concentrated ammonium hydroxide (66:33:1.5); B, 1-butanol–acetic acid–water (10:2:10); C, 2-propanol–HCl–water (170:41:39). For the estimation of enzyme activities blank values were subtracted from the radioactivity or absorbance readings. Radioactivity was estimated with a Packard spectrometer.

*Determination of activity of cytidine triphosphate synthetase [EC 6.3.4.2, UTP-ammonia ligase (ADP)].* The method of Chelbova *et al.* (31) was used with the following modifications. The incubation medium contained 400,000 cpm of [U-$^{14}$C]uridine 5′-phosphate, and 0.2-ml samples of the substrate mixture were combined with 0.3 ml of liver cytosol,

containing 35–45 mg of protein per milliliter. The incubation was terminated after 20 min by the addition of 0.5 ml of 2 N HCl. After separation of precipitated proteins, the supernatant fraction was hydrolyzed in sealed ampoules at 100° for 15 min. Then 40 µl of the hydrolyzed reaction mixtures, together with nonlabeled pyrimidine nucleoside monophosphates, nucleosides, and bases, were placed on chromatographic paper and separated in solvent A. The individual components, corresponding to positions of standards on the chromatogram, were cut out and their radioactivity was determined by liquid scintillation counting in 4 g of 2,5-diphenyloxazole and 100 mg of *p*-bis[2-(5-phenyloxazolyl)]benzene per liter of toluene. The specific activities of cytidine triphosphate synthetase were calculated as percentages of the radioactivity of cytidylic acid and cytidine referred to the total radioactivity of uridylic acid. The specific activity of the enzyme is expressed as nanomoles of cytidine nucleotides per milligram of protein per hour.

*Determination of ATPase (EC 3.6.1.3, ATP phosphohydrolase), UTPase, CTPase, and 5′-nucleotidase (EC 3.1.3.5, 5′-ribonucleotide phosphohydrolase) in microsomal fraction and cytosol.* The incubation mixture contained 50 mM Tris-HCl buffer (pH 7.7), 4.0 mM $MgCl_2$, and 4.0 mM substrate. First 0.3 ml of the substrate mixture was combined with 0.2 ml of microsomal fraction (or cytosol). Nucleoside triphosphatases and 5′-nucleotidases of the microsomal fraction were incubated 4 min, and those of the cytosol, for 20 min. The reaction was terminated by heating at 100° for 1 min. The enzyme activities of CTPase, UTPase and 5′-nucleotidase were determined isotopically by the addition of 80,000 cpm of labeled substrate to the reaction mixture. The individual components were separated in solvent A. ADP and AMP formed during estimation of ATPase activity were eluted with 0.01 N HCl, and absorbance was measured at 259 nm. The nucleoside triphosphatase activities were calculated from the total radioactivities (absorbance) of nucleoside diphosphates and monophosphates, and 5′-nucleotidase, from the total of nucleotides and bases. The specific activities are expressed as micromoles per milligram of protein per hour.

*Estimation of uridine (cytidine) kinase (EC 2.7.1.48, ATP: uridine 5′-phosphotransferase).* The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.7), 4.0 mM ATP, 4.0 mM $MgCl_2$, 0.4 mM uridine (or 0.2 mM cytidine), and 100,000 cpm of labeled uridine (or cytidine). The substrate mixture was combined with 0.1 ml of cytosol. The incubation was carried out for 6 or 15 min, using uridine or cytidine as substrate, respectively. The reaction was terminated by heating at 100° for 1 min. Aliquots of the supernatant fractions were chromatographed together with nonlabeled pyrimidine nucleosides, nucleoside phosphates, and polyphosphates, and separated in solvent A. The specific activities of uridine (cytidine) kinase are expressed in micromoles as the total of all phosphorylated derivatives of uridine (cytidine) per milligram of protein per hour.

*Estimation of uridine phosphorylase (EC 2.4.2.3, uridine:orthophosphate ribosyltransferase).* The reaction mixture contained 20 mM phosphate buffer (pH 7.0), 0.4 mM uridine, and 100,000 cpm of labeled uridine. The substrate mixture was combined with 0.1 ml of the cytosol. Incubation was carried out for 6 min and terminated by adding trichloracetic acid to a final concentration of 5%. The supernatant fraction was applied to chromatographic paper together with nonlabeled uridine and uracil and separated in solvent B. The specific activity of the enzyme is expressed as micromoles of uracil formed per milligram of protein per hour.

*Estimation of cytidine deaminase (EC 3.5.4.5, cytidine aminohydrolase).* The incubation mixture contained 50 mM Tris-HCl buffer (pH 7.7) and 0.02 mM cytidine (500,000 cpm). Substrate mixture (0.2 ml) was combined with 0.3 ml of the homogenate, microsomal fraction, or cytosol. Incubation was conducted for 3 hr. The reaction was terminated by adding trichloroacetic acid to a final concentration of 5%. An aliquot of the supernatant fraction was chromatographed together with pyrimidine nucleosides and bases in solvent A. The specific activity of cytidine deaminase is expressed as picomoles of uridine and uracil formed per milligram of protein per hour.

Protein was determined according to Lowry *et al.* (32), using bovine serum albumin as standard.

## RESULTS

The total amount of uridine bases isolated from the nucleotide pool and expressed per unit weight of tissue remained unaffected after repeated phenobarbital administration. The concentration of cytidine bases was slightly lower, but the difference was not significant. Incorporation of labeled orotic acid into the pool of uridine nucleotides was not affected by repeated administration of the drug; however, the specific activity of the cytidine components was lower in the experimental group (Fig. 1).

Repeated administration of phenobarbital increased the cytidine triphosphate synthetase activity of the liver cytosol, beginning on the second day of administration (Fig. 2). The amination proceeded linearly for 40 min in the presence of both cytosols under comparison. An increase in activity of cytidine triphosphate synthetase in the experimental group was seen after the first minute of incubation. No differences were observed when labeled UTP was used as substrate.

Under the condition used for estimating cytidine triphosphate synthetase activity

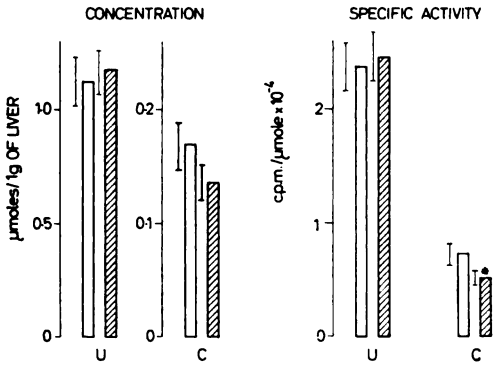


FIG. 1. *Concentrations and specific activities of uridine and cytidine components of acid-soluble pool*

Unshaded columns, controls; hatched columns, 8 days of phenobarbital treatment. The rats were killed 20 hr after labeled orotic acid administration (15 μCi/kg of body weight). Significantly different results ($p < 0.05$) are denoted by asterisks. The brackets indicate the standard errors of six determinations.

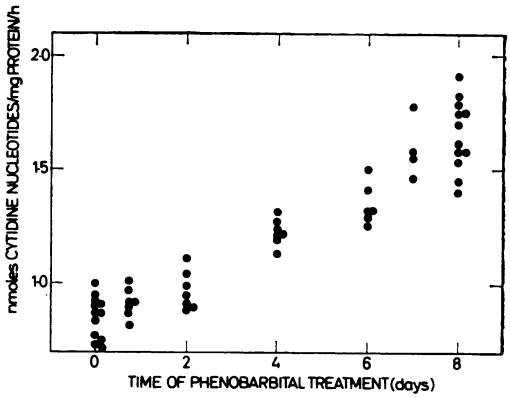


FIG. 2. *Cytidine triphosphate synthetase activity of cytosol*

Each point represents an individual cytosol isolation. The values are the mean of four determinations.

during a 2-min incubation, labeled 5′-UMP was phosphorylated more than 80% to UTP, and no significant differences in its concentration were seen in the two cytosols under comparison (Fig. 3). Similarly, no differences were observed in ATP concentration in the incubation medium.

The specific activities of cytidine triphosphate synthetase of the cytosol were not affected by a 3-hr dialysis. The increased enzyme activity was maintained in the cytosol of phenobarbital-treated animals (Table 1).

The activity of 5′-nucleotidase in the cytosol and microsomal fraction was decreased in the experimental group. The decrease was the same in the presence of 24 mM $MgCl_2$, and hence did not appear to be caused by changes in the activities of nonspecific phosphatase of the liver cytosol (34). On the other hand, the activities of CTPase and UTPase in the cytosol were increased after repeated administration of phenobarbital but were lowered in the microsomal fraction (Table 2).

The stepwise degradation of CTP by liver cytosol of phenobarbital-treated animals showed enhanced CTP dephosphorylation (higher CTPase activity) and 5′-CMP accumulation (lower 5′-nucleotidase activity) (Fig. 4).

The ATPase activity of the liver cytosol was affected only slightly during the course

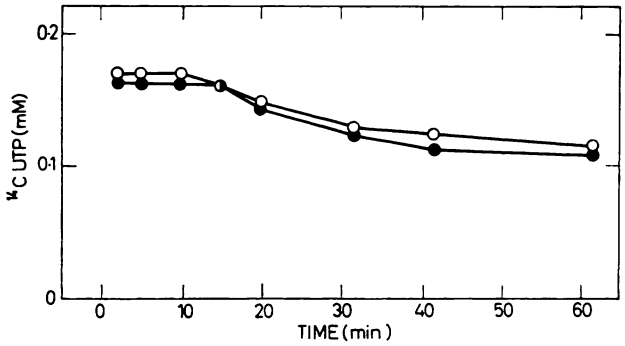


FIG. 3. [$^{14}C$]*UTP level in reaction mixture during determination of cytidine triphosphate synthetase activity*

The composition of the reaction mixture was the same as for the CTP synthetase activity determination, but without glutamine. O——O, controls; ●——●, after 8 days of phenobarbital treatment.

TABLE 1

*Cytidine triphosphate synthetase activity of non-dialyzed and dialyzed cytosol of rat liver*

The dialysis was performed according to Weinfeld and Sandberg (33). The dialyzed cytosol was adjusted to pH 7.3 with 0.2 M Tris, pH 9.0 The phenobarbital was given for 8 days. Values are the means and standard errors of six determinations.

| Rats | Cytosol | |
|---|---|---|
| | Nondialyzed | Dialyzed |
| | *nmoles/mg protein/hr* | |
| Controls | 0.78 ± 0.16 | 0.83 ± 0.14 |
| Phenobarbital-treated | 1.88 ± 0.28 | 1.95 ± 0.36 |

of phenobarbital administration, although it gradually decreased in the microsomal fraction (35) (Fig. 5).

The activity of uridine-cytidine kinase in the cytosol was not altered after repeated phenobarbital treatment, except on the first day after intraperitoneal administration. An analogous course was exhibited by the activities of uridine phosphorylase (Fig. 6). No differences were observed in the radioactivity of nucleoside diphosphates and triphosphates, indicating that no changes in the corresponding kinases were involved.

The activity of liver cytidine deaminase was extremely low. The amounts of uridine and uracil formed in cytosol are about 3 pmoles; in the microsomal fraction, 2 pmoles; and in the total homogenate, 4 pmoles/mg of protein per hour. After 8 days of phenobarbital administration no significant differences in deaminase activity were found in any of these fractions.

None of the enzymes examined was significantly affected by the presence of phenobarbital (sodium salt) at a concentration of 2.0 mM in the incubation medium.

## DISCUSSION

The repeated administration of phenobarbital decreases the incorporation of labeled orotic acid into cytidine nucleotides of rRNA and tRNA and into cytidine nucleotides of the free nucleotide pool (16). The specific radioactivities of uridine nucleotides of cytoplasmic RNAs (after correction for the increased ribosome content in the hypertrophic cells) and of the uridine components of the free nucleotide pool are not affected by repeated administration of phenobarbital. This indicates that the compound probably does not affect the enzyme system involved in the synthesis of pyrimidine nucleotides along the metabolic pathway from orotic acid onward. The preceding reactions are apparently also unaffected. If they were inhibited one would expect increased utilization of exogenous labeled orotic acid. The repeated administration of the drug does not affect the penetration of labeled orotic acid into liver cells, since neither the total radioactivity referred to unit tissue weight (16) nor the specific radioactivity of the free nucleotide pool is significantly altered (16, 17).

For a better understanding of the pheno-

TABLE 2

*Nucleoside triphosphatase and 5'-nucleotidase activities of rat liver microsomal fraction and cytosol*

The rats were given phenobarbital for 8 days. The values are the means and standard errors of eight determinations.

| Rats | UTPase | CTPase | 5'-UMPase | 5'-CMPase |
|---|---|---|---|---|
| | *μmoles nucleoside diphosphate/mg protein/hr* | | *μmoles nucleoside/mg protein/hr* | |
| Microsomal fraction | | | | |
| Controls | 2.3 ± 0.3 | 2.2 ± 0.3 | 3.2 ± 0.4 | 2.1 ± 0.3 |
| Phenobarbital-treated | 1.6 ± 0.2 | 1.4 ± 0.3 | 1.7 ± 0.3 | 1.2 ± 0.2 |
| % of controls | 69% | 63% | 53% | 57% |
| Cytosol | | | | |
| Controls | 0.18 ± 0.03 | 0.15 ± 0.03 | 0.31 ± 0.05 | 0.34 ± 0.04 |
| Phenobarbital-treated | 0.36 ± 0.05 | 0.27 ± 0.04 | 0.19 ± 0.02 | 0.20 ± 0.03 |
| % of controls | 200% | 180% | 61% | 59% |

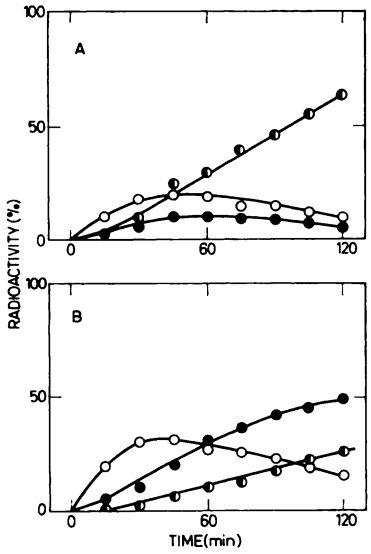


FIG. 4. *Cleavage of [U-$^{14}$C]cytidine triphosphate by cytosol*

A. Controls. B. After 8 days of phenobarbital treatment. The supernatant fractions are adjusted to the same protein concentration. O——O, CDP; ●——●, CMP; ◐——◐, cytidine.

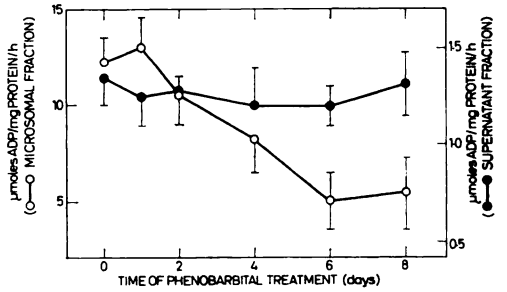


FIG. 5. *ATPase activities of liver microsomal fraction and cytosol*

O——O, microsomes; ●——●, cytosol. The brackets indicate the standard errors of eight determinations.

barbital effect on cytidine nucleotide synthesis in rat liver it is important to take into consideration the following observations. Labeled orotic acid is incorporated into the cytidine moiety of RNA only following a considerable lag (36, 37). The specific activities of uridylic acid and cytidylic acid of rRNA become equal about 10 days after the administration of labeled orotic acid, and the half-life of the cytosine moiety of rRNA is longer than the half-life of uridylic acid (38). The total content of the cytidine components of the free nucleotide pool is about 5–6 times lower than the content of uridine components (39).

The decrease in specific radioactivities of the cytidine moieties of rRNA, tRNA, and the pool after repeated administration of phenobarbital (16) cannot be correlated with the activities of cytidine triphosphate synthetase of the liver cytosol determined *in vitro*. The activities of the enzyme are apparently increased, depending on the time of administration of the drug. This rise of activity is not due to secondary changes of the metabolism of substrates or cofactors of the reaction or to the presence of a low molecular weight activator.

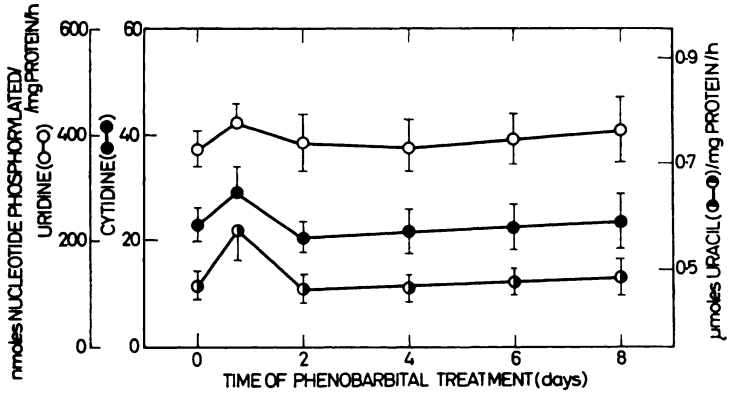


FIG. 6. *Uridine (cytidine) kinase and uridine phosphorylase activities of cytosol* O——O, uridine kinase, ●——●, cytidine kinase; ◑——◑, uridine phosphorylase. The brackets indicate the standard errors of eight determinations.

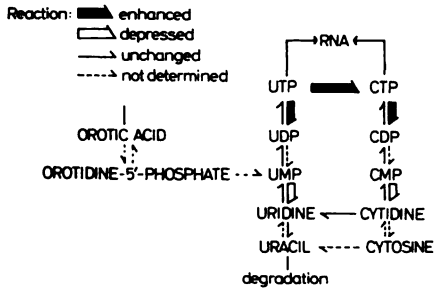


FIG. 7. *Metabolic alterations in pyrimidine synthesis and degradation of liver cytosol after repeated phenobarbital administration*

The properties of the cytidine triphosphate synthetase of mammalian cells and the factors participating in the control of its activity are not known in detail, since the enzyme has not been isolated or partially purified. In the cytosol of liver cells of adult rats, cytidine triphosphate synthetase has a low activity of about 1 nmole of CTP per milligram of protein per hour (40). This fact, together with the low concentration of cytidine components of the free nucleotide pool (39), indicates that the synthesis of cytidine nucleotides is rate-limiting and may represent an important factor in the regulatory mechanism of nucleic acid synthesis and cell proliferation (41, 42). Increased activities of cytidine triphosphate synthetase have been described for tissues with increased proliferative activity (40). Enhanced amination of uridine nucleotides was observed in liver after a single dose of colchicine. In contrast to phenobarbital, it was associated with increased utilization of labeled orotic acid for the synthesis of cytidine RNA nucleotides, while the specific activities of the uridine nucleotides were only slightly affected (33).

The repeated administration of phenobarbital increases the enzyme activities leading to breakdown of CTP and UTP, while the dephosphorylation of ATP in the cytosol is not markedly changed. The activation of CTP synthesis, together with its increased degradation, indicates that administration of the drug may interfere with control mechanisms regulating the steady state of pyrimidine nucleoside phosphates in the cell. On the contrary, the activity of microsomal pyrimidine nucleoside triphosphatases is depressed. The prolonged administration of phenobarbital simultaneously decreases the activities of the enzymes that cleave 5'-CMP and 5'-UMP both in the cytosol and in the microsomal fraction of liver. The decrease in these activities in the liver is inversely related to the growth cycle and can represent one of the factors regulating the synthesis of pyrimidine nucleotides and nucleic acids (43).

The synthesis of pyrimidine nucleotides in the cells is controlled by a number of feedback inhibitions (44–47). Differences in enzyme activities degrading the pyrimidine precursors of nucleic acids suggest that decreased utilization of labeled orotic acid for the synthesis of cytidine nucleotides may be

caused by interference with the regulatory mechanisms of cytidine triphosphate synthetase activity by the inhibitory action of a product (or products) of pyrimidine metabolism whose concentration is changed after the administration of phenobarbital. The activity of partially purified cytidine triphosphate synthetase from *Escherichia coli* is regulated by pyrimidine as well as purine nucleoside triphosphates (48, 49). It was observed that the drop in specific activity of the total pool of free nucleotides after the administration of labeled orotic acid to animals which had received phenobarbital repeatedly proceeds more slowly, beginning 10 hr after the administration of labeled precursor (16). Decreased degradation of uracil was observed even after phenobarbital administration to mice (50).

A summary of the results suggests that the repeated administration of phenobarbital, which is structurally related to pyrimidines, affects the enzyme systems associated with the synthesis and degradation of pyrimidine nucleotides in the cytosol (Fig. 7) and microsomal fraction of liver cells. In experiments *in vivo* these changes are reflected by the decreased utilization of labeled orotic acid for synthesis of the cytidine moiety of the total nucleotide pool as well as of cytoplasmic RNAs, while the synthesis of uridine components is not markedly affected (16). At present we cannot decide whether these changes are associated with the induction of drug-metabolizing microsomal enzymes, with intracellular changes and liver cell hypertrophy, or with the activation of hepatocyte proliferation after repeated administration of phenobarbital (51–53). A more detailed study of these aspects and of the changes related to the composition and metabolism of individual phosphorylated pyrimidine components of the free nucleotide pool after administration of the drug is now under way.

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