MOLECULAR PHARMACOLOGY, 8, 681-690

# Alterations in Liver Nuclear Ribonucleic Acid of Rats Treated with Phenobarbital

JOHN S. WOLD AND WILLIAM J. STEELE

*Department of Pharmacology, Oakdale Toxicology Center, University of Iowa College of Medicine, Iowa City, Iowa 52240*

(Received May 26, 1972)

SUMMARY

Repeated administration of phenobarbital (80 mg/kg twice daily) to immature rats over a 3-day period is attended by increases in the ratio of RNA to DNA of hepatocyte nuclei and whole liver. This effect of phenobarbital does not appear to be mediated by cellular proliferation or an increase in ploidy of hepatocytes, since the DNA content per liver remained almost constant, or by reduced levels of intranuclear ribonuclease activity, since the latter was elevated in phenobarbital-treated rats. The increase in the ratio of nuclear RNA to DNA attains a maximum at 2 days, a time when the incorporation of L-[*methyl*-$^{14}$C]-methionine into methylated nuclear RNAs (rRNA and tRNA) and the relative amounts of preribosomal RNAs also become maximal. The base composition of newly synthesized nuclear RNA shifts to a more guanylic acid–cytidylic acid-rich, ribosomal type of RNA composition after a single day of treatment and remains fairly constant thereafter, whereas the incorporation of $^{32}P_i$ into nuclear RNA is progressively increased over the 3-day period of treatment. The increases in RNA content of nuclei, ratio of nuclear protein to DNA, liver weight, and ratio of liver RNA to DNA become maximal at 3 days after the repeated administration of phenobarbital. These results suggest that one of the effects of phenobarbital may be exerted upon the synthesis of nuclear preribosomal and polydisperse RNAs, which presumably perform an important function in increasing the level of protein components of the hepatic endoplasmic reticulum after phenobarbital administration.

## INTRODUCTION

The induction which occurs in the liver after repeated administration of phenobarbital has been extensively studied in recent years, because phenobarbital provides a reproducible means for increasing the level of a number of drug-metabolizing enzymes and a system for studies of membrane biogenesis. The effect of phenobarbital in increasing the level of protein components of the endoplasmic reticulum has been ascribed mainly to increased rates of synthesis of some, but not all, membrane proteins (1–4) and decreased rates of degradation of certain membrane-associated enzymes (1, 2).

Studies from a number of laboratories suggest that one of the effects of phenobarbital in the liver may occur at the level of transcription. Some of these observations include (a) increases in the efficacy of hepatic chromatin from phenoabrbital-treated rats to synthesize RNA (5), (b) increases in the activity of hepatic nuclear RNA polymerase (6), (c) changes in the DNA-RNA hybridization efficiency of newly

These studies were supported by Grants GM-12675 and GM-01308 from the National Institutes of Health.

synthesized nuclear RNA from the livers of treated rats (7), and (d) inhibition of drug-induced increases of enzyme activity by actinomycin D (8). Moreover, recent studies indicate that repeated administration of phenobarbital to rats is associated with increases in the RNA content of the liver (9, 10), and especially with increases in the RNA content of the smooth microsomal fraction (9, 11, 12). Because it has been demonstrated that the smooth microsomal fraction contains the same ribosomal RNA components as the rough microsomal fraction in addition to a minor membrane RNA component (13) which is depressed by phenobarbital administration (14), ribosomal RNA may be considered established as one of the species of hepatic RNA that is augmented by the administration of the drug. Since the half-life of hepatic ribosomal RNA is about 5 days (15), accumulation of ribosomal RNA could result from an increased rate of synthesis or a decreased rate of degradation. Recently Cohen and Ruddon (16) have reported that following the administration of phenobarbital to rats the isotopic labeling of cytoplasmic ribosomal RNA was increased, whereas the labeling of nuclear RNA was decreased. They have proposed that the increased labeling of ribosomal RNA arises either from decreased degradation of hepatic ribosomal RNA or from increased "processing" and transport of ribosomal RNA to the cytoplasm.

The present studies were designed to investigate effects of repeated administration of phenobarbital on the distribution and metabolism of nuclear RNA in rat liver. The results indicate that the repeated administration of phenobarbital to rats is attended by increases in the ratios of nuclear RNA to DNA and liver RNA to DNA. These changes are accompanied by an increased incorporation of L-[*methyl*-$^{14}$C]methionine and $^{32}P_i$ into nuclear RNA, an early increase in the guanylic acid-cytidylic acid content of newly synthesized nuclear RNA, a relative increase in the preribosomal RNA content of nuclei, and an increase in alkaline ribonuclease activity of isolated nuclei. These findings suggest that one of the effects of phenobarbital may be upon the synthesis of polydisperse and preribosomal RNAs.

## METHODS

Albino male rats (80–120 g) were obtained from the Simonsen Company, Minneapolis, and maintained on Wayne Lab Blox and tap water ad libitum. Groups of 4–10 rats were given a total of two, four, or six intraperitoneal injections of phenobarbital (80 mg/kg) dissolved in 0.9% NaCl, or injections of 0.9% NaCl alone. Doses were given every 12 hr, and each group was killed 12 hr after the last dose.

$^{32}P_i$ (1 mCi/rat, carrier-free) or L-[*methyl*-$^{14}$C]methionine (60 $\mu$Ci/100 g, 11 mCi/mmole) was given 20 min prior to death via the jugular vein to rats that had been lightly anesthetized with ether. At death the livers were perfused *in situ* through the portal vein with ice-cold 0.25 M sucrose and then collected in a bath of the same solution. The livers were freed of connective tissue and minced with a tissue press at 2°. Isolated nuclei were obtained from a 10% homogenate of liver (glass vessel–Teflon pestle; 0.009–0.012-inch clearance; six strokes at 1200 rpm; in 62% sucrose–3 mM calcium acetate by weight) by centrifugation at 40,000 × *g* for 1 hr (17). The pellets were suspended by homogenization as above in 10 volumes of 0.34 M sucrose and sedimented through an equal volume of 1.0 M sucrose–0.5 mM calcium acetate to remove cytoplasmic contaminants (17). With this procedure 55–60% of homogenate DNA was recovered in nuclear pellets. For comparison, nuclei were isolated according to the method of Blobel and Potter (18); with this method 75–80% of the homogenate DNA was recovered in nuclear pellets of both phenobarbital- and 0.9% NaCl-treated rats. Ratios of RNA to DNA in nuclear pellets prepared by either the present procedure or that of Blobel and Potter (18) were nearly identical. Treatment of nuclei with 1% Triton X-100 (18) resulted in the loss of an equivalent amount of RNA (7–10%) from nuclear preparations of both phenobarbital- and 0.9% NaCl-treated rats. However, a comparison of nuclear RNA sedimentation profiles revealed that the Triton X-100 treatment of nuclei resulted in the breakdown of nuclear preribosomal RNAs. For this reason and the fact that the relative changes produced by phenobarbital in the

ratios of nuclear RNA to DNA were not significantly different after Triton X-100 treatment, the detergent was not used in the studies presented here.

RNA was extracted from the pellets of purified nuclei by homogenization with a solution containing 0.3% sodium dodecyl sulfate–0.1 M NaCl–0.05 M sodium acetate, pH 5.0, for 1 min, and then with an equal volume of phenol (90%)–*m*-cresol–water–8-hydroxyquinoline (3:0.6:0.4:4, v/v/v/w) for 30 sec (17). The mixture was agitated vigorously at 55° for 10 min and centrifuged at 25,000 × *g* for 10 min. The aqueous layer was removed and treated with 0.5 volume of phenol-cresol mixture at 25° as before. RNA was recovered from the aqueous layer by precipitation with 2.5 volumes of ethanol containing 2% potassium acetate at 2° overnight. The precipitate of RNA was pelleted and redissolved in a small volume of water. The yield of RNA was 75–80% of the RNA in nuclear pellets for both phenobarbital- and 0.9% NaCl-treated rats. Contaminating $^{32}P_i$ was removed from the $^{32}P$-labeled RNA by gel filtration on a Sephadex G-25 column (1 × 30 cm), which was equilibrated and eluted with a solution of 0.05 M sodium formate–1 mM sodium phosphate, pH 5.0, at 4°. The RNA was recovered from pooled fractions by precipitation with ethanol as described above. Aliquots of nuclear RNA preparations were centrifuged (SW 27 or 27.1 rotor, Beckman Instruments) on linear sucrose gradients (5–40%, w/w, containing 0.1 M NaCl–0.01 M sodium acetate–2 mM EDTA, pH 5.0) at 26,000 rpm for 16 hr at 4°. The gradients were monitored for absorbance at 254 nm and fractionated (0.5- or 1.0-ml fractions) with the aid of an automatic density gradient fractionator (model D, Instrumentation Specialties Company, Lincoln, Neb.). $^{32}P$-Labeled RNA fractions were treated with 0.25 N perchloric acid at 70° for 20 min, diluted by the addition of 1 ml of water and 10 ml of counting medium (19), and counted (LS-250, Beckman Instruments) at about 90% efficiency. $^{14}C$-Labeled RNA fractions were diluted by the addition of 1 ml of water and 10 ml of Biosolv (Beckman)–standard toluene fluor (1:4, v/v) and counted at about 65% efficiency.

Aliquots of $^{32}P$-labeled nuclear RNA (0.5–1 mg) were hydrolyzed with 0.3 N KOH at 37° for 18 hr, and nucleotides were fractionated by gradient elution chromatography (water to 4 N formic acid) on Dowex 1 columns (0.5 × 20 cm) as described previously (19). DNA was determined by the method of Burton (20) after acid hydrolyisis, and RNA, by the method of Fleck and Munro (21) after alkaline hydrolysis. Nuclei were counted as described previously (19).

Alkaline ribonuclease was assayed in a reaction mixture which contained 0.05 M Tris buffer (pH 8.0), 0.5 mM magnesium acetate, 300–350 μg of ribosomal [$^{14}C$]RNA, and nuclei (disrupted by sonic oscillation for 1 min at 0°) equivalent to 300–400 μg of DNA in a volume of 1.0 ml. Incubations were performed at 37° for 20 min and terminated by the addition of 2 ml of cold 7% trichloracetic acid. After cooling in ice for 10 min, the samples were centrifuged and a 1.0-ml aliquot of the supernatant solution was removed for counting (19). The assays were corrected for zero-time values and substrate stability in the absence of nuclei. The results are expressed as micrograms of RNA digested per milligram of DNA (or protein) per 20 min. Protein was determined by the biuret method according to Layne (22), with bovine serum albumin as standard.

## RESULTS

Immature male rats received phenobarbital (80 mg/kg intraperitoneally, twice daily) dissolved in 0.9% NaCl or 0.9 NaCl alone, and were killed 12 hr after the last dose at daily intervals up to 4 days. During this period there was an inverse relationship between the average DNA content per gram of liver and the relative increase in liver weight (Table 1), indicating little change in the DNA content of the liver,[1] whereas the

[1] The amounts (in picograms) of DNA per nucleus are as follows: control, 8.09; phenobarbital (day 1), 8.26; phenobarbital (day 2), 8.40; phenobarbital (day 3), 8.80; phenobarbital (day 4), 9.17. The body weight of rats of the age used in these studies normally increases at a rate of about 5–7 g/day. Since the liver comprises about 4.5% of the body weight and increases in proportion to the latter, liver weight increases at a rate of about

TABLE 1

*Liver RNA, DNA, and relative weight and nuclear RNA, DNA, and protein content of livers of rats treated with phenobarbital or 0.9% NaCl*

Rats received phenobarbital (80 mg/kg) dissolved in 0.9% NaCl, or 0.9% NaCl alone (controls), intraperitoneally every 12 hr, and were killed 12 hr after the last injection. The weights of all animals were measured just prior to treatment and death. Isolated nuclei were prepared and counted as described under METHODS. RNA, DNA, and protein were determined as described in METHODS. The values represent averages of three separate experiments, four rats per group, ± standard errors of the mean. Except for differences in liver and body weight, no significant differences were found for rats treated with 0.9% NaCl for up to 4 days; therefore the values were pooled (controls).

| Treatment | Whole liver | | | | Nuclei | | |
|---|---|---|---|---|---|---|---|
| | RNA | DNA | RNA:DNA | Increase in weight | RNA | RNA:DNA[a] | Protein: DNA |
| | *mg/g* | | | % | *pg/nucleus* | | |
| Controls | 8.02 ± 0.3 | 2.18 ± 0.04 | 3.68 ± 0.10 | | 1.7 ± 0.1 | 0.21 ± 0.01 | 4.5 |
| PB,[b] 1 day | 8.12 ± 0.4 | 1.97 ± 0.06 | 4.12 ± 0.07 | 10 ± 2 | 1.9 ± 0.1 | 0.23 ± 0.01 | 4.7 |
| PB, 2 days | 8.32 ± 0.2 | 1.79 ± 0.04 | 4.65 ± 0.08 | 21 ± 1 | 2.1 ± 0.1 | 0.25 ± 0.01 | 4.9 |
| PB, 3 days | 8.57 ± 0.2 | 1.68 ± 0.05 | 5.10 ± 0.06 | 29 ± 1 | 2.2 ± 0.0 | 0.25 ± 0.01 | 5.0 |
| PB, 4 days | 8.25 ± 0.3 | 1.72 ± 0.03 | 4.80 ± 0.12 | 27 ± 1 | 2.2 ± 0.1 | 0.24 ± 0.01 | 4.9 |

[a] See footnote 1 to the text.

[b] The abbreviation used in this and the following tables is: PB, phenobarbital.

average RNA content per gram of liver increased and attained a maximum (107% of controls) 3 days after the initiation of drug treatment. The ratio of RNA to DNA in the liver also reached a maximum at 3 days, confirming earlier reports of a phenobarbital-induced increase in total liver RNA (9, 10). This change was attended by an increase in the RNA content of nuclei, which attained a maximum (129% of controls) at 3 days, and by increases in the ratios of nuclear RNA to DNA and nuclear protein to DNA, which became maximal 2 and 3 days, respectively, after initiation of drug treatment (Table 1).

The question whether phenobarbital increases the amount of all or only some species of hepatic nuclear RNA was examined by sedimentation studies on RNA extracted from isolated nuclei of liver of rats treated for 1, 2, and 3 days with phenobarbital or 0.9% NaCl. Equal amounts of nuclear RNA (about 85 μg) from the six treatment groups of each experiment were centrifuged in the same rotor on gradients of identical height and composition. The results were compiled from three complete studies of this kind. A comparison of RNA sedimentation profiles for 0.9% NaCl-treated rats (controls) revealed no significant differences in the distribution of nuclear RNAs over the 3-day period of treatment; thus only a single representative profile for the controls is presented in the figures. At 2 and 3 days after repeated administration of phenobarbital, the peaks of preribosomal 33 S, 45 S, and 55 S RNA were augmented relative to the peaks of ribosomal 18 S and 28 S RNA and the slowly sedimenting peak of nuclear RNA (Fig. 1).

In order to estimate the magnitude of the relative increases in preribosomal RNAs, the RNA sedimentation profiles were analyzed with an electronic curve analyzer (model 310, du Pont) and resolved into eight Gaussian peaks with approximate sedi-

0.23–0.32 g/day, or 5–7%/day. The DNA content per nucleus of 1-day-treated rats increased only 2% over controls, i.e., at about one-third the normal rate. Similar results were obtained for 2- and 3-day treated rats; however, the relative increase was greater in 4-day-treated rats. Thus the phenobarbital-induced increases in RNA content per nucleus over the 3-day period of treatment cannot be ascribed to cellular proliferation or polyploidy, since replication is depressed in the liver.

mentation coefficients of 18 S, 22 S, 25 S, 28 S, 33 S, 38 S, 45 S, and 55 S (Fig. 2). For convenience the peak at 28 S is referred to as "ribosomal 28 S RNA"; however, it is understood that the 28 S peak includes both ribosomal 28 S RNA and preribosomal 28 S RNA. The percentage of the total area was

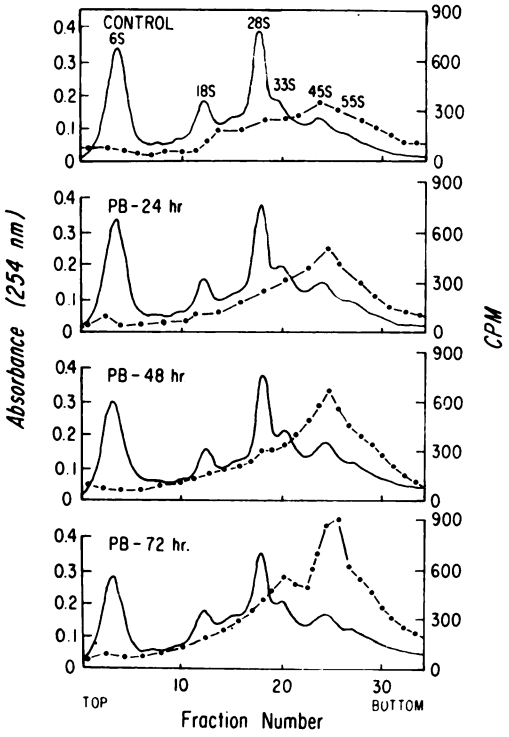


FIG. 1. *Sedimentation profiles of nuclear RNA from livers of 0.9% NaCl-treated (control) and phenobarbital-treated rats to which $^{32}P_i$ (1 mCi/rat) was given 20 min prior to death*

Phenobarbital (PB) (80 mg/kg) or 0.9% NaCl was injected intraperitoneally twice daily for 1, 2, and 3 days; the last dose was given 12 hr prior to death. Equal amounts of RNA (about 85 μg) from the six treatment groups were centrifuged in the same rotor (SW 27.1, Beckman) on linear sucrose gradients (5–40%, w/w, containing 0.1 M NaCl–0.01 M sodium acetate–0.002 M EDTA, pH 5.0) of the same height and composition at 26,000 rpm for 16 hr at 4°. Gradients were monitored for absorbance at 254 nm (——) and divided into 0.5-ml fractions with the aid of an automatic density gradient fractionator (ISCO). The direction of sedimentation is from left to right. Approximate sedimentation coefficients are shown above the peaks (19). ●–●–●, distribution of $^{32}P$ per 0.5-ml fraction. Other details are given in the text.

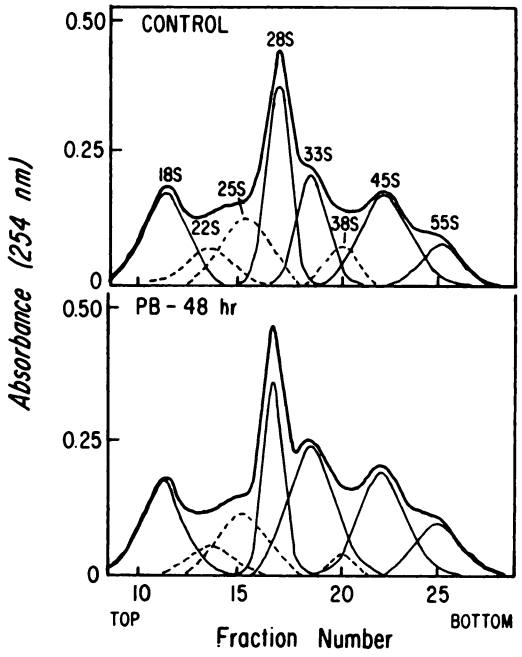


FIG. 2. *Sedimentation profiles of nuclear RNA, ribosomal-preribosomal RNA region, from livers of rats treated with 0.9% NaCl or phenobarbital (80 mg/kg twice daily) for 2 days (upper curves, thick line), and resolution of these profiles into Gaussian peaks (lower curves, thin lines) with du Pont curve analyzer*

Other details are given in the legend to Fig. 1. Although the values obtained by this procedure were only approximations of the true area represented by each RNA component, the analysis provided reproducible results for the same RNA with different inputs, and thus could be used to detect changes in the content of nuclear RNA components.

computed electronically for each peak. The contribution of the preribosomal RNA peaks (33 S, 45 S, and 55 S) to the total ribosomal and preribosomal RNAs (18 S, 28 S, 33 S, 45 S, and 55 S), excluding the three less well-defined RNA components with peaks at 22 S, 25 S, and 38 S, was calculated. The resulting tabulation of the percentages of preribosomal RNAs in phenobarbital- and 0.9% NaCl-treated rats is presented in Table 2. Repeated administration of phenobarbital increased the relative amounts of preribosomal RNAs by factors of 1.2, 1.9, and 1.6 over the controls after 1, 2, and 3 days, respectively, of treatment.

It is likely that this method of analysis would underestimate the magnitude of the change if preribosomal 28 S RNA also accumulates, since this would increase the contribution of "ribosomal 28 S RNA" and thereby partially offset the increases in the more rapidly sedimenting preribosomal RNAs.

To investigate whether changes in RNA distribution were associated with altered metabolism of nuclear RNA, sedimentation studies were performed on RNA extracted from isolated nuclei of liver of rats treated with phenobarbital or 0.9% NaCl for 1, 2, and 3 days prior to a labeling period of 20 min with $^{32}P_i$ or L-[*methyl*-$^{14}$C]methionine. The sedimentation profiles in each experiment were obtained from identical inputs of RNA in order to facilitate the comparison of isotopic labeling data. Although the data of only one experiment are presented for each precursor, each experiment was performed two times. Administration of phenobarbital over a 3-day period increased the incorporation of $^{32}P_i$ into nuclear RNA by factors of 1.1, 1.4, and 1.9 over 0.9% NaCl-treated controls after 1, 2, and 3 days, respectively, of treatment (Fig. 1). The total radioactivity associated with rapidly sedimenting RNAs was increased more than 2-fold at 3 days, when the elevation in RNA content of nuclei reached a maximum. With a labeling period of 20 min the $^{32}$P-labeled RNA molecules of 0.9% NaCl-treated rats sedimented in polydisperse fashion, with the bulk of the radioactivity sedimenting more rapidly than the 28 S species. After 3 days of treatment with phenobarbital the same labeling period gave a sedimentation distribution of $^{32}$P-labeled RNA that was augmented and largely polydisperse; however, prominent peaks at 33 S and 45 S and a shoulder at 55 S were present. Figure 3 shows that phenobarbital treatment also significantly enhanced the incorporation of

TABLE 2

*Distribution of preribosomal RNA from nuclei of liver of rats treated with phenobarbital or 0.9% NaCl*

Rats were treated as described in Table 1. Nuclear RNA was prepared as described in the text. Small amounts of RNA (about 85 μg) from the six treatment groups were centrifuged in the same rotor (SW 27.1, Beckman) on gradients of identical height and composition. Gradients were monitored for absorbance at 254 nm. The resulting sedimentation profiles of nuclear RNA were resolved into Gaussian peaks with the aid of a du Pont curve analyzer (Fig. 2). The fraction of the total area was computed for each peak. The contribution of preribosomal RNA peaks (33 S, 45 S, and 55 S) to the total ribosomal and preribosomal RNAs (18 S, 28 S, 33 S, 45 S, and 55 S), excluding the three less well-defined RNA components (22 S, 25 S, and 38 S), was calculated as described in the text. The values represent the averages of three experiments for each treatment day ± standard errors of the mean.

| Treatment | Preribosomal RNA | Relative increase |
|---|---|---|
| | % | |
| Controls | 52 ± 2 | |
| PB, 1 day | 57 ± 3 | 1.2 |
| PB, 2 days | 67 ± 2 | 1.9 |
| PB, 3 days | 63 ± 2 | 1.6 |

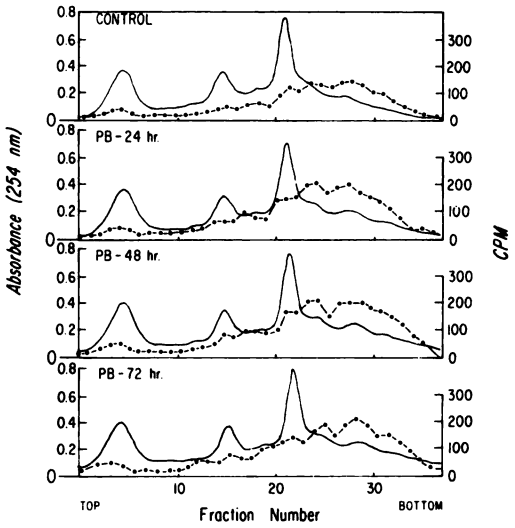


FIG. 3. *Sedimentation profiles of nuclear RNA from livers of 0.9% NaCl-treated (control) and phenobarbital-treated rats to which* L-[*methyl*-$^{14}$C]-*methionine (60 μCi/ 100 g of body weight) was given 20 min prior to death*

The profiles were obtained from equal inputs of RNA (400 μg) after centrifugation (SW 27 rotor, Beckman) at 25,000 rpm for 16 hr at 4°. Gradients were monitored for absorbance at 254 nm (——) and divided into 1-ml fractions. ●–●–●, distribution of $^{14}$C per 1-ml fraction. Other details are given in the legend to Fig. 1 and the text.

isotope from L-[*methyl*-$^{14}$C]methionine into nuclear RNA after a single day of treatment. The specific activities of nuclear RNA from 0.9% NaCl and 1-, 2-, and 3-day phenobarbital-treated rats were 6,500, 11,500, 13,000 and 10,000 dpm/mg, respectively, and were maximal at 2 days (200% of controls), a time when the accumulation of preribosomal RNAs also was greatest (Table 2). With a labeling period of 20 min the $^{14}$C-labeled RNA molecules of both 0.9% NaCl- and phenobarbital-treated rats appeared to be associated largely with peaks and shoulders of the major species of preribosomal RNA.

In order to ascertain whether phenobarbital alters the relative synthetic rates of preribosomal and polydisperse nuclear RNA, the base composition of the $^{32}$P-labeled, newly synthesized nuclear RNA was determined by assaying the distribution of $^{32}$P among the four ribonucleotides released by alkaline hydrolysis (Table 3). The base ratio (A + U):(G + C) of newly synthesized nuclear RNA decreased from a value of 0.91 for the controls to 0.78 after a single day of treatment, and remained fairly constant thereafter. This shift was mainly due to increased radioactivity found in guanylic acid and decreased radioactivity found in adenylic and uridylic acids.

Because an accumulation of preribosomal RNA could result from either a decreased rate of breakdown or an increased rate of synthesis and/or processing, it was important to investigate the effect of phenobarbital administration on nuclear ribonuclease activity. The assays were performed with isolated nuclei from livers of rats treated with phenobarbital or 0.9% NaCl for 1, 2, and 3 days. Administration of phenobarbital over a 3-day period increased the alkaline ribonuclease activity of isolated nuclei by 28% over control values (Table 4) when the data were expressed per milligram of DNA, and by 17% over control values when the data were expressed per milligram of protein. These results are in line with the relative increases in the ratios of RNA to DNA and protein to DNA of nuclei after 3 days of treatment.

## DISCUSSION

These studies show that the repeated administration of phenobarbital (80 mg/kg twice daily) to immature rats is accompanied by increases in the RNA content of hepatocyte nuclei and cytoplasm which become maximal 3 days after initiation of drug treatment. Ratios of nuclear RNA to DNA and liver RNA to DNA also increase, becoming maximal at 2 and 3 days, respectively. This effect of phenobarbital does not appear to be mediated by cellular proliferation or an increase in ploidy of hepatocytes, since the DNA content per gram of liver decreased roughly in proportion to the relative increase in liver weight, indicating little, if any, change in the DNA content per liver. These results are in agreement with those reported by Paulini *et al.* (23), who showed that the repeated administra-

TABLE 3

*Base composition of newly synthesized nuclear RNA of livers of rats treated with phenobarbital or 0.9% NaCl*

Rats were treated as described in Table 1, and $^{32}P_i$ (1 mCi/rat) was injected intravenously 20 min prior to death. Nuclear RNA was prepared, treated to remove $^{32}P_i$, and hydrolyzed with 0.3 N KOH as described in the text. The values for base composition are percentages of the total $^{32}$P in the mixed 2'- and 3'-nucleotides of RNA obtained by ion-exchange chromatography. The values represent averages of duplicate analyses on the samples derived from two separate experiments, ± standard errors of the mean.

| Treatment | Base composition | | | | Base ratio, (A + U):(G + C) |
|---|---|---|---|---|---|
| | C | A | G | U | |
| Controls | 24.4 ± .4 | 25.4 ± .4 | 28.4 ± .1 | 22.7 ± .1 | 0.91 ± 0.00 |
| PB, 1 day | 24.9 ± .4 | 22.3 ± .3 | 30.2 ± .1 | 21.0 ± .2 | 0.78 ± 0.01 |
| PB, 2 days | 26.0 ± .1 | 22.6 ± .1 | 29.9 ± .1 | 21.4 ± .3 | 0.78 ± 0.01 |
| PB, 3 days | 25.2 ± .4 | 22.6 ± .1 | 30.2 ± .4 | 22.3 ± .2 | 0.81 ± 0.01 |

TABLE 4

*Nuclear ribonuclease activity of livers of rats treated with phenobarbital or 0.9% NaCl*

Rats received phenobarbital (100 mg/kg) dissolved in 0.9% NaCl, or 0.9% NaCl alone (controls), every 24 hr and were killed 24 hr after the last injection. Isolated nuclei were prepared as described in the text. Assays were performed in 0.05 M Tris buffer, pH 8.0, at 37°, containing 0.5 mM magnesium acetate, 300–350 μg of ribosomal [$^{14}$C]RNA, and nuclei (disrupted by sonic oscillations for 1 min at 0°) equivalent to 300–400 μg of DNA. Incubations were performed at 37° for 20 min and terminated by the addition of 2 volumes of cold 7% trichloracetic acid. The samples were centrifuged after standing for 10 min at 0°. An aliquot (1 ml) of the supernatant solution was removed for counting as described in the text. The assays were corrected for zero-time values and substrate stability in the absence of nuclei. The data are expressed as micrograms of RNA digested per milligram of DNA or protein during 20 min. The values represent averages of duplicate analyses from four separate experiments ± standard errors of the mean.

| Treatment | Ribonuclease activity | |
|---|---|---|
| | RNA:DNA | RNA:protein |
| | *μg/mg/20 min* | *μg/mg/20 min* |
| Controls | 57.5 ± 2.1 | 14.8 ± 0.7 |
| PB, 1 day | 59.0 ± 1.9 | 14.8 ± 0.4 |
| PB, 2 days | 64.3 ± 1.6 | 15.9 ± 0.6 |
| PB, 3 days | 73.8 ± 1.8 | 17.4 ± 0.5 |

tion of phenobarbital (80 mg/kg twice daily) to immature rats decreases the thymidine labeling index of liver to about 20% of control values. In contrast, they also showed that the repeated administration of lower doses of phenobarbital (40 mg/kg twice daily) to immature rats increases the uptake of thymidine by liver at least 2-fold over controls, confirming earlier reports of a phenobarbital-induced increase in DNA and cell number (24). In addition, the effect of phenobarbital on nuclear RNA content does not appear to be mediated by reduced levels of intranuclear ribonuclease activity, since the latter was elevated in phenobarbital-treated rats.

Accompanying the increase in the ratio of nuclear RNA to DNA was a relative increase in the amounts of nuclear preribosomal RNAs, an increased incorporation of L-[*methyl*-$^{14}$C]methionine and $^{32}P_i$ into nuclear RNA, and a shift in base composition of newly synthesized nuclear RNA to a more GC-rich RNA composition after a single day of treatment. Although the greatest accumulation of nuclear preribosomal RNA was noted 2 days after repeated administration of phenobarbital, it is possible that maximal accumulation occurred later but was obscured by concomitant accumulation of preribosomal 28 S RNA, as discussed above. However, it is also likely that the diminution of preribosomal RNAs at 3 days may have occurred either because of an acceleration of the rate of processing of preribosomal RNA or because of a slackening of the rate of synthesis, as suggested by the decreased incorporation of L-[*methyl*-$^{14}$C]methionine into nuclear RNA at 3 days. The augmentation of GC-rich preribosomal RNA in the nucleus is in agreement with the findings of increased incorporation of L-[*methyl*-$^{14}$C]methionine into nuclear RNA and enriched G + C content of newly synthesized nuclear RNA after 1 and 2 days of drug treatment. Thereafter the over-all base composition of newly synthesized RNA remained fairly constant while the incorporation of $^{32}P_i$ into nuclear RNA was significantly increased, suggesting increased elaboration of both polydisperse and preribosomal nuclear RNAs.

In normal rat liver rapidly sedimenting preribosomal RNAs constitute about 25% of the total nuclear RNA but account for only about 17% of nuclear RNA synthesis after short labeling times (25). The bulk of the rapidly labeled nuclear RNA is AU-rich heterogeneous nuclear RNA (polydisperse RNA), which exhibits a rapid turnover when compared to preribosomal RNA, but, unlike the latter, appears to be unmethylated (26, 27) and retained in the nucleus, where its function is unknown (28). Thus cytoplasmic messenger RNA appears to derive from only a small percentage of the total polydisperse nuclear RNA. The biogenesis of the ribosome begins in the nucleolus with the transcription of a high molecular weight preribosomal RNA, which is methylated (29, 30) and then cleaved in a stepwise, semiconservative fashion into a number of preribosomal RNA intermediates, from which the two ribosomal subunits and

several smaller RNAs eventually emerge. Numerous studies have shown that ribosomal RNA synthesis is elevated by hormones which stimulate proliferation of the endoplasmic reticulum (31) and also by removal of a part of the liver from rats (32). However, it is not known at what step in the complex series of events leading to ribosome formation this increase is accomplished. The rate of ribosome formation may be regulated by changing the rate of synthesis of preribosomal RNA or by maintaining a constant rate of synthesis and altering either the rate of processing or the rate of degradation of preribosomal RNA. These possibilities are not, of course, exhaustive or mutually exclusive.

Repeated administration of phenobarbital appears to exert an increasingly profound effect on hepatic nuclear RNA synthesis, as suggested by the increased incorporation of $^{32}P_i$ and L-[*methyl*-$^{14}$C]methionine into nuclear RNA and the concomitant accumulation of preribosomal RNAs. Since the base composition of newly synthesized nuclear RNA remains essentially unchanged after the first day of phenobarbital treatment, these findings suggest that the same genes are active, but at different rates at 1 and 3 days after treatment with the drug. However, the alterations in the base composition of newly synthesized nuclear RNA may simply reflect changes produced by phenobarbital in nucleotide synthesis or utilization (see below). An early response of hepatic regulatory mechanisms to the administration of phenobarbital is suggested by the findings of increased activity of nuclear RNA polymerase (6), increased transcriptive ability of chromatin (5), and increased incorporation of [$^{14}$C]-orotate into cytoplasmic ribosomal RNA (16) and microsomal RNA (33) within a day of treatment with the drug. In contrast, similar studies (16, 34) have shown that the incorporation of [$^{14}$C]-orotate into nuclear RNA is decreased after a single or repeated administration of the drug. On the basis of the latter observations, it has been suggested (16) that the enhanced labeling of ribosomal RNA may result from effects of phenobarbital either on the stability of cytoplasmic ribosomal RNA or on the processing and transport of ribosomal RNA to the cytoplasm, rather than on the synthesis of ribosomal RNA. Differences in the precursors used for labeling RNA may account for this apparent discrepancy. In this regard, it has been suggested that the hepatic phosphoribosyl pyrophosphate level is depressed by phenobarbital administration and may be rate-limiting for synthesis of pyrimidines *de novo* (35). Consistent with this possibility is the observation that the specific activity of the hepatic nucleotide pool is lowered by phenobarbital when [$^{14}$C]orotate is used as a precursor (16). In contrast, the repeated administration of phenobarbital does not appear to depress the specific activity of the RNA precursor pool with $^{32}P_i$ as a precursor. However, the present studies do not provide information with regard to possible changes in nucleotide synthesis and utilization during the response to phenobarbital administration. This question is presently under investigation. The precursor pools for RNA synthesis in the liver are in equilibrium with nucleotides derived from synthesis *de novo* and also from salvage of products derived from the degradation of RNA and nucleotide coenzymes. Thus, if synthesis *de novo* of pyrimidines is depressed by the administration of phenobarbital, increased phosphorylation of precursors derived from salvage pathways would result in higher labeling of RNA precursors with $^{32}P_i$ than with [$^{14}$C]orotate. In contrast to the behavior of either of the latter precursors, the use of L-[*methyl*-$^{14}$C]methionine to monitor nuclear RNA synthesis provides a distinct advantage, since the incorporation (methylation) into ribosomal RNA (and tRNA) bypasses nucleotide pools and takes place either at the time of transcription or immediately thereafter (29, 30). Moreover, nuclear polydisperse RNA appears to be unmethylated (26, 27); thus we have assumed that the increased incorporation of L-[*methyl*-$^{14}$C]methionine into preribosomal RNAs after phenobarbital administration represents the increasing rates of synthesis of preribosomal RNA in the nucleolus.

The studies reported here suggest that the augmentation of nuclear RNA produced by phenobarbital results from increased elaboration of both polydisperse and preribosomal

RNA. However, the increased incorporation of $^{32}P_i$ into nuclear RNA may be due to fluctuations in the specific activities of nucleotide pools. It is also possible that the accumulation of preribosomal RNAs may have arisen either from an increased rate of processing or from a less wasteful processing of preribosomal RNA. The second possibility seems less likely, since it was shown that nuclear ribonuclease activity is elevated in phenobarbital-treated rats. The present studies do not provide information with regard to the first possibility, which is currently under investigation in this laboratory. However, the results presented here do not appear to be dependent upon any change produced by phenobarbital in the rate of transport of RNA to the cytoplasm, since little of the isotope was found in cytoplasmic RNA after a labeling period of 20 min.

ACKNOWLEDGMENTS

The authors wish to thank Dr. C. A. Swenson for the use of the du Pont curve analyzer, and Mr. A. Campbell, Mrs. S. Rickert, and Mrs. G. Reynolds for technical assistance in part of this work.

REFERENCES

1. H. Jick and L. Shuster, *J. Biol. Chem.* **241**, 5366 (1966).
2. Y. Kuriyama, T. Omura, P. Siekevitz and G. E. Palade, *J. Biol. Chem.* **244**, 2017 (1969).
3. I. M. Arias, D. Doyle and R. T. Schimke, *J. Biol. Chem.* **244**, 3303 (1969).
4. J. L. Holtzman, *Biochem. Pharmacol.* **18**, 2573 (1969).
5. W. N. Piper and W. F. Bousquet, *Biochem. Biophys. Phys. Res. Commun.* **33**, 602 (1968).
6. H. V. Gelboin, J. S. Wortham, and R. G. Wilson, *Nature* **214**, 281 (1967).
7. J. S. Wold and W. J. Steele, *Fed. Proc.* **28**, 484 (1969).
8. S. Orrenius, J. L. E. Ericsson and L. Ernster, *J. Cell Biol.* **25**, 627 (1965).
9. M. R. Juchau and J. R. Fouts, *Biochem. Pharmacol.* **15**, 1453 (1966).
10. T. S. Argyris, *J. Pharmacol. Exp. Ther.* **164**, 405 (1968).
11. H. Remmer and H. J. Merker, *Science* **142**, 1657 (1963).
12. S. Orrenius and L. Ernster, *Biochem. Biophys. Res. Commun.* **16**, 60 (1964).
13. H. W. S. King and W. Fitschen, *Biochim. Biophys. Acta* **155**, 32 (1968).
14. J. Seifert and H. Remmer, *Biochem. Pharmacol.* **20**, 553 (1971).
15. J. R. Loeb, R. R. Howell and G. M. Tomkins, *Science* **149**, 1093 (1965).
16. A. M. Cohen and R. W. Ruddon, *Mol. Pharmacol.* **6**, 540 (1970).
17. W. J. Steele and H. Busch, *Methods Cancer Res.* **3**, 61 (1967).
18. G. Blobel and V. R. Potter, *Science* **154**, 1662 (1966).
19. W. J. Steele, N. Okamura and H. Busch, *J. Biol. Chem.* **240**, 1742 (1965).
20. K. Burton, *Biochem. J.* **62**, 315 (1956).
21. A. Fleck and H. N. Munro, *Biochim. Biophys. Acta* **55**, 571 (1962).
22. E. Layne, *Methods Enzymol.* **3**, 447 (1957).
23. K. Paulini, G. Beneke and R. Kulka, *Beitr. Pathol. Anat. Allg. Pathol.* **144**, 319 (1971).
24. T. S. Argyris and D. Magnus, *Develop. Biol.* **17**, 187 (1968).
25. M. Muramatsu and H. Busch, *J. Biol. Chem.* **240**, 3960 (1965).
26. G. M. Brown and G. Attardi, *Biochem. Biophys. Res. Commun.* **20**, 298 (1965).
27. P. B. Moore, *J. Mol. Biol.* **18**, 38 (1966).
28. R. Soeiro, M. W. Vaughan, J. R. Warner and J. E. Darnell, *J. Cell Biol.* **39**, 112 (1968).
29. R. H. Burdon, *Nature* **210**, 797 (1966).
30. H. Greenberg and S. Penman, *J. Mol. Biol.* **21**, 527 (1966).
31. J. B. Tata, *Nature* **219**, 331 (1968).
32. S. Chandhuri, O. Doi and I. Lieberman, *Biochim. Biophys. Acta* **134**, 479 (1967).
33. R. McCauley and D. Couri, *Fed. Proc.* **29**, 737 (1970).
34. S. J. Smith, R. Gleeson and E. S. Vesell, *Fed. Proc.* **30**, 330 (1971).
35. M. J. Mycek, *Pharmacologist* **13**, 258 (1971).