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Further Studies on Post-transcriptional Stabilization of Ribosomal Precursor Ribonucleic Acid by Phenobarbital[1]

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SUMMARY

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To study the effect of phenobarbital on transcription of hepatic ribosomal precursor RNA (45 S RNA), three experiments were performed in adult male rats receiving single daily intraperitoneal injections of 0.9% NaCl or phenobarbital (100 mg/kg) for 1 or 4 days: measurement of the labeling of hepatic nuclear RNA with L-[*methyl*-^{3}H]methionine, determination of nucleolar RNA polymerase activity, and measurement of alkaline nuclear ribonuclease activity. Labeling of nuclear RNA with [*methyl*-^{3}H]methionine increased approximately 100% and 50% in rats treated for 1 and 4 days, respectively, with phenobarbital. Phenobarbital for 1 or 4 days did not affect nucleolar RNA polymerase activity measured in the presence of high ionic media and α-amanitin. With nonlabeled ribosomal RNA or 45 S [^{3}H]RNA as substrate, nuclear alkaline ribonuclease activity in phenobarbital-treated rats was unaltered. These data confirm our previous finding that phenobarbital-induced stabilization of 45 S RNA molecules accounts for the approximately 2-fold increase in cellular concentration of 45 S RNA in rats treated for 4 days with phenobarbital. A single dose of phenobarbital may stimulate methylation of newly formed 45 S RNA molecules normally degraded in the nucleus, thereby increasing transport of ribosomal subunits to the cytoplasm.

INTRODUCTION

Chronic treatment of rats with phenobarbital increased by approximately 2-fold the cellular concentration of 45 S RNA, the ribosomal precursor RNA (1, 2). No increase in labeling of 45 S RNA occurred in rats treated with phenobarbital for 1 or 4 days after a 10-15-min pulse with [^{14}C]-adenine or [^{3}H]orotic acid (1). In contrast, labeling of cytoplasmic ribosomal RNA with these same isotopes was significantly increased within 24 hr after a single dose (1, 3, 4) but was unchanged after 4 days of drug treatment (1). Using an incubation system *in vitro*, we observed that in nuclei from rats treated for 1 and 4 days with phenobarbital the breakdown of 45 S RNA was significantly decreased as compared to NaCl-treated controls (1). These data suggested that phenobarbital treatment of rats failed to increase transcription of 45 S RNA but

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protected newly synthesized molecules of 45 S RNA from intranuclear degradation, thereby allowing more ribosomal subunits to reach the cytoplasm.

In the light of two reports (2, 5) indicating increased synthesis of ribosomal RNA after treatment of rats with phenobarbital, the present study was designed to clarify this issue and to study the mechanism of phenobarbital-induced post-transcriptional stabilization of 45 S RNA. Three studies were performed in rats treated with phenobarbital for 1 or 4 days: labeling *in vivo* of nuclear RNA with L-[*methyl*-^{3}H]methionine, measurement of nucleolar RNA polymerase activity, and determination of alkaline nuclear ribonuclease activity. These experiments suggest that the phenobarbital-induced elevation of hepatic 45 S RNA concentration arises not from its increased transcription but from enhanced methylation.

MATERIALS AND METHODS

Drug Treatment

Male Sprague-Dawley rats weighing 170–200 g, from Charles River Laboratories, received a single intraperitoneal injection (at 5:00 p.m.) or four daily injections (at 9:00 a.m.) of either phenobarbital (100 mg/kg) or 0.9% NaCl. Liver weight was not altered by 1 day of phenobarbital treatment, but after 4 days of phenobarbital treatment liver weight increased by 20–30%. Body weights of NaCl controls and of drug-treated rats were unchanged after 1 day but were increased to the same extent after 4 days of phenobarbital treatment. In labeling experiments, animals were fasted for 16 hr preceding death. In experiments on ribonuclease and RNA polymerase activities, overnight fasting of animals did not affect the results.

Isotope Labeling

[5-^{3}H]Orotic acid (12.2 Ci/mmole), L-[*methyl*-^{3}H]methionine (4.3 Ci/mmole), and [^{14}C]UTP (384 mCi/mmole) were obtained from New England Nuclear Corporation. The toxin α-amanitin, was a gift from Professor T. Wieland and was also obtained commercially from Henley and Company. In labeling studies each rat received via the portal vein 50 μCi of L-[*methyl*-^{3}H]methionine under light ether anesthesia, and was killed by aortic exsanguination 5 min after injection of the isotope. With a 5-min pulse using the intraportal vein route of injection, L-[*methyl*-^{3}H]methionine labels only the 45 S RNA (6). In all other studies the rats were killed by decapitation. For preparation of labeled nuclear 45 S RNA, each of four rats received an intraperitoneal injection of 20 μCi of [^{3}H]orotic acid and was killed after a 20-min pulse. In all experiments excised livers were placed in ice-cold 0.25 M sucrose, and further steps were carried out at 4°.

Isolation of Nuclei

Isolation of nuclei for labeling studies and ribonuclease assays. For each time point or experimental group, livers from three or four rats were pooled. Minced liver tissue was homogenized in 2.4 M sucrose (1:11, w/v) containing 3.3 mM $CaCl_2$ with four up-and-down strokes in a Teflon-glass homogenizer (0.015–0.020-inch pestle clearance). After successive filtrations through two and four layers of cheesecloth, the homogenate was centrifuged at 40,000 $\times$ g for 75 min to sediment the nuclei (1, 7, 8). In preparation of 2.4 M sucrose to obtain undegraded 45 S RNA, we emphasize the need to make fresh solutions of $CaCl_2$, because microorganisms containing high ribonuclease activity grow readily in $CaCl_2$.

Isolation of nuclei for RNA polymerase assay. The minced liver tissue was placed in a freshly prepared solution of 2.3 M sucrose containing 15 mM $MgCl_2$ and 0.25 mM spermine and homogenized as above (8, 9). The homogenate was centrifuged at 40,000 $\times$ g for 65 min to sediment the nuclei. Nuclei were washed once with 0.25 M sucrose containing 2 mM $MgCl_2$.

Under a phase contrast microscope nuclear preparations obtained by either the sucrose-calcium or sucrose-magnesium procedure were highly purified. No difference was observed between the purity or gross morphology of isolated nuclei of drug-treated rats and NaCl controls.

Extraction and Analysis of RNA

Nuclei were homogenized in a solution containing 0.3% sodium dodecyl sulfate, 0.14 M NaCl, and 0.05 M sodium acetate, pH 5.1 (10, 11), for 1 min (15 strokes) with a loosely fitting Teflon pestle. After addition of 0.05 M sodium acetate-saturated phenol containing 0.1% 8-hydroxyquinoline (11), the sample was homogenized again for 1 min. The suspension was shaken at 65° for 10 min, and then for 20 min at room temperature (10, 12).

The mixture was centrifuged at 17,000 × *g* for 10 min, and the aqueous phase was removed; this was followed by two re-extractions of the aqueous phase with phenol at room temperature. The RNA was precipitated overnight at −20° with 2.5 volumes of ethanol containing 2% potassium acetate (13). The precipitate was dissolved in water and its absorbance at 260 nm was measured; the radioactivity of the solution was then measured in 10 ml of a mixture containing 7 g/liter of 2,5-diphenyloxazole and 100 g/liter of naphthalene, both in dioxane, using a Beckman LS-100 liquid scintillation counter. The specific activity of the total nuclear RNA is expressed as disintegrations per minute per milligram of RNA.

Preparation of 45 S[^{3}H]RNA for Ribonuclease Assay

Between 1 and 2 mg of ^{3}H-labeled nuclear RNA prepared as above were layered over 10–40% sucrose gradients (38 ml) made up in a medium containing 0.1 M NaCl, 1.0 mM EDTA, and 0.01 M sodium acetate, pH 5.1 (14). The gradients were centrifuged in a Spinco SW 27 rotor at 26,000 rpm for 15 hr at 5°. Analysis of gradients was carried out with the aid of an ISCO automatic fractionator system. The fractions constituting the 45 S RNA peak were precipitated with ethanol as above, dissolved in 1.0 ml of H_2O, analyzed for specific activity, and used for the ribonuclease assay.

Nucleolar RNA Polymerase Assay

RNA polymerase was assayed essentially as described by Jacob *et al.* (15, 16). The incubation was carried out in the presence of Mn^{++}–$(NH_4)_2SO_4$ and α-amantin. Since α-amanitin is a specific inhibitor of nucleoplasmic RNA polymerase (form II), the residual activity of the enzyme assayed in high ionic media and in the presence of the toxin represents the actual nucleolar RNA polymerase activity (16–18). The assay mixture contained the following in a final volume of 0.4 ml: Tris-HCl buffer, pH 7.1, 42 μmoles; $MnCl_2$, 0.83 μmole; $(NH_4)_2SO_4$, 150 μmoles; ATP, GTP, and CTP, 0.30 μmole each; [^{14}C]UTP (384 mCi/mmole), 0.0092 μmole; and nuclei from 0.4 g of liver, wet weight. α-Amanitin was included in the reaction mixture at a concentration of 1 μg/ml. The assay was carried out for 50 min.

The reaction was terminated by adding 0.1 mg of yeast RNA as carrier and 1 ml of 10% trichloroacetic acid containing 0.04 M $Na_4P_2O_7$. Each enzyme assay was carried out in triplicate. Blanks were obtained by adding substrates after precipitation with trichloroacetic acid. The reaction mixtures were filtered through Whatman GF/C filter paper under light vacuum and washed four times with 1 ml of 5% trichloroacetic acid containing 0.02 M $Na_4P_2O_7$. The dried filter discs were placed in glass vials, 8 ml of Omnifluor-toluene scintillation fluid were added, and the vials were counted in a Beckman LS-100 scintillation counter. Data are expressed as picomoles of UMP incorporated per milligram of DNA.

Nuclear Ribonuclease Assay Using Nonlabeled Ribosomal RNA as Substrate

Nuclei isolated in sucrose-calcium were washed and suspended in 0.25 M sucrose containing 3 mM $MgCl_2$ and used directly for the assay. The final Mg^{++} concentration in the reaction was 0.5 mM. Alternatively, nuclei isolated in sucrose-calcium were washed and suspended in 0.25 M sucrose (1:0.05, w/v) and were sonicated using a microtip for 2 min in a Branson Sonifier (6 amp) (19). After addition of $MgCl_2$ to a final concentration of 3 mM, the sonic extract was used in the assay.

Ribonuclease activity was determined by a modification (20) of the method of Shortman (21). Each data point was obtained in triplicate. In a final volume of 0.3 ml, the following materials were present in each reaction tube: 0.1 ml of 0.5 M Tris buffer (pH 7.8), 0.1 ml of a 1% purified ribosomal

RNA solution, 0.05 ml of 0.02% bovine serum albumin, and 0.05 ml of liver nuclei (either nonsonicated or sonicated). Nonsonicated nuclei from 0.18 g of liver, wet weight, were used in each tube; for the sonicated nuclei, tissue from 0.09 g of liver was used in each tube. DNA and protein were determined as described below.

The reaction was started by adding nuclei, and the mixture in each tube was shaken in a water bath at 37° for 30 min. The reaction was stopped by immersing the tubes in an ice bath for 1 min, followed by the addition of 0.3 ml of cold 76% ethanol in 1 N HCl. After 15 min in an ice bath, the tubes were centrifuged at 3000 × *g* for 15 min at 4°. A_{260} was measured by dilution of 0.2 ml of the supernatant fluid to 1.0 ml with H_2O. Nuclei of drug-treated rats and NaCl controls were assayed simultaneously. Blanks were treated identically with the other tubes except that nuclei were added after incubation and addition of cold ethanol–HCl. The ribonuclease activity is expressed as A_{260} units of nondiluted supernatant fluid per milligram of DNA and per milligram of protein.

Nuclear Ribonuclease Assay Using Labeled Nuclear 45 S RNA as Substrate

The method of assay was a modification of the procedure of Roth (22). Each data point was obtained in triplicate. Each reaction tube contained the following in a final volume of 3.0 ml: 1.0 ml of 5.6 mM Veronal acetate buffer (freshly prepared), pH 7.9, containing 1.0 mM $MgCl_2$; 1.0 ml of a solution containing 50 μg of 45 S RNA (500 dpm); and nonsonicated liver nuclei from 0.3 g of liver, wet weight, isolated in sucrose-calcium as described above.

The reaction was carried out by adding the buffer, RNA, and nuclei, and shaking the mixture in a water bath at 37° from 2.5 min to 20 min. The reaction was stopped by adding 3.0 ml of a solution of 0.16 M $Na_4P_2O_7$ in 10% trichloroacetic acid. Liver nuclei of drug-treated rats and saline controls were assayed simultaneously. Blanks contained no nuclei and were run at zero time and during the assay. To correct for quenching, 1.0 ml of nuclei from 0.3 g of liver was added to the blanks with the precipitating agent. The reaction tubes were then placed in an ice-water bath for 5 min, filtered through Whatman GF/C filter paper under light vacuum, and washed four times with 1 ml of 5% trichloroacetic acid containing 0.02 M $Na_4P_2O_7$. The dried filters were counted as described above for the RNA polymerase assay by liquid scintillation. Corrections were made for slight variations in the DNA content of nuclei from NaCl controls and drug-treated rats. The data are expressed as the amount of radioactivity (disintegrations per minute) remaining in the acid-insoluble fraction after incubation at the times indicated in Fig. 1.

The reliability of both ribonuclease assays was tested by using different concentrations of substrate and nuclei in each assay system. Increasing or decreasing the amount of RNA (substrate) or nuclei (enzyme) resulted in corresponding changes in measured activity of the ribonuclease.

Determination of DNA and Protein

DNA was extracted from the nuclei by acid hydrolysis and was determined by Burton's modification of the diphenylamine method (23). Protein was determined by the method of Lowry *et al.* (24), using bovine serum albumin as the standard.

RESULTS

Effect of Phenobarbital Treatment of Rats on Labeling of Hepatic Nuclear RNA with L-[*methyl*-^{3}H]*Methionine*

Table 1 shows that treatment of rats for 16 hr and 4 days with phenobarbital significantly increased the incorporation *in vivo* of L-[*methyl*-^{3}H]methionine into total hepatic nuclear RNA. A single dose of the drug increased labeling of the nuclear RNA by more than 2-fold, whereas 4 days of treatment resulted in an enhancement of approximately 50% in incorporation of the isotope.

Effect of Phenobarbital Treatment of Rats on Nucleolar RNA Polymerase Activity

Our earlier data (1) showed no change in the labeling of 45 S RNA with [^{3}H]orotic acid, [^{14}C]guanine, or [^{14}C]adenine after phenobarbital treatment. In order to eliminate potential error in labeling of 45 S RNA

TABLE 1

Effects of single and multiple doses of phenobarbital on labeling of rat liver nuclear RNA with L-[*methyl-*^{3}*H*]*methionine for 5 min*

Phenobarbital (100 g/kg) or 0.9% NaCl was injected intraperitoneally as a single dose or four daily single doses. Sixteen hours after the single dose or 24 hr after the last chronic dose, each rat received an intraportal vein injection of 50 μCi of L-[*methyl-*^{3}H]methionine; the rats were killed by aortic exsanguination after a 5-min pulse. Liver nuclei were isolated, RNA was extracted, and specific activity was determined as described in MATERIALS AND METHODS. The number of experiments is shown in parentheses. Each experiment consisted of two to four rats. Standard errors are indicated.

Phenobarbital treatment	Specific activity of total nuclear RNA		
	Phenobarbital (A)	Control (B)	A:B[a]
	dpm/mg RNA		
100 mg/kg, 16 hr	7160 ± 600 (3)	3360 ± 580 (3)	2.13
100 mg/kg, 4 days	6080 ± 820 (4)	4020 ± 400 (6)	1.51

[a] Difference between experimental and control means: $p < 0.05$ by Student's *t*-test.

TABLE 2

Effects of single and multiple doses of phenobarbital on hepatic nucleolar RNA polymerase activity

Rats were treated with phenobarbital as described in Table 1. Control rats received a single daily injection of 0.9% NaCl for 2 days. Nuclei from three or four rats were isolated in sucrose-magnesium-spermine, and assays were performed as described in MATERIALS AND METHODS. Enzyme activity was linear with increasing concentrations of nuclei from drug-treated rats and NaCl controls. Each assay was carried out in triplicate. The results are the averages of three separate experiments; standard errors are indicated.

Nucleolar RNA Polymerase Activity		
Treatment	Time	UMP incorporated in presence of Mn^{++}–$(NH_4)_2SO_4$ + α-amanitin[a]
		pmoles/mg DNA
Control		650 ± 31
Phenobarbital	16 hr	702 ± 20
Phenobarbital	4 days	660 ± 17

[a] No significant differences between control and phenobarbital-treated animals by Student's *t*-test.

due to fluctuations of precursor pool size (25–28), the effects of phenobarbital treatment on rat liver nucleolar RNA polymerase activity were investigated as an index of transcription. Since measurement of hepatic nucleolar RNA polymerase activity using low ionic media containing Mg^{++} is affected by high nuclear nuclease activity (29), this assay was performed using high ionic media in the presence of α-amanitin, which specifically inhibits the nucleoplasmic RNA polymerase (form II) (16). The enzyme activity, which under these conditions represents only the nucleolar RNA polymerase activity, was unaltered by treatment of rats with phenobarbital, suggesting that phenobarbital exerts no effect on the transcription of 45 S RNA (Table 2). In two experiments, nucleolar RNA polymerase was assayed in low ionic media containing Mg^{++} and α-amanitin; again no significant increase (<15%) in enzyme activity occurred after phenobarbital treatment.

Hepatic Nuclear Alkaline Ribonuclease Activity after Phenobarbital Treatment

Experiments using nonlabeled ribosomal RNA as substrate. Wold and Steele (2) reported that phenobarbital treatment for 3 days increased alkaline nuclear ribonuclease activity; for this reason enhanced stabilization of newly formed 45 S RNA seemed unlikely to these investigators. Alkaline ribonuclease activity was reinvestigated under conditions essentially the same as those employed by Wold and Steele (2), except that nonlabeled ribosomal RNA served as substrate and nuclei from young adult, rather than immature, rats were used. Moreover, in addition to sonicated nuclei, nonsonicated nuclei were used as the enzyme source. The results of this study are shown in Table 3. Under these conditions phenobarbital treatment for 4 days had no effect on alkaline nuclear ribonuclease activity.

Experiments using labeled 45 S RNA as substrate. The use of ribosomal RNA as a substrate for ribonuclease can be criticized because ribosomal RNA, having already been cleaved physiologically from 45 S

TABLE 3

Effect of 4 days of treatment with phenobarbital on liver alkaline nuclear ribonuclease activity with nonlabeled ribosomal RNA as substrate

Rats received four daily single injections of either 0.9% NaCl or phenobarbital (100 mg/kg) and were killed by decapitation 24 hr after the last dose. Nonsonicated nuclei and sonicated nuclei were prepared in sucrose-calcium, and the reactions were carried out as described in MATERIALS AND METHODS. The data are expressed as A_{260} units per milligram of DNA and per milligram of protein; three or four rats were used for each experiment. The number of experiments is shown in parentheses; standard errors are indicated.

Nuclei	Nuclear ribonuclease activity[a]		Protein:DNA
	A_{260}/mg DNA	*A_{260}/mg protein*	
Nonsonicated			
Control	8.64 (2)	3.00 (2)	2.82 (2)
Phenobarital	8.59 (2)	2.86 (2)	3.05 (2)
Sonicated			
Control	10.26 ± 0.84 (4)	3.26 ± 0.90 (4)	4.50 ± 0.52 (4)
Phenobarbital	10.08 ± 1.61 (4)	3.00 ± 0.98 (4)	4.75 ± 0.59 (4)

[a] No significant differences between control and phenobarbital-treated animals by Student's *t*-test.

RNA, is an unsuitable substrate for nuclease(s) acting specifically on the ribosomal precursor RNA. Therefore the activity of alkaline nuclear ribonuclease was investigated utilizing hepatic nuclear 45 S RNA prepared from rats labeled for 20 min *in vivo* with [^{3}H]orotic acid as substrate. Under these conditions almost all of the label represents ribosomal RNA precursor. Figure 1 shows that phenobarbital treatment of rats for 4 days had no effect on the breakdown of nuclear 45 S RNA by alkaline ribonuclease. The same result was obtained for rats treated for 1 day with the drug. In two experiments nucleolar 45 S RNA used as substrate produced similar results.

We eliminated the possibility that 45 S RNA of phenobarbital-treated rats was more stable than control 45 S RNA by using 45 S RNA from drug-treated rats as a substrate. Conditions were identical with those in Fig. 1 except that nuclei and 45 S RNA from rats treated with phenobarbital for 16 hr were incubated with 45 S RNA and nuclei from control rats, respectively. Three experiments were performed; incubations lasted 20 min. No differences were observed from the values shown in Fig. 1. The average radioactivity (± standard error) was: phenobarbital nuclei plus phenobarbital 45 S RNA, 103 ± 4 dpm; control nuclei plus phenobarbital 45 S RNA, 96 ± 8 dpm; phenobarbital nuclei plus control 45 S RNA, 95 ± 6 dpm; control nuclei plus control 45 S RNA, 100 ± 8 dpm. From all these experiments, it appears that phenobarbital treatment augments methylation of 45 S RNA but does not alter alkaline nuclear ribonuclease activity.

DISCUSSION

The results confirm our previous observations that phenobarbital treatment increases the stability and efficiency of processing of 45 S RNA (1). The studies also suggest that a single dose or four daily doses of phenobarbital increase methylation of ribosomal precursor RNA in the nucleolus, but do not significantly alter either nucleolar RNA polymerase activity or nuclear alkaline ribonuclease activity.

In our previous report, using [^{14}C]adenine, [^{14}C]guanine, and [^{3}H]orotic acid, we observed no increase in labeling of 45 S RNA after a single dose or four daily doses of phenobarbital (1). Wold and Steele (2) used $^{32}P_i$ as the isotope; they recognized its technical shortcomings, but reported enhanced labeling of ribosomal precursor RNA with $^{32}P_i$ after 2 days of phenobarbital treatment of immature male rats. Discrepancies in the uptake of $^{32}P_i$ and nucleoside precursors of RNA have frequently been noted (25, 27, 28).

Our present data on the nucleolar RNA

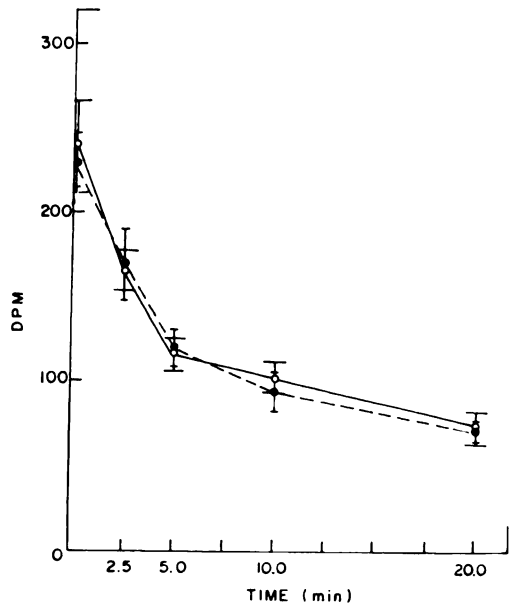

FIG. 1. *Time course of degradation of hepatic nuclear 45 S RNA by alkaline nuclear ribonuclease of rats treated with NaCl or phenobarbital for 4 days*

Rats were treated, nuclei were prepared, and the reactions were carried out as described in MATERIALS AND METHODS. The data are expressed as radioactivity remaining in the acid-insoluble fraction after incubation for the times indicated. The results are the averages of five separate experiments. Standard errors are indicated. ●- - -●, phenobarbital; O——O, NaCl controls.

polymerase assay reveal that phenobarbital treatment produces no significant effect on transcription of 45 S RNA (Table 2), strengthening our earlier data on labeling *in vivo* (1). The 2-fold increase in cellular concentration of 45 S RNA observed after 4 days of phenobarbital treatment (1) apparently results from increased stabilization of newly synthesized 45 S RNA. Furthermore, enhanced labeling of cytoplasmic ribosomal RNA observed within 1 day of treatment with this drug (1, 3, 4) may arise from increased transport of more ribosomal subunits to the cytoplasm.

In agreement with Wold and Steele (2), we observed no change in alkaline nuclear ribonuclease activity after 1 day of phenobarbital treatment, a time when degradation of 45 S RNA decreases (1). However, in contrast to their report of increased alkaline nuclear ribonuclease activity after 3 days of phenobarbital treatment (2), we observed no change in the activity of this enzyme after 4 days of treatment with this drug (Table 3 and Fig. 1). The 28% increase in alkaline nuclear ribonuclease activity after 3 days of twice daily phenobarbital treatment reported by Wold and Steele (2) may be due in part to effects of starvation (22). In several experiments we observed that after 2 or more days of treatment with phenobarbital two daily doses of this drug resulted in a reduction of weight gain in drug-treated rats as compared to NaCl controls. This effect on weight may be due to lethargy at feeding times in rats receiving two daily doses.

A major difficulty in this area of research currently is that the exonucleases and endonucleases that process and cleave 45 S RNA or the messenger RNA precursor cannot be distinguished from nucleases that destroy newly formed nuclear RNA randomly (30–34). Moreover, the possibility exists that the same enzymes process or destroy the precursor, depending on the specificity of the substrate (33, 34). Our results do not rule out the possibility that small amounts of physiologically active subspecies of nuclear ribonuclease are masked in our assay. Phenobarbital inhibition of these nucleases could result in increased RNA polymerase activity when assayed in low ionic media. Meisler and Tropp (35) demonstrated that increasing concentrations of $(NH_4)_2SO_4$ can indeed inhibit ribonuclease activity. This observation partially explains increased RNA polymerase activity measured in high ionic media. Possibly phenobarbital increases RNA polymerase activity assayed in low ionic media by decreasing ribonuclease activity. The phenobarbital-induced increase in Mg^{++}-dependent RNA polymerase activity observed by Gelboin *et al.* (5) might have arisen by this mechanism. Such a concept is supported by the absence of any significant increase in the nucleolar RNA polymerase activity assayed in high ionic media containing α-amanitin. Furthermore, the slight increase in the activity of the Mg^{++}-dependent enzyme cannot explain the almost 2-fold increase in 45 S RNA content after phenobarbital administration (1).

Our experiments on methylation of nuclear RNA (Table 1) generally agree with those of Wold and Steele (2); interpretations differ. We believe that increased methylation of the ribosomal precursor RNA can support either of two hypotheses: enhanced metabolic stabilization or increased synthesis of this RNA. Synthesis and methylation of 45 S RNA occur almost simultaneously; methylation protects 45 S RNA from intranuclear degradation and results in increased production of 28 S and 18 S ribosomal RNA (30, 36). In addition to the fact that only about half the 45 S RNA molecule is conserved during processing (30, 36), 50–80% of 45 S RNA molecules synthesized in eukaryotic cells are inefficiently processed and never reach the cytoplasm as mature ribosomal RNA (1, 37–43).

Our results suggest either increased methylation of each 45 S RNA molecule (hypermethylation), without an increase in the number of molecules, or else the methylation of a greater number of 45 S RNA molecules. If each molecule of 45 S RNA produced after phenobarbital stimulation contained significantly more methyl groups than control 45 S RNA, phenobarbital 45 S RNA should be more resistant to degradation by nuclear ribonuclease than control 45 S RNA (30, 36). That the nuclear ribonuclease assays revealed no such change suggests that no increase in the number of methyl groups per molecule of 45 S RNA occurs. Furthermore, if significantly more methyl groups were incorporated into each molecule of 45 S RNA, the sedimentation characteristics of ribosomal precursor RNA on agarose-acrylamide would be altered because of conformational changes. Phenobarbital treatment produces no difference in sedimentation rates of 45 S RNA, or 35 S, 28 S, or 18 S RNA. Consequently, enhanced stabilization of 45 S RNA after phenobarbital treatment (1) may arise from an increase in the number of 45 S RNA molecules that are methylated.

Little is known about the size and location of *S*-adenosylmethionine pools within the hepatocyte, particularly in the nucleolus, where 45 S RNA is transcribed. Measurement of these pools did not appear practical because such data would be exceedingly difficult to interpret owing to the numerous methyl transfer reactions within the hepatocyte. Moreover, ribosomal RNA methylation represents only a small proportion of these reactions. We are continuing our investigation of the possible role of methylation as a regulatory mechanism for transport of ribosomal RNA to the cytoplasm.

Other mechanisms, such as alterations in the synthesis of regulatory proteins (39) and/or ribosomal proteins (42), may play a role in the phenobarbital-induced stabilization of 45 S RNA. Energy-dependent regulatory mechanisms have been reported which may be involved in the selective transport of newly formed messenger (44) as well as ribosomal RNA (37, 39).

More ribosomal RNA (1, 37–43) and messenger RNA precursor (34, 45–49) are synthesized than are normally required in nondividing or even actively dividing cells. The present studies add to the rapidly growing body of evidence suggesting that post-transcriptional stabilization of newly formed precursors of ribosomal (1, 37–43) and messenger RNA (45–50) may provide a regulatory mechanism for the increased synthesis of specific proteins.

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