MOLECULAR PHARMACOLOGY, 12, 820-831

Phenobarbital-Induced Increases in Methylation of Ribosomal Precursor Ribonucleic Acid

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(Received January 3, 1975)

(Accepted May 18, 1976)

SUMMARY

SMITH, STEVEN J., LIU, DAI KEE, LEONARD, THOMAS B., DUCEMAN, BARRY W. & VESELL, ELLIOT S. (1976) Phenobarbital-induced increases in methylation of ribosomal precursor ribonucleic acid. *Mol. Pharmacol.*, 12, 820-831.

In young adult male rats killed either 6 or 16 hr after a single intraperitoneal injection of phenobarbital (100 mg/kg) or 0.9% NaCl, hepatic nucleolar RNA methyltransferase activity and nucleolar RNA polymerase activity were measured as well as labeling of microsomal RNA *in vivo*. Nucleolar RNA methyltransferase activity was assayed, using isolated nucleoli, by the incorporation of *S*-adenosyl[^{3}H]methionine into RNA. RNA polymerase activity was determined in a similar manner by measuring [^{3}H]UTP incorporation. Incorporation *in vivo* of [^{3}H]orotic acid was used in the cytoplasmic microsomal RNA-labeling studies. By 6 and 16 hr after phenobarbital, incorporation of methyl label into RNA increased by 29% and 36%, respectively. Six hours after a single intraperitoneal injection of phenobarbital, nucleolar RNA polymerase activity was unchanged. Six hours after phenobarbital, incorporation *in vivo* of [^{3}H]orotic acid into cytoplasmic microsomal 18 S and 28 S RNA was stimulated by approximately 50%. The increase in cytoplasmic RNA after phenobarbital administration appears to result from increased stability of nucleolar ribosomal precursor (45 S) RNA due to enhanced methylation rather than from an increase in transcription of 45 S RNA.

INTRODUCTION

Within 6 hr after its administration, phenobarbital increases the activity of hepatic microsomal drug-metabolizing enzymes (1-4). Phenobarbital-induced stimulation of drug-metabolizing enzyme activity requires increased amounts of enzyme protein (1). Since protein synthesis and RNA metabolism are closely associated, knowledge of how cellular RNA is altered by phenobarbital becomes critical to understanding the mechanisms of induction.

Within 16 hr after a single dose of phenobarbital, increased labeling of cytoplasmic ribosomal RNA with [^{3}H]orotic acid can be observed (5-7). In addition, stabilization of newly synthesized ribosomal precursor (45 S) RNA has been shown (5, 8). Previously we observed increased incorporation *in vivo* of [^{3}H]methionine into total hepatic nuclear RNA in phenobarbital-treated rats (8, 9).

These studies were undertaken to investigate the possible function of RNA methylation in relation to the enhanced stabil-

This work was supported in part by Grant MH-24244-01 from the National Institute of Mental Health.

ity of 45 S RNA. To investigate this phenomenon, a nucleolar RNA methyltransferase assay *in vitro* was developed and employed, since experiments *in vivo* using [^{3}H]methionine might be influenced by the effects of phenobarbital on the precursor pool size of *S*-adenosylmethionine or on the labeling of messenger RNA precursors.

Under conditions that minimized precursor pool effects, the present studies reveal that methylation of nucleolar 45 S RNA is increased after phenobarbital treatment. Moreover, this increase in methylation arises from phenobarbital-induced enhancement of nucleolar RNA methyltransferase activity. Although phenobarbital stimulates nucleolar RNA methyltransferase activity and labeling of microsomal RNA *in vivo*, transcription of 45 S RNA, as indicated by RNA polymerase activity, is unchanged.

Therefore the results of this study agree with the hypothesis (5, 8) that phenobarbital treatment increases methylation of some molecules of 45 S RNA, thereby preserving 45 S RNA molecules that would have been degraded intranuclearly in the absence of phenobarbital. This stimulatory effect of phenobarbital on methylation of 45 S RNA provides a mechanism for stabilization of ribosomal precursor RNA.

MATERIALS AND METHODS

Phenobarbital Treatment

Male Sprague-Dawley rats weighing 170–200 g, from Charles River Laboratories, received single intraperitoneal injections of phenobarbital (100 mg/kg) or of NaCl (0.9%). For 6-hr experiments, rats were injected at midnight and killed at 6:00 a.m.; for 16-hr experiments rats were injected at 2:00 p.m. and killed at 6:00 a.m. Rats were fasted for 16 hr prior to death. Overnight fasting of animals did not affect the results.

Isotope Labeling

[5-^{3}H]Orotic acid (12.2 Ci/mmole), [^{3}H]*S*-adenosylmethionine (8.5 Ci/mmole), and [^{3}H]UTP (36 Ci/mmole) were purchased from New England Nuclear Corporation. Unlabeled SAM[1] and UTP were obtained from Sigma and Calbiochem, respectively. In microsomal RNA labeling studies, each rat was injected with 100 μCi of [^{3}H]orotic acid 3 hr before death.

Isolation of Nucleoli

In all experiments, animals were killed by decapitation and excised livers were placed in ice-cold 0.25 M sucrose; further steps were performed at 4°. For each experimental group, livers from five to eight rats were pooled. Minced liver tissue was homogenized in a freshly prepared solution of 2.3 M sucrose (1:11, w/v) containing 15 mM $MgCl_2$ and 0.25 mM spermine, with four up-and-down strokes in a Teflon-glass homogenizer (0.015–0.020-inch pestle clearance). After two filtrations through four layers of cheesecloth, the homogenate was centrifuged at 40,000 × *g* for 60 min to sediment the nuclei (10, 11).

The sedimented nuclei were washed with 0.88 M sucrose containing 5 mM $MgCl_2$, suspended in 0.34 M sucrose (1:1, w/v), and sonicated for 15–20 sec in a Branson Sonifier (9–10 amp) (11, 12). To sediment the nucleoli, 40 ml of the sonicated suspension were layered over 40 ml of 0.88 M sucrose and centrifuged at 2000 × *g* for 20 min.

Under a phase-contrast microscope, nucleolar preparations were highly purified and contained fewer than 1% nuclei. No difference was observed between the gross morphology of isolated nucleoli of drug-treated rats and NaCl controls.

Microsomal RNA Experiments

Isolation of microsomes. To prepare the cytoplasmic microsomal fraction, minced livers from three or four rats were homogenized using a loosely fitting Teflon pestle (0.030–0.040-inch pestle clearance) with three strokes in an ice-cold solution of 0.05 M Tris-HCl (pH 7.6) containing 0.005 M magnesium acetate, 0.025 M potassium chloride, and 0.25 M sucrose (1:10, w/v). After filtration through four layers of

[1] The abbreviations used are: SAM, *S*-adenosylmethionine; PCA, perchloric acid; DTT, dithiothreitol.

cheesecloth, an aliquot (10%) of the suspension was centrifuged twice at 12,000 × *g* for 20 min to remove nuclei, whole cells, and mitochondria. The remaining supernatant fluid was centrifuged at 130,000 × *g* for 90 min to sediment the cytoplasmic microsomes.

Acid-soluble pool. Two milliliters of the 0.25 M sucrose homogenate were mixed with 2 ml of ice-cold 0.4 N perchloric acid, homogenized, and centrifuged at 2000 × *g* for 15 min. The precipitate was washed once with 0.2 N perchloric acid and centrifuged again. The supernatant solutions were pooled and assayed for absorbance at 260 nm and for radioactivity (13); the specific activity of the acid-soluble pool was computed as disintegrations per minute per A_{260}.

RNA extraction. Microsomes were homogenized in a solution containing 0.3% sodium dodecyl sulfate, 0.14 M NaCl, and 0.05 M sodium acetate, pH 5.1 (13, 14), 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 (14), the sample was homogenized again for 1 min. The suspension was shaken for 30 min at room temperature.

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 (15). The precipitated RNA was then dissolved in 0.01 M sodium acetate, pH 5.1, or H_2O, and analyzed spectrophotometrically for absorbance at 260 nm.

Sucrose gradients and analysis of specific activity. Between 1 and 2 mg of RNA were layered over 10–40% sucrose gradients (38 ml) containing 0.1 M NaCl, 1.0 mM EDTA, and 0.01 M sodium acetate, pH 5.1 (16). 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. Absorbance at 254 nm was transcribed with a Honeywell recorder.

The specific activity of the 18 S and 28 S microsomal RNA peaks was determined by pooling the three gradient fractions which constituted the peak and by precipitating them in ethanol overnight as above. The precipitate was dissolved in water, and its absorbance at 260 nm was measured; the radioactivity of the solution was then measured by liquid scintillation (17).

Determination of nucleolar RNA methyltransferase activity. Nucleolar RNA methyltransferase was assayed by a modification of the procedure described by Jacob *et al.* (18) for RNA polymerase. Variations in conditions were investigated systematically to obtain optimal activity. The incubation mixture, in a final volume of 0.5 ml, included Tris-HCl buffer, pH 8.5, 50 μmoles; $MgCl_2$, 1.5 μmoles; NaF, 3 μmoles; dithiothreitol, 0.7 μmole; ATP, 0.4 μmole; GTP, CTP, and UTP, 0.3 μmole each; SAM, 0.005 μmole; [^{3}H]SAM (11.6 Ci/mmole), 0.00028 μmole; and nucleoli containing 0.01–0.02 mg DNA. Tubes were placed in a rotating water bath and incubated for 30 min at 30°. To stop the reaction, 100 μg of unlabeled SAM and 1.5 ml of 0.4 N PCA containing 0.04 M $Na_4P_2O_7$ were added. The tubes were shaken in a Vortex mixer and then placed in an ice-water bath for 20 min. The acid-soluble fraction was centrifuged at 1000 × *g* for 10 min.

RNA was separated from DNA and protein using the procedure of Munro and Fleck (19). The precipitate was washed twice with 0.2 N PCA, and 0.6 ml of 0.3 N KOH was added to the precipitate. After incubation in alkali for 90 min to hydrolyze RNA, the DNA and protein were precipitated with PCA to a final concentration of 0.3 N. The tubes were placed in an ice-water bath for 20 min and then centrifuged to sediment DNA, proteins, and salt. The supernatant was analyzed for incorporation of CH_3- into RNA. DNA and protein were then extracted from the acid-insolbule fraction (19).

Aliquots from each sample were counted by liquid scintillation, using 8 ml of 0.7% 2,5-diphenyloxazole in toluene containing BioSolv [Beckman BBS-3, 1:10 (v/v)]. Activity of the nucleolar methyltransferase

was expressed as picomoles of methyl label incorporated per milligram of DNA.

To estimate the amount of labeled RNA in each fraction, nucleoli were incubated using the nucleolar RNA methyltransferase incubation system described above, in the presence of [^{3}H]UTP instead of labeled SAM. Analysis of the [^{3}H]UTP-labeled fractions showed that 88–92% of the label was present in the RNA fraction, whereas only 8–12% of the label was recovered in DNA and protein.

Using [^{3}H]SAM, 60% of the radioactivity was recovered in the RNA fraction. About 10% of the label was in DNA, and 30% was in protein. No significant difference between control and phenobarbital-treated rats occurred in the amount of label in nucleolar DNA. The amounts of label in protein of nucleoli from control and treated rats were also similar.

Separation of mono- and dinucleotides from nucleolar RNA labeled in vitro with [^{3}H]SAM. DEAE-cellulose was obtained from H. Reeve Angel, Inc. (DE-52), and prepared for chromatography. To separate mononucleotides from alkali-stable dinucleotides, chromatography was carried out using 0.6 × 80 cm columns by a modification of the procedure of Tomlinson and Tener (20).

RNA was labeled *in vitro* using the previously described nucleolar RNA methyltransferase assay system, with either [^{3}H]SAM or [^{3}H]UTP. After the hydrolyzed RNA was prepared as described above, the supernatant was made 1 N with respect to KOH and incubated for 18 hr at 37°. Following this incubation, the tubes were cooled and the pH was adjusted to 7.8 using PCA. To sediment salts that would have interfered with the chromatography, the solutions were cooled at 4° overnight and then centrifuged at 2000 × *g* for 10 min. Between 15,000 and 40,000 cpm of RNA hydrolysate in a 20–25-ml total volume were loaded on the column. The nucleotides were separated using a salt gradient consisting of 150 ml each of 0.025 M Tris-formate, pH 7.8, and 0.2 M Tris-formate, pH 7.8, both containing 7 M urea. The flow rate was 3–4 ml/hr. Fractions of 1 ml were collected, and 0.1-ml aliquots were analyzed for radioactivity as above.

In rats killed 16 hr after receiving a single dose of phenobarbital, nucleolar RNA methyltransferase activity was assayed *in vitro* as described above. For this assay, four tubes, each containing nucleoli isolated from phenobarbital-treated and NaCl-treated controls (0.5-ml total volume per tube), were used. At the end of the 30-min incubation, these tubes were pooled into two groups (2.0-ml total volume per group). The reaction was stopped by adding 5 ml of a solution which yielded a final concentration of 0.3% sodium dodecyl sulfate, 0.14 M sodium chloride, and 0.05 M sodium acetate, pH 5.1. The mixture was homogenized for 10 sec, 7 ml of sodium acetate-saturated phenol containing 0.1% 8-hydroxyquinoline were added, and the suspension was homogenized for 1 min. The sample was then shaken successively for 10 min at 63° and 30 min at room temperature, and centrifuged at 17,000 × *g* for 10 min. The aqueous phase was removed and re-extracted once with phenol at room temperature.

The RNA was precipitated overnight at 20° with 2.5 volumes of 98% ethanol containing 2% potassium acetate. RNA was purified by filtration and reprecipitation with ethanol–potassium acetate as before. The RNA-containing filters were suspended in 3 ml of distilled H_2O for 3 hr at 4°, and the radioactivity in the samples was determined by adding 0.1-ml aliquots to 10 ml of 0.7% 2,5-diphenyloxazole–toluene containing BioSolv (BBS-3, 1:10, v/v). RNA was estimated by measuring absorbance at 260 nm, and the specific activity was calculated as disintegrations per minute per milligram of RNA. RNA was then hydrolyzed in 1 N KOH for 18 hr at 37° and chromatographed as previously described.

Determination of nucleolar RNA polymerase activity. RNA polymerase was assayed as described by Jacob *et al.* (18) in low ionic strength medium containing Mg^{++}. The assay mixture contained the following in a final volume of 0.5 ml: Tris-HCl buffer, pH 8.5, 50 μmoles; $MgCl_2$, 2.5 μmoles; NaF, 3 μmoles; dithiothreitol, 5 μmoles; ATP, GTP, and CTP, 0.3 μmole each; UTP, 0.14 μmole; [^{3}H]UTP (36 Ci/

mmole), 33.6 pmoles; and nucleoli containing 0.1–0.15 mg of DNA. The assay was carried out for 30 min at 37°.

The reaction was terminated by adding 400 μg of UTP and 1.5 ml of 10% trichloracetic acid containing 0.04 M $Na_4P_2O_7$. Each sample was assayed in quadruplicate. Blanks were obtained by adding nucleoli after trichloracetic acid treatment. The acid-insoluble material was collected on Whatman GF/C filters, washed, and counted as described above for the RNA methyltransferase assay. Data are expressed as picomoles of UMP incorporated per milligram of DNA.

The RNA polymerase I assay was investigated using different concentrations of [^{3}H]UTP and nucleoli. A direct relationship between increments in UTP concentrations and the velocity of the reaction was observed from 30 μM to 200 μM. Concentrations of [^{3}H]UTP and enzyme (nucleoli) were used which were not rate-limiting.

Determination of DNA. To estimate DNA content in nucleoli used for the RNA polymerase I assay, aliquots of isolated nucleoli were used. Nucleolar DNA was extracted by acid hydrolysis (19). DNA was determined using Burton's procedure (21). In the RNA methyltransferase assay, aliquots from nucleoli, as well as from the isolated DNA fraction, were taken. DNA was measured using procedures described by Burton (21) and Zamenhof (22).

RESULTS

Conditions for Assay of Nucleolar RNA Methyltransferase Activity

To establish the validity of the rRNA methyltransferase assay, several conditions were investigated. The inset of Fig. 1 shows that at low concentrations of SAM, the enzyme activity is linear. This observation in hepatic nucleoli agrees with that of Liau *et al.* (23). However, at higher SAM concentrations, apparent inhibition by SAM occurs. For this reason, a SAM concentration of 10 μM was used in all assays.

In agreement with other reports (24–26), incorporation of labeled SAM is favored under alkaline conditions; a pH of 8.5 ± 0.1 was employed in all assays (Fig. 2). Temperature sensitivity of the assay is demonstrated in Fig. 3. Previous investigators used either 30° (24) or 37° (25, 26). The standard temperature was 30° in all experiments, because consistently higher

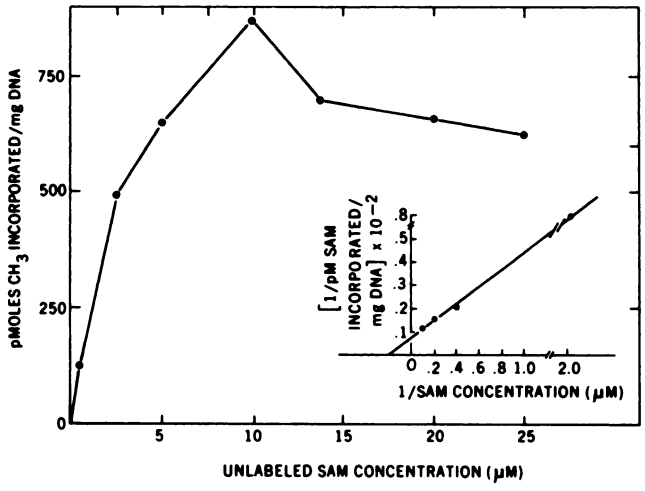

FIG. 1. *Nucleolar RNA methyltransferase assay: SAM concentration curve*

Nucleoli were isolated from the livers of NaCl-treated rats as described in MATERIALS AND METHODS. Nucleolar RNA methyltransferase activity was measured at a constant concentration of [^{3}H]SAM, and the unlabeled SAM concentration was varied from 2.5 to 25 μM. All other conditions were the same as described in MATERIALS AND METHODS. Each data point is the mean of quadruplicate determinations.

enzyme activities were obtained at 30° than at 37°. Figure 4 shows the effect of incubation time on enzyme activity. A 30-min incubation time was utilized in our assay.

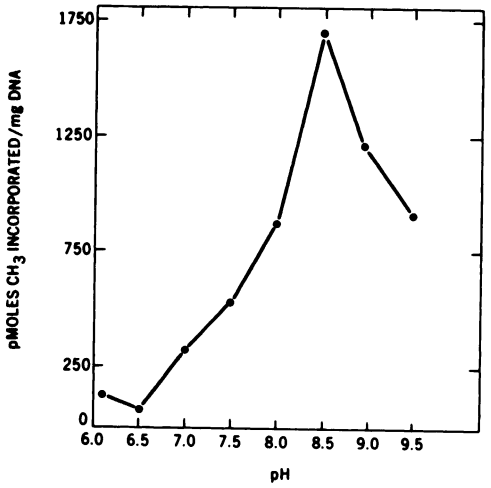

FIG. 2. *Nucleolar RNA methyltransferase assay: pH curve*

Nucleoli were isolated and nucleolar RNA methyltransferase activity was determined as described in MATERIALS AND METHODS. The incubation buffers were adjusted to the designated pH prior to the addition of the nucleoli. Each data point is the mean of four determinations.

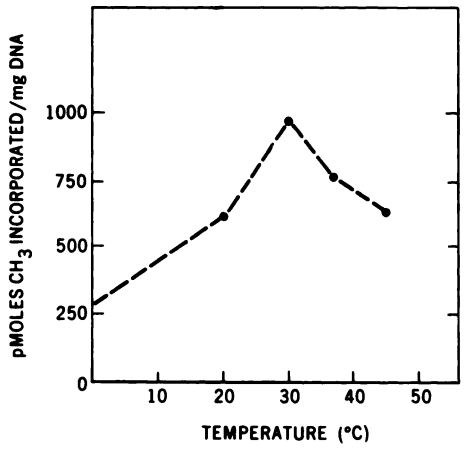

FIG. 3. *Nucleolar RNA methyltransferase assay: temperature curve*

Nucleoli were isolated and assayed for RNA methyltransferase activity as described in Fig. 1. Temperatures were varied by using separate water baths for the incubations. Each data point is the mean of quadruplicate determinations.

Because of the requirement of this enzyme for divalent metal ions (24-27), the effects of variations in magnesium concentration were examined (Fig. 5). Maximum enzyme activity occurred at a concentration of 3 mM $MgCl_2$.

The sulfhydryl agent DTT had previously been reported to exert little, if any,

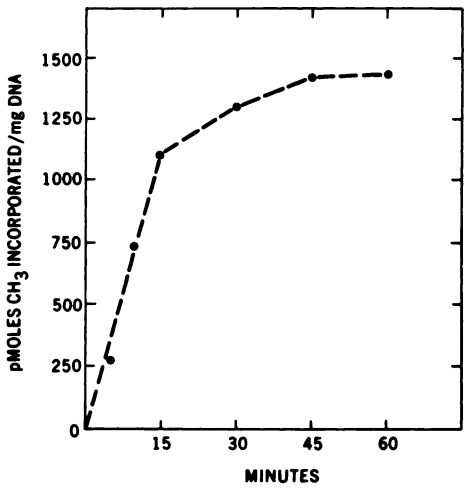

FIG. 4. *Nucleolar RNA methyltransferase assay: incubation time curve*

Nucleolar RNA methyltransferase activity was measured as described in MATERIALS AND METHODS. Each time point is the mean of quadruplicate determinations.

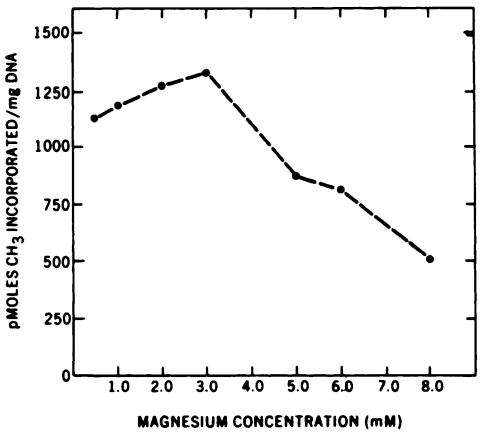

FIG. 5. *Nucleolar RNA methyltransferase assay: $MgCl_2$ concentration curve*

Nucleolar RNA methyltransferase activity was determined as described in MATERIALS AND METHODS. The concentration of $MgCl_2$ in the incubation buffer was varied between 0.5 and 8 mM. Each data point is the mean of quadruplicate determinations.

stimulating effect; some investigators observed an inhibitory effect of DTT on the assay (24, 25, 28). A re-examination of this question revealed that DTT at concentrations below 1.5 mM was required for maximum activity (Fig. 6). However, enzyme activity decreased at concentrations above 1.5 mM under these conditions. Therefore DTT was used in a concentration of 1.3 mM.

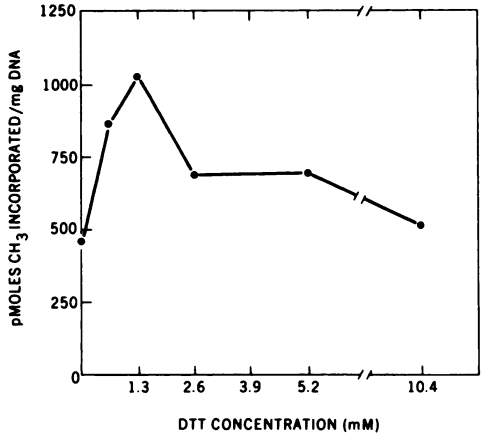

FIG. 6. *Nucleolar RNA methyltransferase assay: DTT concentration curve*

Nucleolar RNA methyltransferase activity was determined as described in MATERIALS AND METHODS. The DTT concentration in the incubation buffer was varied between 0 and 10.4 mM. Each data point is the mean of quadruplicate determinations.

Recovery of Alkali-Stable Dinucleotides Following Assay of Nucleolar RNA Methyltransferase

To demonstrate that 2'-*O*-ribose methylation was occurring during the assay, a modification of the method of Vaughan *et al.* (29) was used. DEAE-cellulose chromatography of nucleotides obtained from RNA labeled *in vitro* with [^{3}H]UTP or [^{3}H]SAM revealed that about 90% of [^{3}H]UTP radioactivity was present as mononucleotides (Fig. 7), whereas 90% of the [^{3}H]SAM radioactivity was recovered as alkali-stable dinucleotides. These observations establish that the product of the nucleolar RNA methyltransferase assay resembles native ribosomal RNA after alkaline hydrolysis (20).

Effect of a Single Dose of Phenobarbital on Nucleolar RNA Methyltransferase Activity

Our earlier data (8) showed that at 16 hr after phenobarbital the incorporation of L-

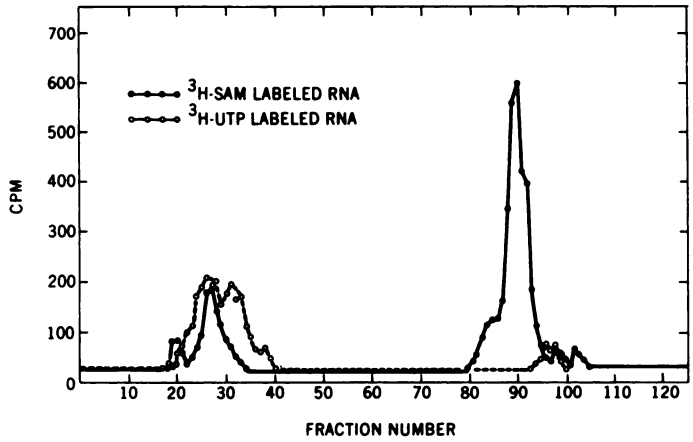

FIG. 7. *DEAE-cellulose chromatography of mono- and dinucleotides produced after alkaline hydrolysis of nucleolar RNA synthesized in vitro and labeled with [^{3}H]SAM or [^{3}H]UTP*

Rats were killed, their livers were removed, and the nucleoli were isolated as described in MATERIALS AND METHODS. Nucleolar RNA methyltransferase activity was assayed as described in MATERIALS AND METHODS. In one group, [^{3}H]UTP was substituted for [^{3}H]SAM in order to compare the incorporation of UMP and methyl groups. Following incubation, RNA was extracted and hydrolyzed in alkali as described in MATERIALS AND METHODS. To separate mononucleotides from alkali-stable dinucleotides, each alkaline hydrolysate was analyzed by DEAE-cellulose chromatography using a linear gradient of Tris-formate, pH 7.8, containing 7 M urea (0.025–0.2 M) as detailed in MATERIALS AND METHODS. Three separate experiments produced similar DEAE-cellulose profiles. This profile is from a single experiment.

[*methyl*-^{3}H]methionine into total hepatic nuclear RNA *in vivo* increased more than 2-fold. Because messenger RNA precursors are methylated and potential error due to phenobarbital-induced fluctuations of precursor pool size *in vivo* could occur, studies were performed *in vitro* under conditions in which SAM and other precursors were present in excess. Table 1 shows that in rats killed 6 and 16 hr after receiving a single injection of phenobarbital (100 mg/kg), a significant increase in nucleolar RNA methyltransferase activity was observed.

Increased incorporation *in vitro* of labeled methyl group into isolated nucleoli of phenobarbital-treated rats should be reflected by an increase of similar magnitude in the amount of labeled dinucleotides per milligram of DNA. In two separate experiments, rats were killed 16 hr after a single dose of phenobarbital or NaCl; nucleoli were isolated, and the assay for nucleolar RNA methyltransferase activity was performed as described in Table 1. Since sugar methylation of a nucleotide confers on adjacent nucleotides resistance to alkaline digestion, alkali-stable dinucleotides containing methyl groups are formed after incubation of ribosomal RNA for 18 hr in 1 N sodium hydroxide. Table 2 shows that increased nucleolar RNA methyltransferase activity in phenobarbital-treated rats is reflected by similar elevations in labeled, alkali-stable dinucleotides.

Effect of a Single Dose of Phenobarbital on Nucleolar RNA Polymerase Activity

Table 3 shows that 6 hr after phenobarbital treatment there was no increase in the incorporation of [^{3}H]UTP into isolated nucleoli. This assay was carried out in low ionic strength medium in the presence of Mg^{++}. These results are identical with those reported earlier for 16 hr and 4 days of treatment with phenobarbital (8). Earlier studies *in vivo* on labeling of hepatic 45 S RNA with various labeled purines and pyrimidines gave similar results (5). These studies demonstrate that phenobarbital has no significant effect on transcription of ribosomal precursor RNA.

TABLE 1

Effect of a single dose of phenobarbital on hepatic nucleolar RNA methyltransferase activity

Phenobarbital (100 mg/kg) or 0.9% NaCl was injected intraperitoneally as a single dose. Six or sixteen hours after injection of the drug, the rats were killed. Liver nucleoli were isolated and assayed as described in MATERIALS AND METHODS. Each assay was carried out in quadruplicate. The number of experiments is shown in parentheses. Standard errors are indicated.

Treatment	Time	Nucleolar RNA methyltransferase activity	
		SAM incorporation	Ratio of phenobarbital to control[a]
	hr	*pmoles/mg DNA*	
Control	6	980 ± 28 (4)	
Phenobarbital	6	1263 ± 80 (4)	1.29
Control	16	718 ± 51 (6)	
Phenobarbital	16	976 ± 34 (6)	1.36

[a] Differences between control and treated rats at 6 and 16 hr are significant by Student's *t*-test ($p < 0.01$).

Effects of a Single Dose of Phenobarbital on Labeling of Microsomal RNA with [^{3}H]Orotic Acid

Table 4 shows that 6 hr after phenobarbital treatment, labeling of 18 S and 28 S microsomal RNA increased by approximately 50%. These increases in labeling of microsomal RNA 6 hr after treatment of rats with phenobarbital were similar to those observed after 16 hr of phenobarbital treatment (5). A single dose of phenobarbital has no effect on the specific activity of the total acid-soluble nucleotides. These data support the possibility that increased methylation of 45 S RNA molecules may be a mechanism by which phenobarbital treatment enhances the post-transcriptional stability of this RNA and increases the specific activity of labeled ribosomal RNA.

DISCUSSION

These studies demonstrate that a single dose of phenobarbital increases methylation *in vitro* of newly synthesized ribosomal precursor RNA. Furthermore, these results obtained *in vitro* agree with pre-

vious labeling experiments *in vivo* (5). Since nucleolar RNA methyltransferase activity was assayed with saturating concentrations of precursors (Table 1), the possibility that precursor pool sizes might influence the results obtained after phenobarbital (5, 8) was minimized. The present studies support the hypothesis which emerged from previous investigations (5, 8), that phenobarbital enhances labeling of cytoplasmic ribosomal RNA (5–7) through post-transcriptional stabilization of 45 S RNA (5, 8) rather than through transcriptional effects (9).

In mammalian cells, 50–80% of newly synthesized ribosomal RNA is degraded intranuclearly and thus never reaches the cytoplasm (5, 30–34). Ribosomal RNA is methylated primarily in the 2'-hydroxyl position of the ribose moiety concurrently

TABLE 2

Effect of a single dose of phenobarbital on rat hepatic nucleolar RNA polymerase activity

Rats were treated with phenobarbital as described in Table 1. Six hours after injection of the drug, the rats were killed by decapitation. Liver nucleoli from five to eight rats were isolated by sonication, and assays were performed as described in MATERIALS AND METHODS, in low ionic strength medium containing Mg^{++}. Each assay was carried out in quadruplicate. The number of experiments is shown in parentheses. Standard errors are indicated.

Treatment	Time	Nucleolar RNA polymerase activity	
		UMP incorporation[a]	Ratio of phenobarbital to control
	hr	*pmoles/mg DNA*	
Phenobarbital	6	1023 ± 104 (6)	
Control	6	998 ± 154 (6)	1.03

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

TABLE 4

Recovery of alkali-stable dinucleotides from nucleolar RNA labeled in vitro with S-adenosyl-L-[^{3}H]methionine

Rats were killed 16 hr after receiving a single dose of phenobarbital or 0.9% NaCl. Nucleoli were prepared using the sonication technique, and the assay was carried out as described in MATERIALS AND METHODS. RNA methylated *in vitro* was extracted with sodium dodecyl sulfate and phenol. After alkyline hydrolysis of the purified RNA, the nucleotides were eluted by DEAE-cellulose chromatography. Each fraction was collected and analyzed for radioactivity. The results shown are the averages of two separate experiments.

Treatment	Nucleolar RNA methyltransferase		Recovery of alkali-stable dinucleotides	
	Activity	Ratio of phenobarbital to control	Activity	Ratio of phenobarbital to control
	pmoles SAM/mg DNA		*cpm/mg DNA*	
Control	659	1.00	14,079	
Phenobarbital	1,046	1.59	22,673	1.61

TABLE 3

Effect of single doses of phenobarbital on labeling of microsomal RNA with [5-^{3}H]orotic acid

Rats were treated with phenobarbital as described in Table 1. After 3 hr, each rat received 100 μCi of [5-^{3}H]orotic acid intraperitoneally. The rats were decapitated 3 hr later, and specific activity was determined for 28 S and 18 S RNA as described in MATERIALS AND METHODS. Each value is the mean ± standard error of five experiments; three or four rats were used for each experiment.

Treatment	Specific activity of acid-soluble nucleotides: ratio of phenobarbital to control	28 S RNA		18 S RNA	
		Specific activity[a]	Ratio of phenobarbital to control	Specific activity[a]	Ratio of phenobarbital to control
	dpm/A_{260}	*dpm/mg RNA*		*dpm/mg RNA*	
Phenobarbital, 6 hr	1.02	8531 ± 690	1.51	7570 ± 490	1.52
Control		5650 ± 430		4980 ± 390	

[a] Differences between experimental and control means were significant by Student's *t*-test ($p < 0.05$).

with its nucleolar synthesis (26, 35–37). Sugar methylation of the ribosomal RNA precursor confers stability and protects against degradation by nucleases (29, 36–40). Although direct proof is lacking, circumstantial evidence suggests that undermethylated 45 S RNA exists in eukaryotic cells (24, 29). Undermethylated RNA which accumulates in nucleoli of methionine-starved HeLa cells is not processed normally into cytoplasmic ribosomal subunits (29). In the absence of RNA synthesis after treatment of these cells with actinomycin D, methylation of undermethylated RNA occurred when methionine levels returned to normal. Moreover, addition to the nucleolar rRNA methyltransferase assay of undermethylated ribosomal RNA precursor from Novikoff hepatoma cells starved for methionine produced an approximately 2-fold increase in activity. Furthermore, the presence of 2′-*O*-methylated nucleotides in synthetic RNA polymers greatly reduces the hydrolysis of these polynucleotides by exonucleases (41).

Phenobarbital treatment produces only small increases in nucleolar RNA methyltransferase activity (Table 1). Although the number of methyl groups per molecule of newly formed 45 S RNA is known (36), the number of methyl groups necessary for normal stability and maturation of each molecule of 45 S RNA is not. If a limited number of specific sites that are methylated on the 45 S RNA molecule are essential for stability, only small decreases in methylation per molecule would render the 45 S RNA molecule unstable.

Hypermethylation of existing 45 S RNA may also occur in phenobarbital-treated rats (8). However, our observations suggest that phenobarbital raises to normal levels the methylation of some undermethylated 45 S RNA molecules, which otherwise would be most susceptible to intranuclear degradation. Phenobarbital apparently confers stability on these 45 S RNA molecules, enabling them to be "salvaged" and to be transported to the cytoplasm as ribosomal subunits. The mechanism by which phenobarbital stimulates methylation is unclear (6). From these experiments, methylation of 45 S ribosomal precursor RNA appears to be one regulatory step for the stability and transfer of ribosomal subunits from the nucleus to the cytoplasm.

Although no decrease was observed in nuclear RNase activity in rats treated with phenobarbital (8), alterations in specific exonucleases (42–44) and endonucleases (45–49) could also be involved in the metabolic stabilization of 45 S RNA. Phenobarbital treatment of rats may also alter the phosphorylation of nucleolar ribosomal proteins (50). Changes in these and other nuclear proteins may affect the stability and maturation of nascent nucleolar preribosomal subunits (38, 50).

Like the results obtained 16 hr after phenobarbital treatment (5, 8), the present experiments reveal that phenobarbital influences rRNA metabolism 6 hr after phenobarbital treatment (Tables 1–4), the time at which significant induction of drug-metabolizing enzymes is first observed (1–10). Studies performed both *in vivo* (6, 51) and *in vitro* (52) suggest that RNA synthesis may be a rate-limiting step during initial stages of the induction process.

In two separate experiments, nucleolar RNA methyltransferase activity was studied in rats killed at 6:00 a.m. This activity was 35% higher in rats receiving 0.9% NaCl intraperitoneally at midnight than in rats receiving it at 2:00 p.m. or rats that were neither injected nor handled. There is a consistent difference between the control enzyme activities 6 hr and 16 hr after phenobarbital treatment ($p < 0.05$ by Student's *t*-test). This difference may be due to stress-induced effects as influenced by diurnal variations (see Fig. 4 of ref. 53). Phenobarbital treatment has no effect on diurnal variations in the levels of plasma corticosterone (53).

To establish unequivocally the effects of phenobarbital on rRNA metabolism, enzymes involved in processing 45 S RNA, such as nucleolar RNA methyltransferase and various nuclear nucleases, must be solubilized and characterized. Thus far, attempts to solubilize nucleolar RNA methyltransferase have been unsuccessful (25).

Although nuclear nucleases have been solubilized, they have not been fully characterized (42–49).

Experiments performed in other laboratories (54) suggest that phenobarbital treatment stimulates aggregate RNA polymerase II activity in isolated nuclei. It is possible that phenobarbital treatment might also affect messenger RNA synthesis, its post-transcriptional stability, or both.

ACKNOWLEDGMENTS

We are grateful to Dr. S. T. Jacob for helpful discussions during the course of this work, and to Mr. Howard Beittenmiller for technical assistance.

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