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The Effect of Acute and Chronic Phenobarbital Treatment on the Activity of Rat Liver Deoxyribonucleic Acid-Dependent Ribonucleic Acid Polymerases

THOMAS J. LINDELL,[1] ROYAL ELLINGER, JAMES T. WARREN, DAVID SUNDHEIMER, AND ANTHONY F. O'MALLEY[2]

Department of Pharmacology, University of Arizona College of Medicine, Tucson, Arizona 85724

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SUMMARY

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Eukaryotic DNA-dependent RNA polymerases (I, II, and III) have a diurnal rhythm which is altered by the administration of a single dose of phenobarbital (60 mg/kg) in young rats on a 12-hr light, 12-hr dark lighting schedule. Constant lighting essentially abolished the pattern of activity of RNA polymerases I and III and produced a minor variation in the rhythm of RNA polymerase II. Administration of phenobarbital (100 mg/kg) to animals maintained in constant light altered the diurnal rhythm of RNA polymerase II but did not change the activities of RNA polymerases I and III. No significant alteration in the activity of any of the RNA polymerases was observed by comparison of phenobarbital-treated rats with NaCl-treated controls under either normal or constant lighting when individual points across the 24-hr period were averaged. Under both lighting protocols phenobarbital produced an increase in plasma corticosteroid 6-9 hr after administration, which could not be correlated with changes in the activities of the RNA polymerases. No relationship could be established between the normal pattern of plasma corticosteroid and RNA polymerase activities. Chronic administration of phenobarbital (100 mg/kg/day) did not stimulate nuclear RNA polymerases on days 1-4 of administration; only on day 5 was stimulation observed. The 5-day average RNA polymerase II activity was significantly depressed in phenobarbital-treated compared with control animals. No change in liver RNA polymerases was observed in older rats given phenobarbital in their drinking water (1 mg/ml) for 10 days. The results suggest that hypertrophy induced in the liver by phenobarbital is not accompanied by a discernible increase in hepatic transcription as manifested by increases in RNA polymerase activity, and support other studies in which decreased turnover and/or stabilization of hepatic RNA was found.

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[1] To whom requests for reprints should be directed.

[2] Recipient of a fellowship (CA-05363) from the National Institutes of Health.

INTRODUCTION

Phenobarbital is a barbiturate which has the additional capability of being able to induce hepatic drug-metabolizing enzymes (1). The consequence of this action

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is an increased metabolism of phenobarbital itself as well as other xenobiotics and endogenous compounds (2). This induction of drug-metabolizing enzymes by barbiturates has been widely studied and found to be correlated with an increase in smooth endoplasmic reticulum (3–6); however, specific molecular events in this induction remain to be elucidated. As an inducer, phenobarbital has been found to affect stimulation of DNA-dependent RNA synthesis (7) and the synthesis of specific mRNA(s), as evidenced by the inhibition of this induction by actinomycin D (5, 8, 9) and, more recently, α-amanitin (10). Some investigators have suggested that there is an increase in ribosomal RNA (11, 12) and/or an increased post-transcriptional stabilization of 45S RNA or cytoplasmic RNA (11, 13–18). Since there is a general increase in cytoplasmic ribosomes (19) and an increased protein synthetic capacity (19, 20), we wondered whether we could observe any effect on the activity of the nucleolar DNA-dependent RNA polymerase I activity in rat liver nuclei isolated from phenobarbital-treated compared with control animals. Other tissue-specific hormones are known to cause changes in the activity of nucleolar RNA polymerase, which is thought to be involved in the ultimate hypertrophic response in the target tissue (21).

It was felt that a reinvestigation of the effect of phenobarbital on eukaryotic transcription was warranted for two reasons. Earlier studies which suggested stimulation of RNA polymerase activity (7) did not permit any conclusion as to which RNA polymerase was stimulated, owing to the method of assay. These studies are now feasible through the use of α-amanitin, a specific inhibitor of RNA polymerase II (22–24), in nuclear assays to assess the contributions of RNA polymerases II and I plus III (22). Second, we wished to determine the possible effect of a xenobiotic on the diurnal variation in hepatic RNA polymerases (25).

We have confirmed that DNA-dependent RNA polymerases display a diurnal variation but have found that the administration of a single dose of phenobarbital (60 or 100 mg/kg) alters this diurnal pattern. While RNA polymerase activities may vary from controls at certain times of the day, the mean activities of RNA polymerases across the period of study are generally unaltered or depressed. This result suggests that while specific inductive effects are observed, these effects are not manifested in a general increase in the activity of liver RNA polymerases. Similar results are presented for two different protocols of chronic treatment with phenobarbital.

METHODS

Animals. All rats employed in these experiments were male animals of the Sprague-Dawley strain and were obtained from the Division of Animal Resources at the University of Arizona College of Medicine. Rats were bred within this facility from stock obtained from Charles River Laboratories and were maintained on Purina rat chow ad libitum unless otherwise stated. Animals were obtained as weanlings and housed three per cage. They were maintained on a 12-hr light, 12-hr dark (normal) lighting schedule (light from 6:00 a.m. to 6 p.m.) or constant lighting for at least 2 weeks prior to any study. Sodium phenobarbital (Lilly) was dissolved in 0.15 M NaCl and injected intraperitoneally in doses specified in the various experiments. When given in the drinking water, phenobarbital was dissolved at a concentration of 1 mg/ml. Under all conditions when plasma corticosteroid was to be measured, the animals were handled as little as possible prior to killing.

Isolation of rat liver nuclei. Animals were killed by decapitation, and blood was collected where indicated. Individual livers were weighed and nuclei were isolated by a modification of the method of Blobel and Potter (26), as described by Lindell (27). Nuclear pellets were resuspended in a volume of 1 M sucrose, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10 mM Tris-HCl, pH 7.5, equivalent to one-half the weight (w/v) of the whole liver.

RNA polymerase assays in isolated liver

nuclei. RNA polymerase assays were performed in liver nuclei as described by Thut and Lindell (28). Incorporation was linear over the time of the assay (5 min), and this linearity was still evident when twice the amount of nuclei was used in the assay. Nuclear DNA of samples ranged from 30 to 80 μg/50 μl. α-Amanitin- (Henley and Company, New York) insensitive activity is termed RNA polymerase I plus III activity (22) because the amount of α-amanitin employed (0.1 μg/assay) was not sufficient to inhibit RNA polymerase III (29). The amount of RNA polymerase III in routine nuclear assays is never over 20% (30), and often much lower.[3] All activities are expressed in picomoles of UMP incorporated in 5 min per milligram of DNA. DNA was determined by the method of Burton (31), using calf thymus DNA (Sigma type II) as a standard.

Determination of plasma corticosteroid. Blood from individual decapitated animals was collected in heparinized tubes. Plasma was prepared by centrifugation of the heparinized blood and pipetting the supernatant to a plastic tube. Individual samples were frozen until corticosteroid determination could be performed. Corticosteroid assays were done on 50-μl samples by the method of Glick *et al*. (32) as modified by Brown and Hedge (33).

RESULTS

Effect of acute phenobarbital (60 mg/kg) on hepatic RNA polymerases of rats maintained under normal lighting and fed ad libitum. Because of the inconsistent results we had obtained in past experiments after single injections of phenobarbital and isolation of nuclei at various times, we felt it necessary to examine the activities of RNA polymerases in isolated liver nuclei at numerous times during a 24-hr period after a single injection of phenobarbital (60 mg/kg) relative to animals which had been injected with 0.15 M NaCl alone. Fig. 1 shows that at various times there were significant differences in the activities of RNA polymerases I plus III and II relative to the NaCl-injected control animals, although no trend of stimulation could be observed throughout the 24-hr period.

Effect of acute phenobarbital (100 mg/kg) on hepatic RNA polymerases of rats maintained on constant lighting. The data for control and phenobarbital-treated rats in Fig. 1 were obtained on separate days. In order to compare the experimental and control animals more exactly, we decided to examine them on the same day. Also, it was felt that if the diurnal variation in RNA polymerase activities could be eliminated, a clearer relationship could be established for the possible role of phenobarbital in hepatic RNA transcription. We found that adrenalectomy and hypophysectomy did not abolish the diurnal rhythm of these enzyme activities (data not shown). We did find, however, that constant lighting for 2 weeks prior to phenobarbital injection (100 mg/kg) produced much less variation in RNA polymerase II activity and virtually eliminated the diurnal rhythm of RNA polymerase I plus III activities (Fig. 2). Phenobarbital did not produce any significant elevation or depression in RNA polymerase II activity across the 24-hr period studied. There were two time points (2:30 p.m. and 5:30 a.m.) where significant changes in RNA polymerase I plus III activity were seen. RNA polymerase I plus III activities were depressed at 2:30 p.m. and elevated at 5:30 a.m., but 24-hr averages showed no discernible stimulation or depression of either RNA polymerase II or I plus III by phenobarbital (Table 1).

Effect of chronic phenobarbital treatment (100 mg/kg/day for 5 days) on activity of hepatic RNA polymerases. Phenobarbital (100 mg/kg) was administered to rats at 8:30 a.m. for 1–5 days. Controls received 0.15 M NaCl. Each day three phenobarbital-treated and three control animals were killed while the remaining animals received additional injections of phenobarbital or NaCl. All nuclei were immediately prepared and frozen in liquid nitrogen. Assays of all nuclei were performed at the same time. On days 1–4 the ratio of RNA polymerase activities in nuclei from phenobarbital-treated relative to control rats was depressed below 1.0. Only on day

[3] R. G. Roeder, personal communication.

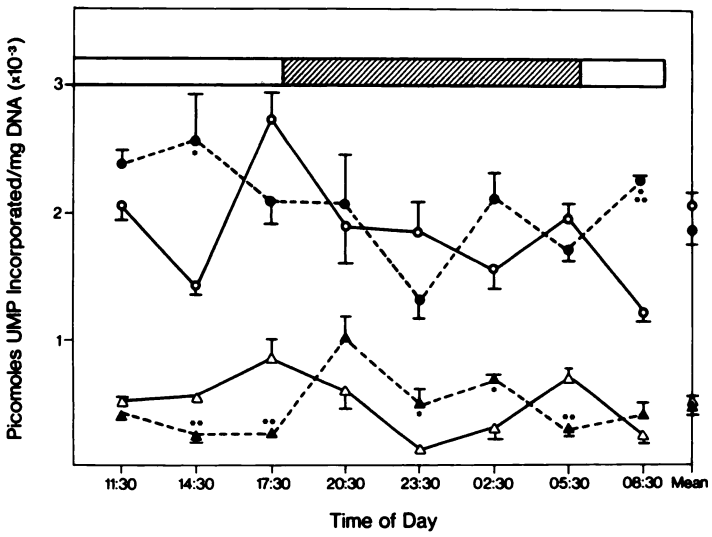

FIG. 1. *Effect of phenobarbital on diurnal variation of RNA polymerases in nuclei isolated from rat liver*

Weanling rats were obtained from a local breeding colony and housed three per cage for 2 weeks with complete access to food and water as described in METHODS. The hatched area indicates the dark part of the cycle. Experiments with NaCl-injected control and phenobarbital-treated animals (60 mg/kg) were performed on separate days. Injections were administered at 8:30 a.m., and the first time point was 11:30 a.m. At the time the rats were killed, control animals weighed 142 ± 2.96 g while phenobarbital-treated animals weighed 145 ± 3.27 g. Animals were killed (three per point) at the times specified, their livers were weighed and homogenized, and nuclei were isolated, suspended, and assayed immediately after suspension as described in METHODS. RNA polymerase activities are expressed as picomoles of UMP incorporated during 5 min per milligram of DNA. All points are the mean of the RNA polymerase activities from three separate animals ± standard error of the mean. Where error bars are not evident, they lie within the symbols. RNA polymerase II activity: ○——○, control; ●---●, phenobarbital. RNA polymerases I plus III: △——△, control; ▲---▲, phenobarbital. Values with asterisks are significantly different from controls. Mean RNA polymerase activities per day ± standard errors are plotted at the right side of the graph.

* $p < 0.05$; ** $p < 0.02$; *** $p < 0.001$.

5 was the activity of RNA polymerases from the treated animals greater than controls. The ratio for RNA polymerases I plus III was 1.57, while that for RNA polymerase II was 1.20. There was no increase in RNA polymerase I plus III activities when averaged over the 5-day period of study (Table 1). There was, however, a significant depression ($p < 0.05$) of RNA polymerase II activity in phenobarbital-treated animals relative to NaCl-injected controls.

Effect of phenobarbital in drinking water (1 mg/ml for 10 days) on hepatic RNA polymerases of older rats. Phenobarbital and food were allowed ad libitum to older rats (heavier than 300 g) for 10 days, with a normal lighting schedule. Three phenobarbital-treated and three control rats were killed on day 10 at 10:30 a.m. and 3:30 p.m. (Fig. 3). Only at the earlier time point was any significant change from controls observed. Phenobarbital depressed the activity of RNA polymerases I plus III ($p < 0.005$), but when phenobarbital and control data were respectively averaged, there was no significant difference between the two groups (Table 1).

Effect of phenobarbital on plasma corticosteroid levels after acute phenobarbital (60 mg/kg) administration. To assess the possible role of endogenous corticosteroid on the diurnal rhythm of hepatic RNA polymerases in both control and phenobarbital-treated animals, the level of this steroid was measured in plasma prepared individually from the same rats as in the experiments of Fig. 1. Figure 4 shows a diurnal variation in plasma corticosteroid which was significantly higher than con-

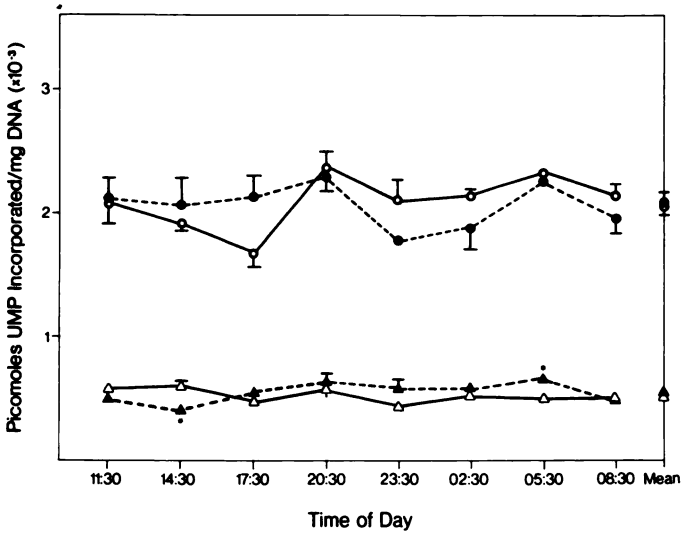

FIG. 2. *Effect of constant lighting on diurnal rhythm of RNA polymerases of rats treated with NaCl (controls) or a single acute injection of phenobarbital (100 mg/kg)*

Forty-eight animals were housed in groups of three and treated exactly as described in the legend to Fig. 1 and METHODS, except that they were maintained under constant lighting conditions for 2 weeks prior to the start of the experiment. Animals were weighed the night before the study was begun (138 ± 1.36 g), and phenobarbital or NaCl was injected at 8:30 the following morning. Animals were killed (six per time point; three control and three phenobarbital) beginning at 11:30 a.m., and nuclei were isolated and assayed as described in METHODS. RNA polymerase activities are expressed as in the legend to Fig. 1. Each point represents the mean RNA polymerase activity of three separate animals ± standard error. Where error bars are not evident, they lie within the symbols. RNA polymerase II activity: O——O, control; ●---●, phenobarbital. RNA polymerases I plus III activities: Δ——Δ, control; ▲---▲, phenobarbital. Mean RNA polymerase activities per day ± standard errors are plotted at the right.

*Significantly different from control ($p < 0.05$).

trol levels 6 hr (2:30 p.m.) after the administration of an acute dose of phenobarbital. There was also a significant depression below controls at 12 hr (8:30 p.m.). However, the mean plasma corticosteroid level in phenobarbital-treated animals across the 24-hr period of the study was not significantly different from controls (18.4 ± 1.41 and 20.3 ± 2.23 μg/100 ml of plasma in control and phenobarbital-treated rats, respectively). This rise in plasma corticosteroid was also observed in the phenobarbital-treated rats 6 hr after injection in the constant lighting study of Fig. 2 (data not shown). We could not establish a correlation between plasma corticosteroid levels and RNA polymerase activities assayed in nuclei isolated from either rats treated with phenobarbital or controls.

DISCUSSION

Numerous hormones produce hypertrophy as part of their action in target tissues (21), and this is accompanied by a prior increase in nucleolar RNA polymerase I activity (34–44). It was felt that since phenobarbital is a classic inducer of liver hypertrophy, a prior increase in RNA polymerase I might be observed in assays of nuclei from phenobarbital-treated animals relative to control animals. A problem, however, was encountered in assessing the effect of phenobarbital on the activity of hepatic RNA polymerases, since there is a diurnal rhythm in the activity of these enzymes in normal animals (25). We have observed an alteration in this rhythm after the administration of 60 mg/kg of phenobarbital (Fig. 1). The rhythm observed in the phenobarbital-treated animals was unique compared with controls and did not represent a simple shift due to the administration of a hypnotic dose of phenobarbital. While there were time points which showed significant stimulation of these enzyme activities, there were also time

TABLE 1

Effect of phenobarbital on rat liver nuclear RNA polymerase activities

Normal lighting refers to 12 hr of light and 12 hr of darkness. All injections were administered at 8:30 a.m. Results are means and standard errors.

Protocol	Weight	No. of rats	RNA polymerase activity	
			I + III (ratio)[a]	II (ratio)[a]
	g		*pmoles UMP incorporated/mg DNA*	
Acute (60 mg/kg), normal lighting				
Control	142 ± 2.96	27	495 ± 48.4	1860 ± 96.6
Phenobarbital	145 ± 3.27	24	475 ± 60.1 (0.96)	2050 ± 101 (1.10)
Acute (100 mg/kg), constant lighting				
Control	138 ± 1.36	24	518 ± 14.7	2090 ± 55.2
Phenobarbital		23	538 ± 22.0 (1.04)	2050 ± 57.3 (0.98)
Chronic (100 mg/kg × 5 days), normal lighting				
Control	130 ± 3.28	15	765 ± 67.0	1640 ± 105
Phenobarbital		15	650 ± 44.0 (0.85)	1310 ± 97.0[b] (0.80)
Chronic (1 mg/ml × 10 days), normal lighting				
Control	315 ± 4.17	6	980 ± 126	2180 ± 236
Phenobarbital		6	870 ± 45.6 (0.89)	1910 ± 88.9 (0.88)

[a] Ratio of phenobarbital to control.

[b] Significantly different from control ($p < 0.05$).

points where significant depression was observed relative to controls. When these time points were averaged (Table 1), there was no difference between the activities of RNA polymerases in phenobarbital-treated and control rats.

Since rats are nocturnal, hypnotic doses of phenobarbital were always administered in the morning, during the light period, to minimize alterations in feeding behavior induced by a hypnotic dose of this drug. This is an important consideration, since feeding alters hepatic RNA polymerase activities and fasting has been found to abolish their diurnal rhythm (45). Fasting causes a decrease in rRNA transcription (46) and the activity of nucleolar RNA polymerase I (47). Therefore, we felt that fasting was too drastic a measure to attempt to abolish the diurnal rhythm of RNA polymerases as suggested by Barbiroli *et al.* (45). In order to obtain a more unequivocal assessment of the effect of phenobarbital on hepatic RNA polymerases, however, it was thought necessary to minimize the diurnal variation seen in Fig. 1. Constant lighting has been observed to abolish the diurnal rhythm in the endoplasmic reticulum and hexobarbital oxidase activity (48, 49) in rat liver. We therefore maintained rats under constant lighting conditions for 14 days prior to the injection of a single dose of phenobarbital (100 mg/kg) or NaCl (Fig. 2). The activity patterns of both RNA polymerases II and I plus III were different from the control data of Fig. 1. While RNA polymerase II still displayed a slight rhythm, RNA polymerases I plus III were markedly changed, showing little or no rhythm. The administration of phenobarbital produced no significant trend of stimulation in any of the RNA polymerases relative to controls.

Although the constant lighting protocol may have inherent disadvantages, it can be seen from Table 1 that the mean activities of the RNA polymerases compare favorably with those of the rats maintained on a normal lighting schedule. This indicates that the activities of these enzymes were within the normal range for animals of this size and age. Furthermore, within the 2-week period of this study, the animals' growth was within normal limits, indicating that they consumed normal

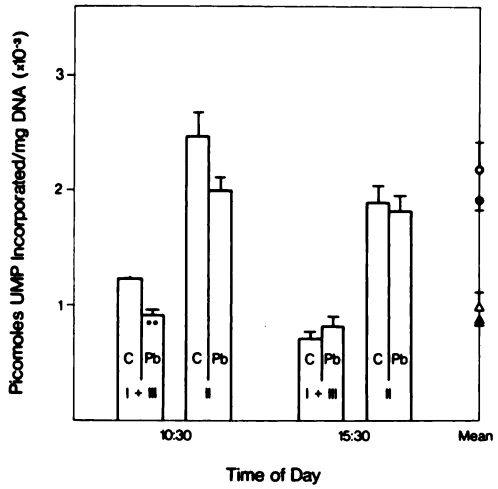

FIG. 3. *Effect of chronic phenobarbital in drinking water on activity of RNA polymerases assayed in liver nuclei*

Adult male rats (315 ± 4.17 g) were given phenobarbital (1 mg/ml) in drinking water for 10 days, at which time three control and three phenobarbital-treated animals were killed at 10:30 a.m. and 2:30 p.m. Nuclei were isolated and assayed immediately after resuspension in buffer as described in METHODS. The activity of RNA polymerases is expressed as in the legend to Fig. 1. Data for NaCl-injected controls (C) and phenobarbital (Pb)-treated rats are depicted side by side, RNA polymerases I plus III at the left and RNA polymerase II at the right in each group. Each group represents the mean of three separate animals ± standard error of the mean. Where error bars are not evident, they lie within the symbols.

**Significantly different from control ($p < 0.005$).

quantities of food even though diurnal feeding patterns may have been affected. The two chronic phenobarbital regimens gave essentially the same result, in that no significant stimulation of RNA polymerase activities was observed. Only in the chronic injection experiment (Table 1) was there any significant difference between phenobarbital-treated and control animals; this was a depression of RNA polymerase II activity.

These studies suggest that phenobarbital does not increase the activity of nucleolar RNA polymerase I in the manner of a number of hormones in target tissues (34–44). While phenobarbital does increase the number and types of polyribosomes (16, 19, 20, 50) and protein synthesis (51), suggest-

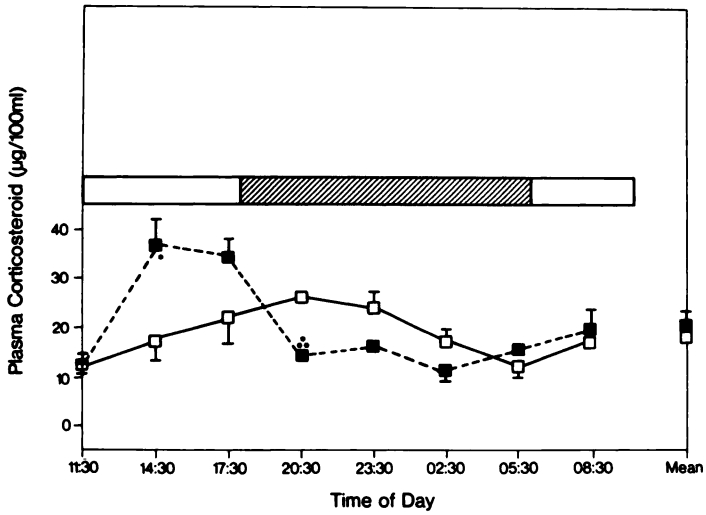

FIG. 4. *Effect of acute phenobarbital treatment (60 mg/kg) on diurnal plasma corticosteroid levels*

Blood was collected individually from the same rats as in the experiment depicted in Fig. 1. The hatched area indicates the dark part of the cycle. Plasma corticosteroid was determined as described in METHODS. Corticosteroid levels are expressed as micrograms per 100 ml of plasma. Each point represents the mean corticosteroid determination on three separate animals ± standard error of the mean. Where error bars are not evident, they lie within the symbols. □——□, NaCl-injected control rats; ■---■, phenobarbital-treated rats. Values with asterisks are significantly different from controls. Mean plasma corticosteroid levels per day are displayed at the right side of the graph.

* $p < 0.05$; *** $p < 0.001$.

ing hypertrophy, it does not appear that the primary stimulus is on the transcription of rRNA. Similar results were reported by Smith *et al.* (14, 52), who saw no increase in RNA polymerase I in nucleoli isolated from phenobarbital-treated rats. This study lends further support to post-transcriptional events, such as the stabilization of RNA against turnover. Smith *et al.* (52) suggested that this stabilization may be due to increased methylation of 45S RNA, whereas Steifert and Vacha (15) and Louis-Ferdinand and Fuller (17) observed a decrease in cytoplasmic RNase activity. The mechanism of this decrease in cytoplasmic RNase activity is not known.

The observation of an altered diurnal rhythm of enzyme activities induced by a xenobiotic is an important consideration for studies in which RNA polymerase and other enzyme activities are measured after the administration of drugs and carcinogens, as originally suggested by Glasser and Spelsberg (25). The use of animals maintained under constant lighting may be an important consideration in such studies. The virtual abolition of a diurnal rhythm of RNA polymerases I plus III in constant lighting suggests a potential relationship of RNA polymerase I activity to the normal diurnal rhythm of the endoplasmic reticulum. A comprehensive study comparing the changes in endoplasmic reticulum with RNA polymerase I activity would be of interest in exploring this possible relationship.

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