MOLECULAR PHARMACOLOGY, 6, 540-547

The Metabolism of Ribonucleic Acid in Rat Liver after Phenobarbital Administration

A. M. COHEN[1] AND R. W. RUDDON[2]

Departments of Pharmacology and Oral Biology, The University of Michigan Medical and Dental Schools, Ann Arbor, Michigan 48104

(Received April 4, 1970)

SUMMARY

Incorporation of orotic acid-5-^{3}H (100–200 μCi) into hepatic nuclear and cytoplasmic RNA extracted from purified nuclei or cytoplasmic fractions and separated on linear sucrose gradients was determined after intraperitoneal administration of phenobarbital (75 mg/kg) to rats. Pulses of 1-, 2-, 4-, and 12-hr duration were administered 2 hr after injection of phenobarbital or 0.9% NaCl in order to examine alterations in cytoplasmic RNA metabolism early in the induction process, i.e., before any marked increase occurred in the levels of microsomal drug-metabolizing enzymes. The data indicated that there was a 20–30% increase above controls in the specific activity of 28 S and 18 S ribosomal RNA, which became apparent after a 2-hr pulse dose of labeled precursor administered 2 hr after phenobarbital treatment, and which was maintained for 12 hr. The augmented labeling appeared to be restricted to ribosomal RNA species. Analysis of nuclear RNA after pulses of ½, 1, 2, and 4 hr, given 2 hr after phenobarbital, indicated that there was no detectable increase in synthesis of ribosomal RNA or ribosomal RNA precursors. It is suggested that phenobarbital may enhance the stability of hepatic ribosomal RNA or increase the "processing" and transport of this substance to the cytoplasm, and that these events, which occur early in the induction process, may be involved in the elevation of microsomal oxidase activity observed after phenobarbital administration.

INTRODUCTION

Phenobarbital is the prototype of one of the major categories of chemical substances capable of increasing the level of mammalian hepatic microsomal drug-metabolizing enzymes (1). However, the mechanism by which phenobarbital exerts this inductive effect has not yet been well defined. Studies employing inhibitors of DNA-dependent RNA synthesis or of protein synthesis (2–4) have indicated that the increase in enzyme activity following phenobarbital treatment is due to an increased synthesis of enzyme. Additional evidence for this has come from experiments which have shown that phenobarbital treatment results in an increased ability of hepatic microsomes from treated animals to incorporate amino acids into protein *in vitro* (5). Moreover, it has been reported that hepatic nuclei (6) and chromatin (7) isolated from phenobarbital-treated rats have an enhanced capacity to synthesize RNA *in vitro*.

[1] Predoctoral Fellow of the National Institute of Mental Health (5 F01 MH 37949).

[2] Research supported by Grant NIH-DE-07231 from the National Institutes of Health. Address requests for reprints to R. W. Ruddon, Department of Oral Biology, Pharmacology Section, New Dental Building, Ann Arbor, Michigan 48104.

In addition to these effects on RNA and protein synthesis, other investigations have indicated that phenobarbital treatment may result in partial to complete inhibition of the degradation of various hepatic microsomal constituents. The studies of Holtzman and Gillette (8) indicate that the increased phospholipid content of the microsomes from phenobarbital-treated rats may be due, in part, to inhibition of phospholipid catabolism. Similarly, the studies of Shuster and Jick (9, 10) and of Omura *et al.* (11) indicate that daily treatment with phenobarbital results in inhibition of the breakdown of hepatic microsomal NADPH–cytochrome *c* reductase and cytochrome b_5 and in partial inhibition of the degradation of other microsomal proteins. It appears, therefore, that phenobarbital may exert its inductive effect both by increasing the synthesis of microsomal enzymes and by inhibiting their breakdown.

Whereas the effect of phenobarbital on the turnover of microsomal proteins has been the subject of several investigations, its actions on the various species of RNA concerned with protein synthesis have not been extensively examined. To gain a better understanding of the relationship between RNA metabolism and enzyme induction, we have studied the effects of a single dose of phenobarbital on the incorporation of labeled precursor into hepatic nuclear and cytoplasmic RNA during the initial stages of induction.

METHODS

Male Sprague-Dawley rats (Spartan Farms, Lansing, Mich.) weighing 60–70 g were given a single intraperitoneal injection of either phenobarbital (75 mg/kg) dissolved in 0.9% NaCl or 0.9% NaCl alone. Two hours later each rat received a pulse dose (100 or 200 μCi) of orotic acid-5-^{3}H (940 or 2810 mCi/mmole, New England Nuclear Corporation) via the tail vein. Four control and four phenobarbital-treated rats were killed at either ½, 1, 2, 4, or 12 hr after receiving the labeled precursor. Food was withheld from the animals during the pulse periods. The livers from the rats in each group were removed and pooled in 5 volumes of ice-cold 0.32 M sucrose–3 mM $MgCl_2$, and then homogenized in a glass homogenization tube fitted with a Teflon pestle. The resulting homogenates were centrifuged at 1000 × *g* for 10 min to obtain a crude nuclear pellet and the cytoplasmic supernatant fraction. Nuclei were further purified by resuspension and centrifugation through 2.4 M sucrose–1 mM $MgCl_2$. RNA was extracted from the purified nuclei and cytoplasmic fraction according to methods outlined by Steele and Busch (12). Briefly, the purified nuclei were mixed with a volume of buffer (0.3% sodium dodecyl sulfate–0.1 M NaCl–0.05 M sodium acetate, pH 5) equivalent to the original wet weight of liver, and then an equal volume of phenol–*m*-cresol mixture (4:1), containing 0.1% 8-hydroxyquinoline and saturated with the previous buffer, was added. This combination was homogenized in a glass tube fitted with a Teflon pestle and transferred to a water bath at 65°. The RNA was extracted by vigorous agitation of the phenol-buffer mixture for 10 min in a water bath, followed by 10 min of shaking at room temperature in a vibrating shaker. The aqueous phase, containing the extracted RNA, was separated from the phenol phase by centrifugation at 20,000 × *g* in a Sorvall centrifuge. The aqueous phase was then removed by pipette and subjected to two further extractions at room temperature with fresh phenol mixture.

The phenol phase from the first extraction was combined with an equal volume of fresh 0.3% sodium dodecyl sulfate–0.1 M NaCl–0.05 M sodium acetate buffer at pH 5 and agitated at room temperature to extract any remaining RNA. The aqueous phase from this re-extraction was separated as before and washed once with fresh phenol mixture. To the combined aqueous phases, 2.5 volumes of absolute ethanol containing 2% potassium acetate were added, and the RNA was allowed to precipitate overnight at 0°. The resulting RNA was pelleted by low-speed centrifugation, and the ethanol phase was decanted. A volume of 0.01 M NaCl equal to that of the combined aqueous phases was then added to dissolve the RNA and extract any low molecular weight,

water-soluble contaminants. Subsequently 2.5 volumes of the absolute ethanol solution were again added to precipitate the RNA over a 1–2-hr period at −20°. The resulting RNA pellet was dissolved in 0.1 M NaCl–0.01 M sodium acetate, pH 5, for analysis on a linear sucrose gradient.

Cytoplasmic RNA was extracted from the postnuclear supernatant fraction by a similar procedure, except that the volume of the sodium dodecyl sulfate-containing buffer added originally was equal to the volume of the cytoplasmic fraction and the original extraction was carried out at room temperature.

The resulting nuclear or cytoplasmic RNA fractions contained no detectable protein [by the method of Lowry *et al.* (13)], and the DNA content, determined by the diphenylamine reaction, was not more than 7% of the total nucleic acid of the extracts. Samples containing 1 mg of RNA (as determined by the orcinol reaction) were layered on linear sucrose gradients made up in 0.3% sodium dodecyl sulfate–0.1 M NaCl–0.05 M sodium acetate buffer, pH 5. The gradients were centrifuged at 25° in the SW 25.1 rotor on a model L2-65B Spinco ultracentrifuge. After centrifugation, the tubes containing the gradients were punctured, and the resulting effluent was allowed to pass through a flow cell adapter in a Beckman DB recording spectrophotometer set to record at 260 mμ. Fractions (0.8 ml) were collected directly in scintillation counting vials. To each fraction, 15 ml of Bray's solution (14) containing 6% Cab-O-Sil were added, and the radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer. The resulting radioactivity profile was then correlated with the optical density pattern recorded at 260 mμ, and all results were recorded as counts per minute per absorbance unit at 260 mμ.

The specific activity of the acid-soluble nucleotide pool was determined after pulse doses of 50 μCi of orotic acid-^{3}H for 10, 20, 30, 60, 120, or 240 min. The labeled precursor was injected intravenously 2 hr after phenobarbital (75 mg/kg) or 0.9% NaCl. The livers were removed and homogenized in 3 volumes of 0.27 N perchloric acid. The homogenate was centrifuged at 20,000 × *g* for 10 min, and the resulting supernatant fraction was analyzed for absorbance at 260 mμ and radioactivity.

RESULTS

Previous studies in our laboratory have shown that the induction of hepatic microsomal ethylmorphine *N*-demethylase activity produced by a single dose of phenobarbital can be inhibited by actinomycin D only if the latter drug is given within the first 8 hr following the barbiturate (15). When the inhibitor of DNA-dependent RNA synthesis was administered more than 8 hr after phenobarbital, the extent of enzyme induction was the same as in animals given phenobarbital alone. This finding indicated that RNA synthesis was a rate-limiting event only during the initial stages of the induction process. However, this type of study does not clearly answer the question whether phenobarbital produces its inductive effect through stimulation of RNA synthesis or via inhibition of RNA degradation with synthesis proceeding normally, since in either case continued synthesis of at least a basal amount of RNA would be essential to maintain the protein-synthesizing apparatus necessary for synthesis of microsomal enzymes, and inhibition of enzyme induction by actinomycin D would not distinguish between these two alternatives. In order to determine more directly which of these mechanisms operates during the initial stages of phenobarbital induction, hepatic nuclear and cytoplasmic RNA fractions were labeled with orotic acid-^{3}H for periods ranging between 1 and 12 hr after treatment with either 0.9% NaCl or phenobarbital. The labeled RNA species were then separated and analyzed on linear sucrose gradients. In this way it was possible to examine the effects of phenobarbital on the various types of RNA and also to correlate events occurring in the nuclei with those in the cytoplasm at a given time. We felt that this approach would enable us to differentiate between the effects of phenobarbital on the synthesis of RNA and effects of the drug on "post-transcriptional" events.

Figures 1, 2, and 3 show the optical

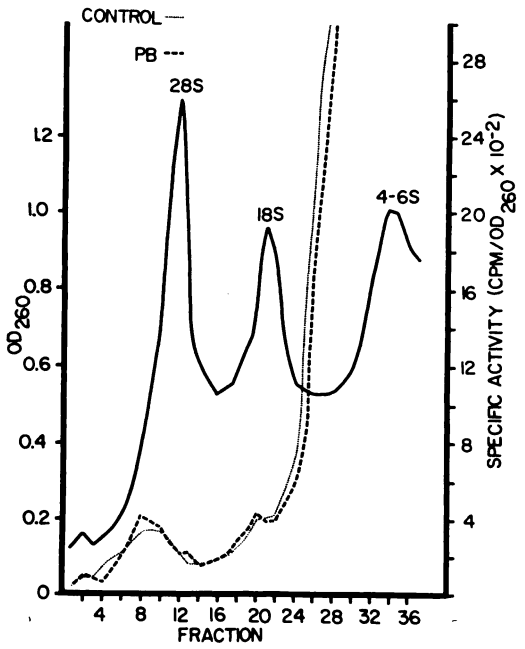

FIG. 1. *Sucrose gradient analysis of cytoplasmic RNA: 1-hr pulse*

Samples (1 mg) of hepatic cytoplasmic RNA, extracted and pooled from four control or four phenobarbital (PB)-treated rats after a 1-hr pulse dose of 200 μCi of orotic acid-^{3}H, were layered over linear 10–30% (w/w) sucrose gradients made up in 0.3% sodium dodecyl sulfate–0.1 M sodium chloride–0.05 M sodium acetate, pH 5. Gradients were centrifuged at 24,000 rpm in the Spinco SW 25.1 rotor for 16 hr at 25°. Tubes were then punctured, and the absorbance at 260 mμ as well as the radioactivity of the resulting fractions were determined. Results are reported as counts per minute per absorbance unit at 260 mμ. The solid line represents the absorbance profile.

density and corresponding specific radioactivity profiles of hepatic cytoplasmic RNA from rats treated with phenobarbital or NaCl 2 hr prior to pulse-labeling with orotic acid-^{3}H for 1, 2, and 4 hr, respectively. As can be seen, at the end of the 1-hr pulse the specific radioactivity profiles from control and phenobarbital-treated rats were almost identical, with the possible exception of the area under the 28 S ribosomal RNA peak, where there was a suggestion of enhanced labeling in the phenobarbital-treated rats. However, after 2 hr of labeling there was a definite increase in the specific activities of the 28 S and 18 S ribosomal RNAs. This effect was not seen

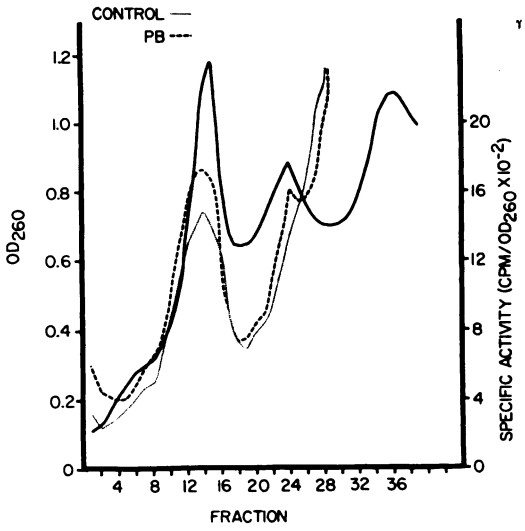

FIG. 2. *Sucrose gradient analysis of cytoplasmic RNA: 2-hr pulse*

Conditions were the same as in Fig. 1, except that the cytoplasmic RNA was extracted after a 2-hr pulse with orotic acid-^{3}H.

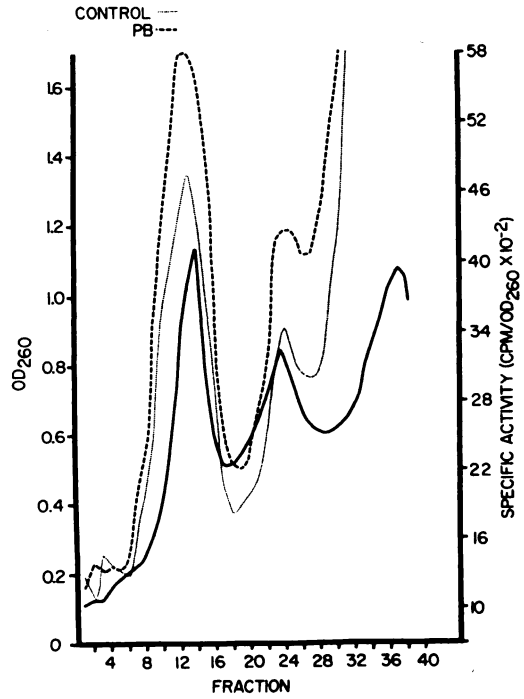

FIG. 3. *Sucrose gradient analysis of cytoplasmic RNA: 4-hr pulse*

Conditions were the same as in Fig. 1, except that the cytoplasmic RNA was extracted after a 4-hr pulse with orotic acid-^{3}H.

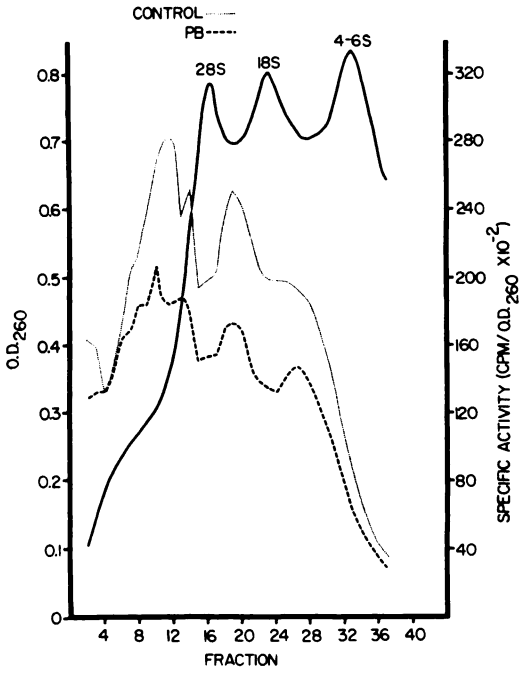

FIG. 4. *Sucrose gradient analysis of nuclear RNA: 30-min pulse*

Samples (1 mg) of hepatic RNA, extracted from purified nuclei pooled from four controls or four phenobarbital (PB)-treated rats after a 30-min pulse with 100 μCi of orotic acid-^{3}H, were layered over linear 10–35% (w/w) sucrose gradients made up in 0.3% sodium dodecyl sulfate–0.1 M sodium chloride–0.05 M sodium acetate, pH 5. Centrifugation and fractionation procedures were identical with those in Fig. 1.

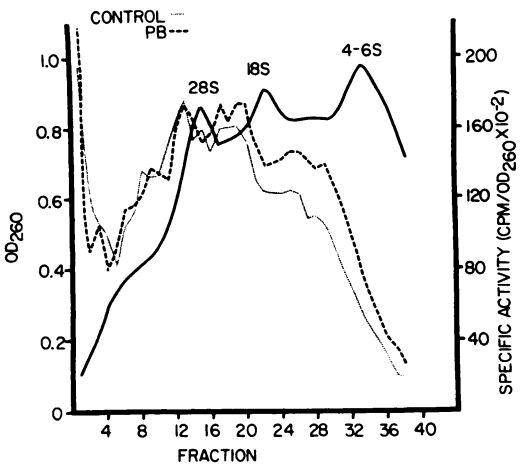

FIG. 5. *Sucrose gradient analysis of nuclear RNA: 1-hr pulse*

Conditions were the same as in Fig. 4.

elsewhere along the gradient, and therefore seemed to be rather specific for the ribosomal class of RNA. After a 4-hr pulse the increase in the specific activities of ribosomal RNA from the phenobarbital-treated rats was even more evident. This elevation was seen in three out of three experiments with a 4-hr pulse of orotic acid-^{3}H, and the range of increased specific activities was 20–30% at the peaks of the 28 S and 18 S species. Thus, these results indicate that one of the early effects of phenobarbital, which occurs prior to elevation of microsomal oxidase activity, is an alteration in either the synthesis, "processing," or stability of ribosomal RNA.

In order to determine which mechanisms might be responsible for the increased labeling of these species, purified hepatic nuclear RNA was similarly labeled and analyzed. These results are shown in Figs. 4–7, which indicate that in general there was no difference in the labeling pattern of nuclear RNA between control and phenobarbital-treated rats following 1-, 2-, or 4-hr pulses with orotic acid-^{3}H. Analysis of nuclear RNA after a 30-min pulse (Fig. 4), however, indicated that there was a decrease in the labeling of nuclear RNA

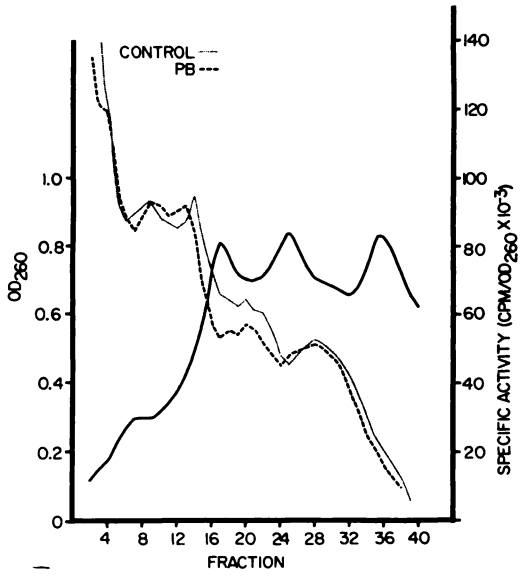

FIG. 6. *Sucrose gradient analysis of nuclear RNA: 2-hr pulse*

Conditions were the same as in Fig. 4.

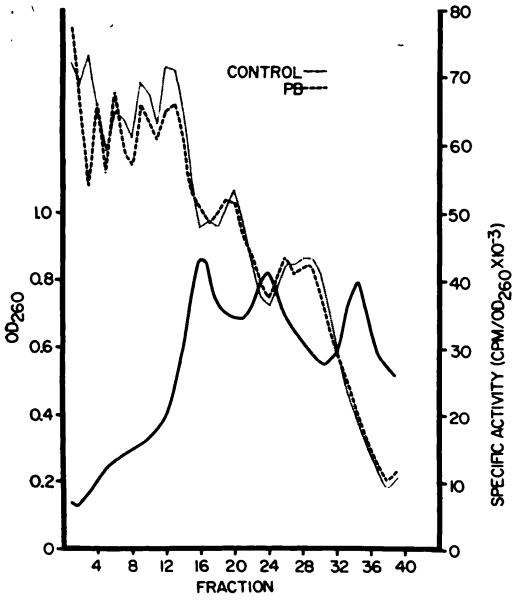

FIG. 7. *Sucrose gradient analysis of nuclear RNA: 4-hr pulse*

Conditions were the same as in Fig. 4.

species following phenobarbital treatment. This might reflect the somewhat lower specific activity of the nucleotide pool in phenobarbital-treated rats following a short pulse (Table 1). In addition, there was an apparent enhanced labeling of species smaller than 18 S in the phenobarbital-treated rats following a 60-min pulse dose of the labeled precursor. This may be significant, since it is in this size range that many types of messenger RNA are thought to exist. In any event, these results would tend to rule out an increased synthesis of ribosomal RNA as being responsible for the increased labeling of these species seen in the cytoplasm at the corresponding times. The specific activity of the acid-soluble nucleotide pool was not markedly altered by phenobarbital treatment with pulses up to 60 min (Table 1). There appeared to be a decrease in specific activity of the pool from phenobarbital-treated rats with pulses of 2 and 4 hr. This decrease might reflect a decreased turnover of RNA, which would be consistent with the idea that the stability of RNA was enhanced in phenobarbital-treated animals.

In order to determine the effects of phenobarbital on RNA metabolism at a time when the inductive process was well under way, cytoplasmic RNA was analyzed following a 12-hr pulse dose of orotic acid-^{3}H given 2 hr after either 0.9% NaCl or phenobarbital, and nuclear RNA was analyzed following a 30-min pulse with the labeled precursor given 12 hr after 0.9% NaCl or phenobarbital. The data in Fig. 8 indicate that the enhanced labeling of cytoplasmic ribosomal RNA from phenobarbital-treated rats was maintained for at least 12 hr following a pulse of orotic acid-^{3}H. Moreover, the specificity of this action of phenobarbital was apparent, since it can be seen that although all cytoplasmic RNA species were well labeled at this time, only the ribosomal forms from phenobarbital-treated animals showed enhanced labeling. In contrast, there was a decreased incorporation of labeled orotate into nuclear RNA following a 30-min pulse administered 12 hr after phenobarbital; the data were virtually identical with those obtained with a 30-min pulse 2 hr after phenobarbital (Fig. 4). The results from these experiments also indicate that the enhanced specific radioactivity of cytoplasmic ribosomal RNA cannot be explained on the basis of an increased synthesis of these species, even as late as 12 hr after phenobarbital treatment. Thus our observations suggest that the increased

TABLE 1

Effect of phenobarbital on specific activity of acid-soluble nucleotide pools at various times after injection of orotic acid-^{3}H

NaCl (0.9%) or phenobarbital (75 mg/kg) dissolved in 0.9% NaCl was administered intraperitoneally at zero time. Two hours later each rat received an intravenous pulse of orotic acid-^{3}H, and the specific activity of the acid-soluble pool was determined as described in METHODS. Samples from two control and two treated rats were pooled for analysis at each time.

Treatment	10 min	20 min	30 min	60 min	120 min	240 min
	(cpm/A_{260}) × 10^{-2}					
Control	172	231	198	195	171	161
Phenobarbital (75 mg/kg)	165	186	182	196	135	119

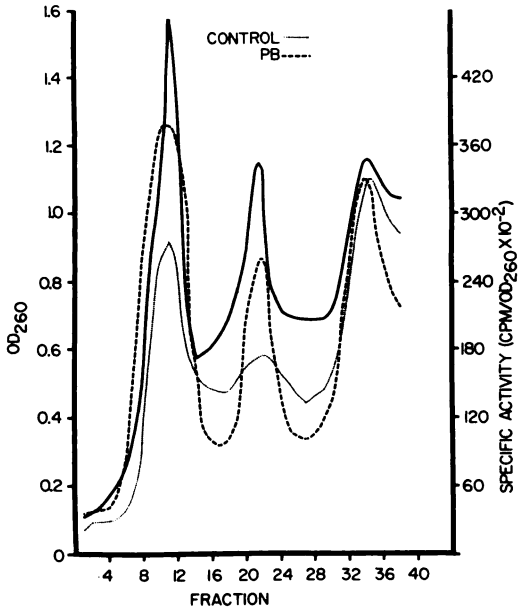

FIG. 8. *Sucrose gradient analysis of cytoplasmic RNA: 12-hr pulse*

Conditions were the same as in Fig. 1.

hepatic microsomal enzyme activity seen after phenobarbital injection is correlated with enhanced stability of ribosomal RNA rather than with increased synthesis of these or other types of RNA.

DISCUSSION

The present investigation has examined the effects of a single dose of phenobarbital (75 mg/kg) on the metabolism of several species of hepatic RNA during the first 12 hr after administration of the drug. We have previously determined that by 12 hr a 50% increase in hepatic microsomal ethylmorphine *N*-demethylase activity is produced following a single dose of the barbiturate in the young rats (60–80 g) employed in our experiments. In addition, actinomycin D studies (15) have indicated that RNA synthesis is a rate-limiting event in the induction process only during the first 8 hr. As a result of these findings, we felt that it was important to examine more directly the effects of phenobarbital on RNA metabolism within this apparently critical period of the induction process. Accordingly, changes in the specific radioactivity of both hepatic nuclear and cytoplasmic RNA were analyzed by labeling these species with orotic acid-^{3}H for various periods of time following treatment with either NaCl or phenobarbital.

Phenobarbital was shown to produce a consistent enhancement of the specific radioactivity of both 28 S and 18 S cytoplasmic ribosomal RNA when rats received pulse doses for 2 hr or longer (Figs. 2, 3, and 8). This increase ranged from 20 to 30% at the peaks and was most evident only for the ribosomal species, although in some experiments there was an indication of an elevated specific activity of RNA less than 18 S.

We obtained no evidence for an increased incorporation of labeled precursor into any of the more distinct species of nuclear RNA extracted from phenobarbital-treated rats with pulses of orotate-^{3}H ranging from 30 min to 4 hr (Figs. 4–7). Thus, the elevated specific radioactivity of hepatic cytoplasmic ribosomal RNA cannot be explained on the basis of enhanced synthesis of these species in the nuclei. It therefore appears that phenobarbital may somehow act to increase the rate of "processing" of ribosomal RNA and its transport to the cytoplasm or to prevent the degradation of these species once they enter the cytoplasm.

Recent reports tend to support the latter conclusion. McCauley and Couri (16) have reported a higher specific radioactivity of total microsomal RNA extracted from rats after 12 and 18 hr of phenobarbital treatment. In addition, prolonged treatment with phenobarbital (5–6 days) was shown to result in marked inhibition of hepatic microsomal ribonuclease activity (17). Finally, the half-life of liver ribosomal RNA was found to be increased after rats were treated with phenobarbital for 5 days and then exposed to labeled orotic acid (18). The 20–30% increase in specific activity of ribosomal RNA observed in our experiments most likely represents a relative change in the balance between synthesis and degradation, rather than an absolute increase of rRNA in the phenobarbital-treated rats. In fact, since the half-life of total ribosomal RNA is thought to be of the order of magnitude of 120 hr (19), an absolute increase

in these species would become apparent only after several days of phenobarbital administration. In addition, it should be emphasized that in our studies pulses of labeled orotate were administered as early as 2 hr after phenobarbital. Thus, the effects of the drug on RNA metabolism are very early events and appear before any significant increase in microsomal enzyme activity.

It has not yet been possible to connect the apparent effects of phenobarbital on the stabilization of ribosomal RNA directly with the marked induction of microsomal enzyme activity produced by the drug. At this point only conjecture about the relationship between the two is possible. The enhanced stability of the ribosomal RNA species may actually be indicative of a decreased rate of degradation of entire mRNA-polyribosome complexes. A coordinated increase in the stability of membrane phospholipids (8) and ribosomes might reflect the assembly and stabilization of whole protein-synthesizing units that may be involved in the fabrication of various types of proteins whose synthesis is increased by "inducing" agents such as phenobarbital. It is clear that additional experimentation is necessary before any definitive conclusions can be reached as to the relationship between the induction of hepatic enzyme activity and the alterations in hepatic RNA metabolism produced by phenobarbital.

ACKNOWLEDGMENT

The authors wish to thank Mrs. Cynthia Rainey for her excellent technical assistance and for her help in the preparation of the manuscript.

REFERENCES

1. A. H. Conney, *Pharmacol. Rev.* **19**, 317 (1967).
2. S. Orrenius. J. L. E. Ericsson and L. Ernster, *J. Cell Biol.* **25**, 627 (1965).
3. A. H. Conney and A. G. Gilman, *J. Biol. Chem.* **238**, 3682 (1963).
4. J. M. Fujimoto and G. L. Plaa, *J. Pharmacol. Exp. Ther.* **131**, 282 (1961).
5. H. V. Gelboin and L. Sokoloff, *Science* **134**, 611 (1961).
6. H. V. Gelboin, J. J. Wortham and R. G. Wilson, *Nature* **214**, 281 (1967).
7. W. N. Piper and W. F. Bousquet, *Biochem. Biophys. Res. Commun.* **33**, 602 (1968).
8. J. L. Holtzman and J. R. Gillette, *J. Biol. Chem.* **243**, 3020 (1968).
9. L. Shuster and H. Jick, *J. Biol. Chem.* **241**, 5361 (1966).
10. L. Shuster and H. Jick, *J. Biol. Chem.* **241**, 5366 (1966).
11. T. Omura, U. Kuriyama, P. Siekevitz and G. E. Palade, *in* "Microsomes and Drug Oxidations" (J. R. Gillette, A. H. Conney, G. J. Cosmides, R. W. Estabrook, J. R. Fouts, G. J. Mannering, eds.), p. 475. Academic Press, New York, 1969.
12. W. J. Steele and H. Busch, *in* "Methods in Cancer Research" Vol. III, p. 61. Academic Press, New York, 1967.
13. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
14. G. A. Bray, *Anal. Biochem.* **1**, 279 (1960).
15. A. M. Cohen and R. W. Ruddon, *Pharmacologist* **11**, 313 (1969).
16. R. McCauley and D. Couri, *Fed. Proc.* **29**, 2781 (1970).
17. R. T. Louis-Ferninand and G. C. Fuller, *Biochem. Biophys. Res. Commun.* **38**, 811 (1970).
18. W. J. Steele, *Fed. Proc.* **29**, 2779 (1970).
19. T. Omura, P. Siekevitz and G. E. Palade, *J. Biol. Chem.* **242**, 2389 (1967).