The effect of phenobarbital on the transcriptional activity of liver

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The effect of phenobarbital on the transcriptional activity of liver was studied by measuring the synthesis of RNA by suspensions of hepatocytes isolated from rats treated with phenobarbital for various time periods. The absolute rates of RNA synthesis by isolated hepatocytes were determined by measuring the incorporation of [³H]orotic acid into RNA as UMP and the specific radioactivity of the UTP pool. The specific radioactivity of the UTP extracted from hepatocytes isolated from phenobarbital-treated rats was consistently lower than that of the UTP pool of hepatocytes from untreated rats. Phenobarbital treatment increased the rate of RNA synthesis 10-fold over that observed for hepatocytes from untreated rats. The maximum rate of RNA synthesis was observed 16–18 h after phenobarbital administration. Phenobarbital treatment also affected the nuclear–cytoplasmic transport of RNA by isolated hepatocytes. Immediately after phenobarbital treatment, the transport of RNA decreased; however, 24 h after phenobarbital administration, the transport of RNA was increased 4-fold. An increase in the synthesis of RNA *in vivo* by liver was found 18 h after phenobarbital treatment, and the incubation of suspensions of hepatocytes with various concentrations of phenobarbital increased RNA synthesis significantly.

Phenobarbital is a sedative, which has been shown to be a potent tumour-promoting agent. Phenobarbital induces the synthesis of a variety of enzymes in the liver, including the mixed-function oxidase system. Unlike 3-methylcholanthrene and the steroid hormones, which initiate the induction process by binding to receptors and interacting with chromatin, no receptor for phenobarbital has been found (Liberator & Bresnick, 1981). Over the past 20 years, several research groups have studied the role of RNA synthesis and RNA metabolism in the induction of liver enzymes by phenobarbital. Phenobarbital treatment has been found to increase the amount of translationally active mRNA for cytochrome *P*-450 (Colbert *et al.*, 1979; Dubois & Waterman, 1979; Lechner *et al.*, 1979), NADPH-cytochrome *P*-450 reductase (Gonzalez & Kasper, 1980a), epoxide hydratase (Gonzalez & Kasper, 1980b; Pickett & Lu, 1981) and glutathione transferase (Daniel *et al.*, 1977; Kalinyak & Taylor, 1982). However, the molecular mechanism responsible for the increased amounts of translationally active mRNA is not known.

Abbreviations used: poly(A)⁺ RNA, polyadenylated RNA; poly(A)[−] RNA, RNA that does not contain a poly(A) segment.

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Several lines of evidence suggest that phenobarbital treatment induces RNA synthesis. Procacini & Bresnick (1975) observed an increase in the acetylation of histones in the liver after phenobarbital treatment, a phenomenon that has been temporally correlated with an increase in transcriptional activity (Ruiz-Carrillo *et al.*, 1975). The administration of actinomycin D or α -amanitin before phenobarbital treatment decreases the induction of cytochrome *P*-450 (Nebert & Gielen, 1971; Jacob *et al.*, 1974). Multiple injections of phenobarbital have been shown to increase the poly(A)⁺ RNA content of liver polyribosomes (Colbert *et al.*, 1979). However, Lindell *et al.* (1977) and Liberator & Bresnick (1981) were unable to detect any increase in the transcriptional activity of isolated liver nuclei after phenobarbital treatment. More recently, Kumar *et al.* (1980) reported that phenobarbital does not stimulate the synthesis of RNA *in vivo* by liver tissue. Because an increase in RNA synthesis has not been found, the increase in the amount of translationally active mRNA in the liver after phenobarbital treatment has been attributed to: (a) an increase in the processing and/or transport of nuclear RNA (Gonzalez & Kasper, 1980a; Phillips *et al.*, 1981); (b) an increase in the stability of RNA (Cohen & Ruddon, 1971; Venkatesan & Steele,

1975) because of increased methylation (Smith *et al.*, 1973, 1976); and (c) a change in mRNA structure that increases the efficiency of translation (Gonzalez & Kasper, 1980a; Colbert *et al.*, 1979).

The purpose of the present study was to re-examine the effect of phenobarbital on the transcriptional activity of liver. Using suspensions of freshly isolated hepatocytes, which Castle *et al.* (1979) have shown are transcriptionally active and synthesize species of RNA similar to those synthesized *in vivo*, we measured the absolute rates of RNA synthesis before and after phenobarbital treatment. Phenobarbital treatment increased the rate of RNA synthesis by hepatocytes 10-fold over the rate of RNA synthesis observed with hepatocytes isolated from untreated rats. The incubation of hepatocytes with phenobarbital *in vitro* also increased the rate of RNA synthesis. Therefore our results demonstrate that phenobarbital treatment increases the transcriptional activity of liver. A preliminary report on some of this work has been presented (Hardwick & Richardson, 1982).

Materials and methods

Isolation of hepatocytes

Male Fischer F344 rats weighing 250–300 g were purchased from Harlan Industries, Indianapolis, IN, U.S.A. Rats were given phenobarbital (80 mg/kg body wt.) intraperitoneally. At various times after phenobarbital administration, hepatocytes were isolated from the rats between 08:00 and 09:00 h to minimize diurnal variation. The hepatocytes were obtained by the perfusion of the liver *in situ* with Hanks solution containing 0.05% (w/v) collagenase as described by Englemann *et al.* (1981). The hepatocyte population consisted almost exclusively of parenchymal cells. The cell viability was routinely 94–99%, as determined by Trypan Blue exclusion, and the ATP/ADP ratio of the hepatocytes was 2.8–4.6. The yield of hepatocytes ranged from 30 to 50 million cells/g of liver.

Measurement of RNA synthesis by suspensions of hepatocytes

This was determined as described by Castle *et al.* (1979). Hepatocytes (3×10^6 cells/ml) were incubated at 37°C under O_2/CO_2 (19:1) in modified Hanks medium that was buffered with 15 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] (pH 7.4) and contained 26 μ M-[5- 3 H]orotic acid (0.1 μ Ci/nmol). This concentration of orotic acid saturated the system. Hepatocytes were collected after various incubation periods by low-speed centrifugation (20 g for 1 min). The cell pellet was homogenized in 0.5 M-HClO₄. The homogenate was centrifuged at 10000 g for 10 min, and the supernatant and precipitate were kept.

The supernatant was used to measure the specific radioactivity of the acid-soluble UTP pool of the hepatocytes. Nucleotides were extracted from the supernatant by using Alamine/Freon as described by Riss *et al.* (1980). The precipitate was homogenized in 0.5 M-HClO₄, centrifuged at 10000 g for 10 min, washed with 95% ethanol, and dried. The precipitate was then resuspended in 0.3 M-KOH and incubated at 37°C for 24 h to hydrolyse the RNA in the precipitate. The KOH hydrolysate was acidified by adding HClO₄ to a final concentration of 0.5 M. Nucleotides were extracted from the acid-soluble fraction of the KOH hydrolysate with Alamine/Freon as described above, and the amounts of radioactivity incorporated into UMP and CMP were determined by using high-performance liquid chromatography.

In those experiments in which the incorporation of radioactivity into nuclear and cytoplasmic RNA was determined, the nuclear and cytoplasmic fractions of hepatocytes were obtained as described by Atryzek & Fausto (1979), and the incorporation of UMP into the cytoplasmic and nuclear fractions was determined as described above.

Separation and quantification of nucleotides

The nucleotides in the acid-soluble extracts from hepatocytes were eluted from a Partisil SAX 10/25 column (0.46 mm $\times$ 25 mm) with a potassium phosphate gradient, which is a modification of the high-performance liquid-chromatography system described by Riss *et al.* (1980). UTP is resolved from the other nucleotides with this system. The radioactivity that was eluted as UTP was determined with a liquid-scintillation counter (Richardson *et al.*, 1982), and the amount of UTP in the sample was calculated from the peak height of the A_{254} .

The nucleoside 2'- and 3'-monophosphates extracted from the KOH hydrolysates were separated with an isocratic elution system with 10 mM-KH₂PO₄ (pH 3.5) at a flow rate of 2 ml/min. The pyrimidine nucleoside 2'- and 3'-monophosphates are eluted as a single peak, whereas the 2'- and 3'-isomers of the purine nucleoside monophosphates are resolved. The radioactivity that was eluted as 2'- or 3'-CMP or 2'- or 3'-UMP was determined with a liquid-scintillation counter.

Results

To measure RNA synthesis by suspensions of hepatocytes, we used the radioisotope [3 H]orotic acid; the liver rapidly and efficiently incorporates orotic acid into RNA (Dahnke, 1975). Orotic acid serves as a precursor for both the cytidine- and uridine-containing nucleotides; therefore we first measured the amount of radioactivity incorporated into RNA as CMP and UMP. In general, approx.

Fig. 1. Incorporation of $[^3\text{H}]$ orotic acid into RNA as CMP by suspensions of hepatocytes

The percentage of radioactivity incorporated into RNA as CMP when hepatocytes were incubated with $[^3\text{H}]$ orotic acid was determined for hepatocytes isolated from untreated (○) rats and from rats 12 (◐) and 18 (●) h after phenobarbital treatment. Each point is the mean of four determinations from two rats.

90% of the radioactivity was incorporated into RNA as UMP; however, as Fig. 1 shows, the percentage of radioactivity incorporated into RNA as CMP increased with increasing incubation time. Because the synthesis *de novo* of the pyrimidine nucleotides proceeds from UTP to CTP, one would expect to find a greater percentage of radioactivity in CTP with increasing incubation time. Fig. 1 also shows that the percentage of radioactivity incorporated into RNA as CMP by hepatocytes isolated from phenobarbital-treated rats was greater than that observed for hepatocytes from untreated rats. Therefore we measured RNA synthesis specifically as the amount of radioactivity incorporated into RNA as UMP.

To measure RNA synthesis accurately in cell suspensions with $[^3\text{H}]$ orotic acid, the specific radioactivity of the precursor, UTP, must be determined. Fig. 2(a) shows the specific radioactivity of the UTP extracted from hepatocytes at various times after incubation with $[^3\text{H}]$ orotic acid. The specific radioactivity of the UTP pool of hepatocytes isolated from phenobarbital-treated rats was much lower than for hepatocytes isolated from untreated rats. The decrease in the specific radioactivity of the UTP pool appears to arise from an expansion of the UTP pool after phenobarbital treatment. The size of the UTP pool (after 60 min of incubation) was 1352 ± 228 pmol of UTP per million cells (mean $\pm$ s.e.m. for four animals) and 2026 ± 205 pmol of UTP per million cells (mean $\pm$ s.e.m. for 11 animals) for hepatocytes isolated from untreated rats and

Fig. 2. Effect of phenobarbital on the incorporation of $[^3\text{H}]$ orotic acid into UTP and RNA by isolated hepatocytes. Hepatocytes from untreated rats (○) and from rats 3 (●), 9 (◐), 12 (△), 16 (▲), 18 (◻) and 24 (■) h after phenobarbital treatment were incubated with $[^3\text{H}]$ orotic acid for various lengths of time. The specific radioactivities of the UTP pools from the acid-soluble extracts of the hepatocytes are shown in (a). The time course of RNA synthesis (b) was determined by dividing the amount of radioactivity incorporated into RNA as UMP by the specific radioactivity of the UTP pool.

Table 1. *Effect of phenobarbital on the rates of RNA synthesis and the accumulation of newly synthesized RNA in the nuclear and cytoplasmic fractions of isolated hepatocytes*

Hepatocytes were isolated from rats at various times after one injection of phenobarbital, except for one group of rats ($\times 3$), which were given phenobarbital for 3 consecutive days (the rats were killed 24 h after the last injection). Hepatocytes were incubated with [^3H]orotic acid, and the radioactivity incorporated into RNA as UMP was divided by the specific radioactivity of the UTP pool. The rates (pmol of UMP/min per 10^6 cells) of RNA synthesis and appearance of newly synthesized RNA in the nuclear and cytoplasmic fractions of the hepatocytes were determined in the same cell samples during the initial 10–20 min of incubation, when the incorporation of UMP into RNA by hepatocytes was linear with respect to time. Each value represents the mean $\pm$ S.E.M. for data obtained from duplicate samples from three or four animals.

Time after phenobarbital administration (h)	Rate of RNA synthesis	Rate of appearance of RNA		Cytoplasm/nucleus ratio
		Nucleus	Cytoplasm	
(untreated)	24 ± 3	15 ± 1	13 ± 1	0.87 ± 0.06
3	20 ± 2	12 ± 4	$4 \pm 1^*$	$0.33 \pm 0.04^*$
6	24 ± 4	15 ± 3	$6 \pm 3^*$	$0.40 \pm 0.05^*$
9	$43 \pm 7^*$	$27 \pm 3^*$	$7 \pm 1^*$	$0.26 \pm 0.01^*$
12	$45 \pm 5^*$	$34 \pm 4^*$	$11 \pm 1^*$	$0.32 \pm 0.03^*$
16	$251 \pm 31^*$	$169 \pm 10^*$	$52 \pm 2^*$	$0.31 \pm 0.06^*$
18	$165 \pm 15^*$	$140 \pm 21^*$	$74 \pm 4^*$	$0.52 \pm 0.09^*$
24	$152 \pm 10^*$	$37 \pm 6^*$	$136 \pm 6^*$	$3.68 \pm 0.50^*$
$\times 3$	$53 \pm 6^*$	19 ± 3	33 ± 4	$1.74 \pm 0.19^*$

* These values are significantly different from the value of the untreated animals at the $P < 0.01$ level.

rats 12–24 h after phenobarbital administration respectively.

The time course for the synthesis of RNA by hepatocytes isolated from phenobarbital-treated and untreated rats is shown in Fig. 2(b). Both the rate and extent of RNA synthesis after 60 min of incubation were increased after phenobarbital treatment. The initial rates of RNA synthesis by hepatocytes after phenobarbital treatment are shown in Table 1. No significant change in rate of RNA synthesis was observed during the first 6 h after phenobarbital administration. However, an increase in the extent of RNA synthesis was observed within 3 h after phenobarbital administration (Fig. 2b). A significant increase in the rate of RNA synthesis was observed 9 h after phenobarbital administration. The rate of RNA synthesis was maximum 16 h after phenobarbital treatment and was 10 times the rate of RNA synthesis by hepatocytes isolated from untreated rats. Between 16 and 24 h after phenobarbital administration, a decrease in the absolute rate of RNA synthesis was observed. It is noteworthy that the rate of RNA synthesis by hepatocytes isolated from rats given phenobarbital for 3 successive days (a regimen generally used for the induction of the mixed-function oxidase system) was only one-fifth the rate of RNA synthesis by hepatocytes isolated from rats 16 h after a single administration of phenobarbital.

By using oligo(dT)-cellulose affinity chromatography, we compared the synthesis of poly(A)⁺ RNA and poly(A)[−] RNA by hepatocytes isolated

from untreated rats and from rats 18 h after phenobarbital administration. Fig. 3 shows that the synthesis of both poly(A)⁺ RNA and poly(A)[−] RNA was increased after phenobarbital treatment; however, the increase in that latter was much greater than the increase in the former.

Because Kumar *et al.* (1980) reported that phenobarbital treatment affected the transport of RNA from the nucleus to the cytoplasm, we also measured the rates of the appearance of newly synthesized RNA in the nuclear and cytoplasmic fractions of hepatocytes (Table 1). During the first 6 h after phenobarbital treatment, no significant change in the rate of appearance of RNA in the nucleus was observed; however, the rate of appearance of newly synthesized RNA in the cytoplasm of hepatocytes for the first 9 h after phenobarbital administration was 50% of that observed for hepatocytes from untreated rats. A significant increase in the rate of appearance of newly synthesized RNA in the nucleus was observed 9 h after phenobarbital treatment, and the rate was maximal 16–18 h after phenobarbital administration. However, the rate of appearance of new RNA in the cytoplasm from hepatocytes was not significantly greater than that of untreated rats until 16 h after phenobarbital administration. Between 18 and 24 h after phenobarbital treatment, the rate of appearance of newly synthesized RNA in the nucleus of hepatocytes decreased by approx. 80%, whereas the rate of appearance of RNA in the cytoplasm increased by 80%.

Fig. 3. Effect of phenobarbital treatment on the synthesis of poly(A)⁺ RNA and poly(A)⁻ RNA

Hepatocytes isolated from untreated (O) rats and from rats 18 h after phenobarbital treatment (●) were incubated in the presence of [³H]orotic acid. At various times, the RNA was extracted from the hepatocytes with guanidium thiocyanate as described by Chirgwin *et al.* (1979), and the RNA was fractionated into poly(A)⁺ RNA and poly(A)⁻ RNA by oligo(dT)-cellulose chromatography (Bantle *et al.*, 1976). The pmol of UMP incorporated into poly(A)⁺ RNA (a) and poly(A)⁻ RNA (b) were obtained by dividing the radioactivity incorporated into the RNA as UMP by the specific radioactivity of the UTP pool and the mg of RNA. Each value represents the mean of data collected from three animals.

To compare the nuclear-cytoplasmic transport of newly synthesized RNA by hepatocytes isolated from rats before and after phenobarbital treatment, the data in Table 1 were expressed as a ratio of the rate of appearance of newly synthesized RNA in the cytoplasm to the rate of appearance in the nucleus. The ratio decreased dramatically after phenobarbital treatment and remained low until 24 h after phenobarbital administration, even though the rate of appearance of newly synthesized RNA in the nucleus increased 10-fold during this time period. The ratio of the cytoplasmic to nuclear rates of appearance of RNA increased almost 10-fold between 18 and 24 h after phenobarbital administration. Hepatocytes from rats treated with phenobarbital for 3 successive days also had a significantly higher ratio than hepatocytes from untreated rats.

The effect of phenobarbital treatment on the synthesis of RNA by the liver *in vivo* was also studied with a limited number of animals. Rats (three in each group) were given [³H]orotic acid intraperitoneally before and 18 h after phenobarbital treatment. At 60 min after the administration of the [³H]orotic acid, the amount of radioactivity incorporated into RNA as UMP by the liver and the specific radioactivity of the acid-soluble UTP extracted from the liver were determined as described in the Materials and methods section. The synthesis of RNA *in vivo* during the 60 min period was

30.9 ± 2.9 nmol of UMP/g of liver for untreated rats and 97.1 ± 5.4 nmol/g for the phenobarbital-treated rats. These results show that the synthesis of RNA *in vivo* by the liver was increased significantly ($P < 0.01$) by phenobarbital treatment.

In a series of experiments, we also found that the transcriptional activity of hepatocytes was increased by the incubation of suspensions of hepatocytes *in vivo* with phenobarbital. The absolute rates of RNA synthesis (determined during the first 10–20 min of incubation as described in Table 1) by hepatocytes were 49 ± 4 and 23 ± 5 pmol of UMP/min per million cells in the presence and absence of phenobarbital respectively. Because *p*-hydroxyphenobarbital, the major metabolite of phenobarbital, does not induce either liver hypertrophy or liver enzymes (Cresteil *et al.*, 1980), we compared the synthesis of RNA by hepatocytes incubated with *p*-hydroxyphenobarbital and phenobarbital (Fig. 4). A slight decline in RNA synthesis was observed with increasing concentrations of *p*-hydroxyphenobarbital; however, incubation of hepatocytes with concentrations of phenobarbital above 0.3 mM resulted in a continuous increase in RNA synthesis with increasing concentrations of phenobarbital.

Discussion

Using suspensions of hepatocytes isolated from rats at various times after phenobarbital treatment,

Fig. 4. Effect of phenobarbital and *p*-hydroxyphenobarbital on RNA synthesis by isolated hepatocytes

Hepatocytes were incubated with various concentrations of phenobarbital or *p*-hydroxyphenobarbital, and RNA synthesis was determined by dividing the amount of radioactivity incorporated into UMP by the specific radioactivity of the UTP pool. (a) Time course of RNA synthesis when hepatocytes were incubated with 2.58 mM-phenobarbital (●) or 2.58 mM *p*-hydroxyphenobarbital (Δ); each point represents the mean of experiments from three animals. (b) RNA synthesis after incubation of hepatocytes with [³H]orotic acid for 20 min with various concentrations of phenobarbital (●) or *p*-hydroxyphenobarbital (Δ). Each point represents the mean of data collected from two animals. The RNA synthesis by hepatocytes incubated in the absence of phenobarbital and *p*-hydroxyphenobarbital is shown by the dashed line.

we carefully measured the effect of phenobarbital on the transcriptional activity of liver in order to gain an insight into the molecular mechanism of phenobarbital induction. Although experimental conditions can be carefully controlled in suspensions of hepatocytes, we were cognizant of the potential problems in measuring RNA synthesis by cells in suspension. Phenobarbital treatment was found to alter several parameters that would affect the measurement of RNA synthesis. For example, the incorporation of [³H]orotic acid into RNA as CMP increased approx. 100% after phenobarbital treatment (Fig. 1), and the specific radioactivity of the UTP pool decreased significantly after phenobarbital treatment (Fig. 2a).

A significant increase in the absolute rate of RNA synthesis was observed within 9 h after phenobarbital administration, and the absolute rate of RNA synthesis was maximal 16–18 h after phenobarbital treatment (Table 1). The synthesis of both poly(A)⁺ RNA and poly(A)[−] RNA was increased 18 h after phenobarbital administration (Fig. 3); however, the latter was induced to a much greater extent than the former. The increase in transcription by hepatocytes after phenobarbital treatment

reflects the situation in the whole animal, because we found that the synthesis of RNA *in vivo* by liver was increased 18 h after phenobarbital treatment. An increase in the absolute rate of RNA synthesis was also observed when hepatocytes were incubated with phenobarbital. Interestingly, the incubation of hepatocytes with *p*-hydroxyphenobarbital had no effect on RNA synthesis.

The results of our study are not in agreement with several previous studies, which showed that phenobarbital treatment had no effect on the transcriptional activity of liver. It appears that the contradictory observations arise primarily from the different induction protocols used. In our study, RNA synthesis was measured at several time intervals during the first 24 h after the administration of one dose of phenobarbital as well as after multiple injections of phenobarbital. Lindell *et al.* (1977) and Liberator & Bresnick (1981) measured the transcriptional activity of liver nuclei isolated from rats after 4 and 5 days of phenobarbital treatment. Our data show that the rate of RNA synthesis by hepatocytes isolated from rats treated with phenobarbital for 3 days was only one-fifth the rate observed for hepatocytes isolated from rats 16 h

after one administration of phenobarbital (Table 1). In addition, we found that the rates of appearance of newly synthesized RNA in the nuclei of hepatocytes isolated from rats treated with phenobarbital for 3 days or from untreated rats were the same. Therefore it appears that the transcriptional activity of liver after chronic phenobarbital treatment is less than that observed after the administration of a single dose of phenobarbital. In the initial study by Gelboin *et al.* (1967), in which an increase in transcription was observed, nuclei were isolated from rats 20h after phenobarbital administration. Kumar *et al.* (1980) also reported that phenobarbital treatment had no effect on the synthesis of RNA *in vivo*; however, they only measured RNA synthesis during the first 8h after phenobarbital administration. We found that a significant increase in the rate of RNA synthesis was not observed until 9h after phenobarbital administration. In addition, Kumar *et al.* (1980) did not measure the specific radioactivity of the UTP pool in their study.

Kumar *et al.* (1980) proposed that the primary effect of phenobarbital was to promote the transport of RNA from the nucleus, because they found that the release of radioactively labelled RNA from isolated nuclei *in vitro* was stimulated in the presence of cytosol isolated from phenobarbital treated rats. Using hepatocytes from phenobarbital-treated rats, we also found that phenobarbital treatment had an effect on the appearance of newly synthesized RNA in the cytoplasm. Immediately after phenobarbital administration, we found that the rate of appearance of newly synthesized RNA in the cytoplasm of hepatocytes was decreased significantly; however, it is possible that specific mRNA species might be selectively transported during this period. A dramatic increase in the transport of newly synthesized RNA into the cytoplasm was observed 24h after phenobarbital administration. Although phenobarbital treatment for 3 days had no effect on the rate of appearance of newly synthesized RNA in the nucleus of hepatocytes, the rate of appearance of newly synthesized RNA in the cytoplasm was approx. 2.5-fold greater for hepatocytes from rats treated with phenobarbital for 3 days than for untreated rats.

In conclusion, our data show that phenobarbital affects RNA metabolism at several steps. Initially, a decrease in the nuclear–cytoplasmic transport of RNA is observed, which is followed by an increase in the rate of transcription. Transcription appears to be maximal 16–18h after phenobarbital administration. A decline in transcription is observed 24h after phenobarbital administration; however, at this time, an increase in the nuclear–cytoplasmic transport of RNA is observed. Chronic phenobarbital treatment only increased RNA synthesis slightly. On the other hand, chronic phenobarbital treatment had

a pronounced affect on the nuclear–cytoplasmic transport of RNA.

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