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ALTERATIONS IN RABBIT LIVER PROTEIN SYNTHESIS AS A RESULT OF PHENOBARBITAL TREATMENT.[1]

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SUMMARY - Phenobarbital dramatically alters rabbit liver polysome and polysomal poly(A+)mRNA driven in vitro protein synthesis. These alterations are due to a movement of poly(A+)mRNA from the cytoplasmic pool into the polysomes, to a decrease in mRNA degradation and to de novo mRNA synthesis. Our observations suggest that a major portion of the effects attributable to phenobarbital occurs at steps other than transcription.

INTRODUCTION - The administration of phenobarbital to rats (1), rabbits (2), mice (3) and other animals (4) is known to cause liver hypertrophy, proliferation of the endoplasmic reticulum and specific enzyme induction. Previous studies have shown that phenobarbital can increase the transport of RNA from nucleus to cytoplasm (5,6), decrease cellular nuclease activity (7) and also increase mRNA synthesis (8). It is not clear whether phenobarbital alters protein synthesis primarily by a) increasing the synthesis of required mRNA's, b) increasing the rate of translation of already existing mRNA's, c) increasing the stability of the mRNA's, or d) a combination of these possibilities. In an effort to clarify the mechanisms of phenobarbital action, we have examined the molecular changes occurring in rabbit liver as a result of phenobarbital treatment. Our data suggests that phenobarbital acts by a) increasing the formation of polysomes from cytoplasmic poly(A+)mRNA and ribosomes, b) decreasing the degradation of polysomal poly(A+)mRNA, c) increasing overall poly(A+)mRNA synthesis and d) increasing the number of mRNA's that are "capped" (9). The first two of these events are apparent within 6 hours of phenobarbital treatment.

MATERIALS AND METHODS - Male rabbits (NZ whites, 2-2.5kg) were obtained from a local rabbitry and kept under normal vivarium conditions. A phenobarbital solution (Sigma) was injected i.p. (80mg in saline/kg bw) every 24 hours for 4 days. Rabbits were killed 6, 12, 18 and 24

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hours after the first injection, 12 and 24 hours after the second injection, and 24 hours after the fourth injection. These are referred to as 6, 12, 18, 24, 36, 48 and 96 hour rabbits. Zero hour rabbits received no injections. Rabbits were killed by KCl injection, the livers rapidly removed, trimmed free of connective tissue and gall bladder, and portions taken for the preparation of polysomes, polysomal poly(A+)mRNA and total poly(A+)mRNA.

Polysomes were prepared as described by Palmiter (10). The polysomes used for translation studies were centrifuged through a 0.5M sucrose pad (10)and solubilized in 20mM HEPES[2] (pH7.6), 5mM $MgCl_2$. Otherwise, the polysomes were used to prepare polysomal RNA by phenol-chloroform extraction (10). For the isolation of total RNA, tissues were homogenized in 5 volumes of 0.01 M sodium acetate (pH5.2), 0.01 M Na_2EDTA, 0.5% sodium dodecyl sulphate then extracted repeatedly with phenol-chloroform. Polysomal RNA and total RNA samples were chromotographed twice on oligo(dT)cellulose (11) yielding polysomal and total poly(A+)mRNA fractions, respectively.

The translations were carried out at 30° for 60 min. using wheat germ lysate (12) containing 60μc[^{3}H]leucine/ml and 10 to 50μg of polysomes, or 1 to 4μg mRNA, a range over which the translation assays were linearly dependent on exogenous mRNA. Radioactivity was determined in the hot TCA insoluble precipitates as described (12). The 5' labelling of mRNA was carried out using [γ^{32}P]ATP and T_4-RNA kinase as described by Chen and Roe (13).

RESULTS AND DISCUSSION - When liver polysomes were translated using wheat germ lysate (Fig. 1), the amount of protein synthesized per μg polysome input increases with time of phenobarbital treatment up to 18 hr, then decreases by 24 hr. After the second injection of phenobarbital at 24 hr, there is a further increase in protein synthesis for the first 12 hr (i.e., 36 hr polysomes), then a decrease at 24 hr (i.e. 48 hr polysomes), and finally a further decrease at 72 hr (i.e. 96 hr. polysomes) approaching zero hr. polysome values. The data for the 0-24 hr polysomes agrees with the results of Colbert et al. (14), and is consistant with the hypothesis that the decrease at 24 hr is due to the phenobarbital being cleared from the system, thereby diminishing its biological effect. The increase in protein synthesis following the second injection could be due either to an increase in the amount of mRNA associated with polysomes or due to the mRNA becoming a more efficient template for protein synthesis. In order to test which event was causing this observed increase in protein synthesis polysomal poly(A+)mRNA was isolated (the amount calculated as a percentage of total polysomal RNA), and translated. The results of these experiments are shown in Table 1 and Fig. 2A (solid line). It can be seen that the poly(A+)mRNA content of the polysomes increases with time of phenobarbital treatment, suggesting that one of the effects of phenobarbita

2. HEPES = N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid.

could be to shift poly(A+)mRNA from a non-translatable cytoplasmic pool into the polysomes. At 24 hr, the polysomal poly(A+)mRNA levels decrease but a

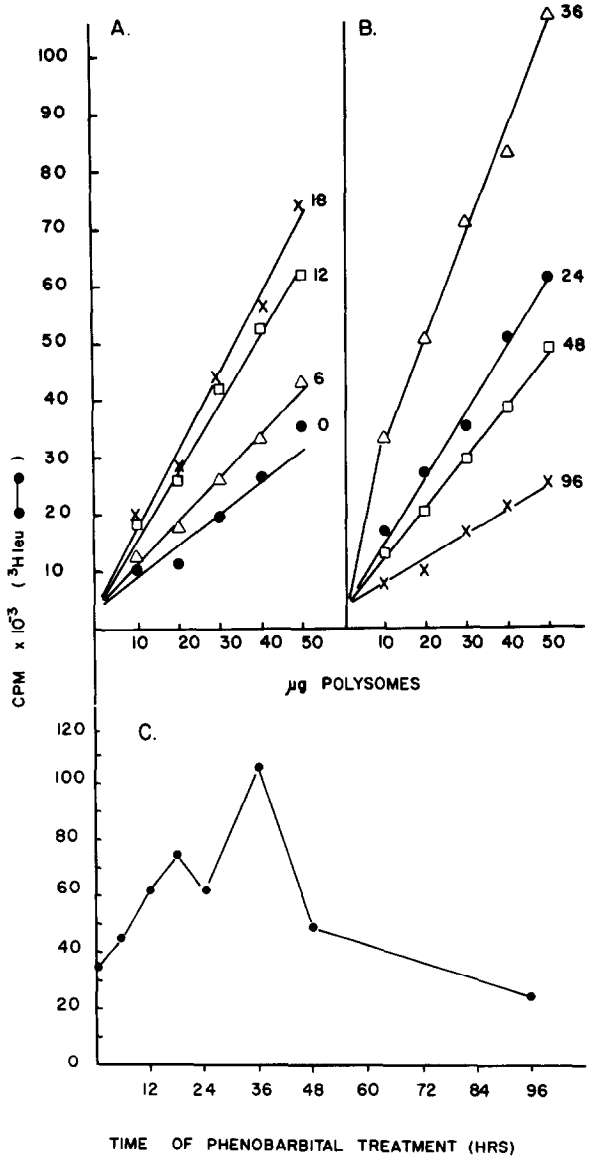

Figure 1. Translation of liver polysomes using wheat germ lysate.

A. Polysomes were isolated from rabbit livers 6, 12 and 18 hr. after administration of phenobarbital (see materials and methods). Zero hour represents non-treated or control rabbits. All polysome solutions were adjusted to $50A_{260}$/ ml and increasing quantities of polysomes translated. One A_{260} of polysomes is taken as approximately 100µg of polysomes (10). B. Same conditions as A. except polysomes were isolated from 24, 36, 48 and 96hr rabbits. C. The data in A and B has been replotted to show changes in protein synthesis/50µg of polysomes occurring with time of phenobarbital treatment.

TABLE 1.

mRNA Content of Liver RNA Samples with Different Times of Phenobarbital Treatment(hrs)

Source of RNA	0+	6	12	18	24	36	48	96
Polysomal RNA	0.19*	0.90	0.75	1.15	0.68	1.72	0.42	0.48
Total liver RNA	1.0*	1.3	1.9	1.8	2.5	1.2	1.4	1.7

+0,6,12 etc. represent number of hours after phenobarbital treatment. Animals were reinjected at 24, 48, and 72 hrs (see Materials and Methods).

*represents the percentage of poly(A+)RNA recovered by oligo(dT) chromatography of the RNA sample.

SG3:B

second injection of phenobarbital leads to an increase in poly(A+)mRNA levels at 36 hr; however 48 and 96 hr levels approach zero hr. values. In contrast, levels of poly(A+)mRNA present in total liver RNA do not alter appreciably until 12 hr (in agreement with studies of Jacob et al., [8]) reach a maximum at 24 hr, then decline again. This increase in total liver poly(A+)mRNA might be due to an increase in de novo mRNA synthesis (8,15) as it is clear that in control liver, most of the mRNA is not associated with polysomes (Table 1), and that the subsequent phenobarbital treatment increases polysomal poly(A+)mRNA content and total liver poly(A+)mRNA content.

The translation of polysomal poly(A+)mRNA is shown in Fig. 2A (solid line). At 18 hr there is a dramatic increase in protein synthesis/2μg poly(A+)mRNA, a slight decrease at 24 hr, an increase at 36 hr, and then a decline at 48 and 96 hr. towards zero hr. values. The increases in polysomal protein synthesis activity (e.g., 18 hr and 36 hr) could be due to the increase in polysomal poly(A+)mRNA content and to the increase in the translational efficiency of the associated mRNA. In order to determine how this latter process occurs, we considered the possibility of decreased 5' terminus degradation of the mRNA since phenobarbital has been shown to decrease nuclease activities (7). This decrease in degradation would increase the translational efficiency of the mRNA, since translation proceeds 5'-3' and most eucaryote mRNA's require the presence of 5' modified guanosine base, i.e. a "cap" (9). If degraded, the 5' terminal end of the mRNA should be readily phosphorylated by T_4RNA kinase and [γ^{32}P]ATP; if it was not degraded, then it would be a poor sustrate for

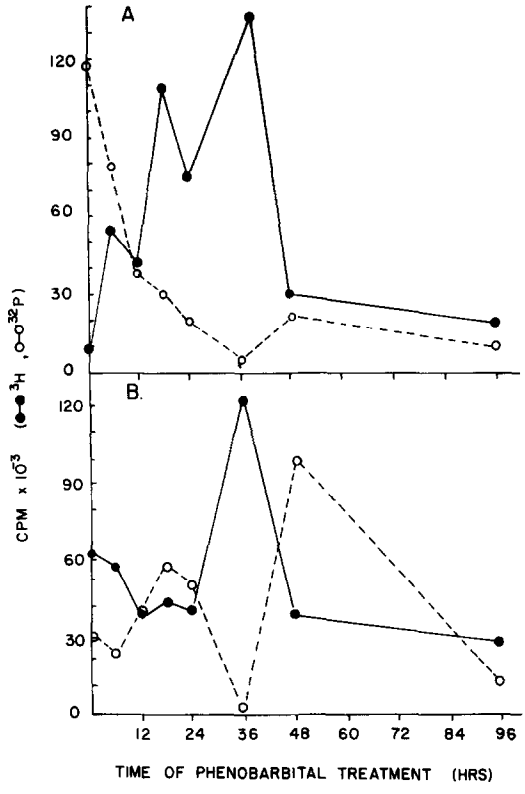

Figure 2. Alterations in mRNA liver protein synthesis and mRNA 5' terminus degradation with time of phenobarbital treatment.

A. Polysomal poly(A+)mRNA was isolated from the polysomes used in Figure 1 and translated. Only the values for 2μg mRNA/50μl reaction are given (●————●). The phosphorylation of the 5' terminus of the mRNA represents [^{32}P] incorporated/ 0.001 A_{260} of mRNA (o-----o) (13). B. Same conditions as for A., except total poly(A+)mRNA was isolated from the livers used in Figure 1 and Figure 2A.

this phosphorylation (13). The results of this study (Fig. 2A broken line) show that there is a direct correlation between the translational efficiency of the mRNA and the amount of phosphorylation. Consistant with this conclusion is the observation the best template for phosphorylation and the worst template for in vitro protein synthesis is the control (zero hr.) mRNA. Within 6 hr there is a decrease in phosphorylation, continuing until 24 hr. then leveling off, whereas protein synthesis is elevated at 18 and 36 hr. Samples of each of the [^{32}P] 5' labelled mRNAs were examined by 15% polyacrylamide gel electrophoresis, followed by autoradiography. All the mRNA's had the same size distribution suggesting that neither degradation or phenobarbital treatment had any significant effect on the overall size distribution of the mRNA. (Data not shown). Similar

results were obtained when male rabbits were injected with only 8mg phenobarbital/kg body weight, i.e., alterations occurred in polysomal poly(A+)mRNA translational efficiences and in 5' end phosphorylation (data not shown). The data presented in Fig. 2A suggests that one of the early effects of phenobarbital is to decrease mRNA 5' terminus degradation. This was also studied indirectly by translating 2μg of 0 and 18 hr polysomal poly(A+)mRNA in the presence of 0 or 0.8mM S-adenosyl homocysteine (SAH). The addition of SAH to the translation reaction inhibited protein synthesis by 70% and 45%, respectively (total protein synthesis in the absence of SAH was taken as 100%), suggesting that the endogenous "capping" activity of wheat germ lysate (9) can recognize rabbit mRNA as a suitable template and that 0 hr polysomal poly(A+)mRNA is less "capped" than 18 hr mRNA, i.e., 0 hr mRNA is more degraded which agrees with the data in Fig. 2A. The translation of globin mRNA was completely unaffected by the addition of SAH, agreeing with previous studies (9).

In contrast to the data shown in Fig. 2A for polysomal poly(A+)mRNA, neither the translational efficiency nor the 5' terminus phosphorylation of total poly(A+)mRNA, changed with time of treatment, except for the 36 hr sample (Fig. 2B). Once again there was a correlation between the translational efficiency of the mRNA and the 5' terminus degradation. It is not clear why 36 hr total poly(A+)mRNA is the best template for protein synthesis, although it is possible that at 36 hr most of the cytoplasmic mRNA is associated with polysomes; hence, total poly(A+)mRNA is the equivalent of polysomal poly(A+)mRNA. This is consistent with the data in Fig. 1 and Table 1. Alternatively, the effect of phenobarbital is maximal at 36 hr, which is speculative until further studies are carried out.

Overall, our data demonstrates that the mechanism of action of phenobarbital is complex and involves multiple cellular processes, including alterations in mRNA synthesis and stabilization. However, we have only studied changes occuring in the poly(A+)mRNA populations and it is possible that phenobarbital also affects levels of poly(A-)mRNA. The increased translational efficiences of polysomes (Fig. 1) compared to polysomal poly(A+)mRNA (Fig. 2A) may be due to the former containing poly(A-)mRNA (16,17). Some of the effects noted in this report may be biological responses to trauma associated with phenobarbital treatment. Hurley and Irwin (18) recently reported that polysomes isolated from rat liver 24 hr post-skinburn were nearly twice as active for *in vitro* protein synthesis as control polysomes.

When female rabbits were injected with phenobarbital (80 mg/kg body weight) the translational efficiencies of both liver polysomes and liver polysomal poly(A+)mRNA did not alter to the same extent as found for treated male rabbits (data not shown), e.g. 18 hr polysomal poly(A+)mRNA was only 3 times as active as 0 hr mRNA. This suggests that many of the effects reported here are sex dependent as well as phenobarbital induced.

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