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A POSSIBLE ROLE OF LIVER MICROSOMAL ALKALINE RIBONUCLEASE IN THE STIMULATION OF OXIDATIVE DRUG METABOLISM BY PHENOBARBITAL, CHLORDANE AND CHLOROPHENOTHANE (DDT)

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Abstract—The administration of phenobarbital (80 mg/kg), chlordane (100 mg/kg) and DDT (100 mg/kg) to adult male Wistar rats, produced a constant and early significant reduction in the liver microsomal alkaline RNase that preceded the rise of cytochrome P-450, aminopyrine oxidative demethylase, aniline hydroxylase activities and liver weight.

During chronic phenobarbital administration a low stable RNase activity was found in parallel with an increased steady state of P-450 hemoprotein, related enzyme activities and liver weight.

When prolonged phenobarbital treatment was stopped RNase activity rose sharply reaching values near 100 per cent before P-450, aniline and aminopyrine metabolizing capacities started to decrease.

It is concluded that microsomal alkaline RNase may play a role in the regulation of protein synthesis in liver cell and in the mechanisms of stimulation of oxidative drug metabolism by inducers of the phenobarbital group.

PRETREATMENT of animals with phenobarbital and many other drugs and foreign substances results in an increased activity of the mixed-function oxidases of the liver, a proliferation of the endoplasmic reticulum and a net increase in its protein content.[1–4]

The exact mechanism of induction remains to be elucidated but evidence based on the use of known inhibitors of protein synthesis and on the incorporation of labeled amino-acids demonstrates that inductive process involves the synthesis of increased amounts of drug metabolizing enzymes. The effects of phenobarbital are prevented by ethionine,[5] puromycin,[6] actinomycin,[7] substances known to block protein synthesis by different mechanisms.

Gelboin and Sokoloff[8] showed that livers from phenobarbital induced animals metabolize drugs at an increased rate and are better able to incorporate amino-acids into microsomal protein *in vitro*. Kato *et al.*[9] showed that the treatment of rats with phenobarbital stimulated the *in vitro* and *in vivo* incorporation of [^{14}C]leucine into microsomal protein. Microsomes from phenobarbital treated rats are more active in L-[^{14}C]phenylalanine incorporation in the absence of polyuridylic acid. After the removal of endogenous messenger RNA by a pre-incubation, the microsomes from phenobarbital treated rats are more than twice as sensitive as control microsomes to

polyuridylic acid directed L-[^{14}C]phenylalanine incorporation. Kato *et al.*[10] have concluded that this can be due to a phenobarbital induced increase in both endogenous microsomal messenger RNA content and the number of ribosomal binding sites available to polyuridylic acid. In fact it is known that phenobarbital treatment affects the total amount of microsomal RNA[7] increasing ribosomal RNA,[11] as an early event produced before any significant increase in microsomal drug metabolizing enzyme activity.[12] These facts have been presented as an explanation for the enhancement in protein synthesis.[13] However, no difference in amino-acid incorporation was observed in ribosomes from control and phenobarbital treated rats indicating that deoxycholate soluble factors, components of the endoplasmic reticulum are important in protein synthesis and are altered by phenobarbital treatment.[10]

Liver microsomes contain an alkaline RNase known to be able to degradate RNA *in vitro* and presumed to act *in vivo*.[14] RNase and its cellular inhibitor have been proposed as important control factors in protein synthesis in animal cells in a general way.[15]

In the present work the modifications in alkaline RNase activity in microsomes, isolated from rats during the treatment with phenobarbital and after its suspension were studied. While this investigation was in progress Louis-Ferdinand and Fuller[16] reported a suppression of hepatic RNase in rats during phenobarbital stimulation of drug metabolism.

In order to ascertain if the variations found were a constant factor in the phenomena produced by inducing agents of the phenobarbital group, the alkaline RNase activities were determined in liver microsomes from animals treated with a single dose of chlordane and DDT. Concomitantly the variations in liver weight, aniline hydroxylase, aminopyrine demethylase and cytochrome P-450 were studied in order to establish their time relationships, and possible interdependence.

MATERIAL AND METHODS

Male Wistar rats, 3–3·5 months old were used throughout this investigation. The animals were starved for 24-hr period, receiving water *ad lib.* before they were weighed and sacrificed. Control and experimental animals were kept under the same experimental conditions, within each experiment. Groups of three rats were used for each sample.

Phenobarbital treated animals were given 80 mg/kg body weight, daily, in aqueous solution (16 mg/ml) intragastrically, for a maximum of 11 days. The rats were killed at different times afterwards (between 12 hr after the first administration and 24 hr after the last, and along 13 subsequent days, after chronic treatment was suspended).

In experiments where other inducing agents have been used, one single dose was given orally, in corn oil solution, 100 mg chlordane, 100 mg DDT/kg body weight and the rats weighed and sacrificed 10, 24 and 48 hr after the administration of each drug.

After decapitation and exsanguination of the animals, livers were quickly removed weighed, pooled and 5 g tissue homogenized immediately in 6·5 vol. of 0·25 M sucrose–1 mM (EDTA). Homogenization and all further operations were carried out at low temperature, and sample containers kept in ice baths.

Liver microsomes were prepared according to the method described by Schenkman *et al.*[17] by centrifuging the homogenate successively at 500 *g* for 10 min, 7000 *g* for

10 min and 12,500 *g* for 10 min to remove cell debris, mitochondria and light mitochondria. The resultant supernatant was centrifuged for 1 hr at 105,000 *g* and the microsomal pellet resuspended in an equal volume 0·15 M KCl 25 mM tris buffer (pH 7·5) and resedimented to remove traces of hemoglobin. The washed microsomes were then suspended in 2 vol. of the same buffer mixture, and adjusted to a protein concentration of 6·67 mg/ml determined by the biuret method.

Cytochrome P-450 was determined in microsomal suspensions containing 2 mg protein/ml as described by Remmer *et al.*[3] from the CO-difference spectrum of dithionite treated microsomes and using the molar extinction coefficient of 91 mM^{-1} cm^{-1} for Δ_A (450–490 mμ) determined by Omura and Sato[18] for calculations and expressed in mμmoles per mg microsomal protein.

Aniline hydroxylase and aminopyrine demethylase activities were determined using an incubation mixture consisting of tris buffer 0·05M (pH 7·5), $MgCl_2$ 5μmoles, Na-isocitrate 8 μmoles, NADP-Na 1 μmole, isocitric deshydrogenase (type IV "Sigma") 20 μl and 8 μmoles of substrate for 1 mg of microsomal protein. Incubations were performed in air, at 37° in a Dubnoff metabolic shaker, for 5 min when the substrate was aminopyrine and for 20 min when aniline was used. Aliquots were precipitated in the cold with TCA, centrifuged and formaldehyde or *p*-hydroxyaniline formed during the reaction, determined in the supernatant with NASH reagent,[19] (consisting of 150 g ammonium acetate, 3 ml glacial acetic acid, 2 ml acetylacetone, made to 500 ml with distilled water, and pH adjusted to 6·00), and with phenol reagent, respectively.

Aminopyrine demethylase and aniline hydroxylase activities were calculated in mμmoles of HCHO, and in mμmoles *p*-aminophenol formed per minute per mg microsomal protein.

For the determination of RNase activity, 1·8 ml microsomal suspensions were treated with 0·2 ml of 5% sodium desoxycholate solution, for 30 min at room temperature. An aliquot of 0·1 ml (corresponding to 0·6 mg microsomal protein) was then incubated for 30 min at 37° in a thermostable shaking bath, with 0·25 ml of 0·25 M tris–HCl buffer pH 7·5, 0·1 ml of 20 mM EDTA and 0·25 ml of 1·2% RNA solution. The purified RNA used as substrate was prepared from a commercial RNA (yeast RNA "B.D.H.") that was treated by the method of Kirby[20] and further dialysed against 0·01 M EDTA as described by Shortman.[21]

Immediately after the incubation, the sample tubes were transferred to an ice bath and precipitated with an equal volume of cold 0·75% uranyl-acetate in 25% perchloric acid.

The assays were run in duplicate as well as blank tests. After complete precipitation the soluble fractions were separated by centrifugation at 0° for 30 min at 12,500 *g*.

0·2 ml of the clear supernatants were diluted with 5 ml of distilled water in a test tube and absorbances determined at 260 mμ. RNase activities were expressed in units per minute per milligram microsomal protein; one unit corresponds to an increase in absolute absorption value of 1000, within the range of linearity.[22]

RESULTS

Rats treated with phenobarbital showed a progressive rise in cytochrome P-450, aminopyrine demethylase and aniline hydroxylase, attaining maximal values at the third day after the initiation of the treatment. Steady state induced conditions were

established after the fourth day. Increased values were kept approximatively at the same level until 48 hr after the last phenobarbital administration, decreasing then gradually to normal values.

RNase was significantly reduced 12 hr after the initiation of the treatment reaching lower stable values at 48 hr. After the prolonged phenobarbital treatment was suspended, microsomal alkaline RNase activity showed a sharp rise, as it is represented in Fig. 1.

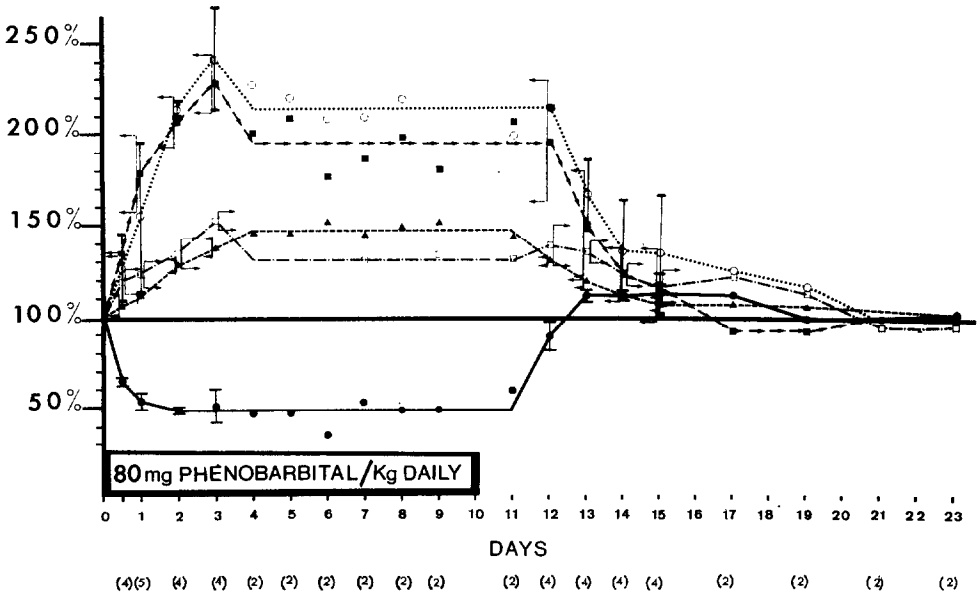

FIG. 1. Plot of the mean percentual values ± S.D. obtained in phenobarbital treated rats, ——●——●——●—— RNase; – –▲– – –▲– – –▲– – liver weight; →■→→■→→■→ cytochrome P-450 levels; · · ·○· · · ·○· · · ·○· · aminopyrine demethylation; – · –□– · – · –□– · – · –□ – · – aniline hydroxylation. Figures in brackets represent the number of experiments for each point.

Control values were: RNase, 0·177 ± 0·031 units per min per mg microsomal protein; g liver per 100 g body weight, 2·78 ± 0·13; cytochrome P-450, 0·88 ± 0·10 mμmoles/mg microsomal protein; aminopyrine demethylation, 3·14 ± 0·25 mμmoles HCHO liberated per min per mg microsomal protein; aniline hydroxylation 0·178 ± 0·034 mμmoles hydroxy-aniline formed per min per mg microsomal protein.

The yield of microsomal protein recuperated per gram liver was 31·04 mg ± 3·6 for control ($n = 10$). A significant increase was only found in rats treated for 4 consecutive days, when the values reached 130 per cent.

Livers from phenobarbital treated rats are bigger and more friable mainly because of the rise in phospholipids of the membranes and consequent increase in cells size. On account of that, the yield of microsomes and microsomal protein in cell fractionation may be affected, as the behavior of the tissue is different, during homogenization and differential centrifugation. Meanwhile, it can be concluded that the decrease in RNase specific activity observed is not due to a dilution effect as it is produced in the first hours after phenobarbital treatment, when no significant increase in microsomal protein was found (103 per cent for 12 hr treatment, 101 per cent for 24 hr, 104 per cent for 48 hr).

Animals treated with a single dose of chlordane, showed to have significantly increased capacities of oxidative drug metabolism, and P-450 hemoprotein, 24 hr after the administration of the drug, and even more strongly at 48 hr, while RNase activity was reduced to less than 50 per cent of the normal 10 hr after the rats were treated, and during the whole period studied (Fig. 2).

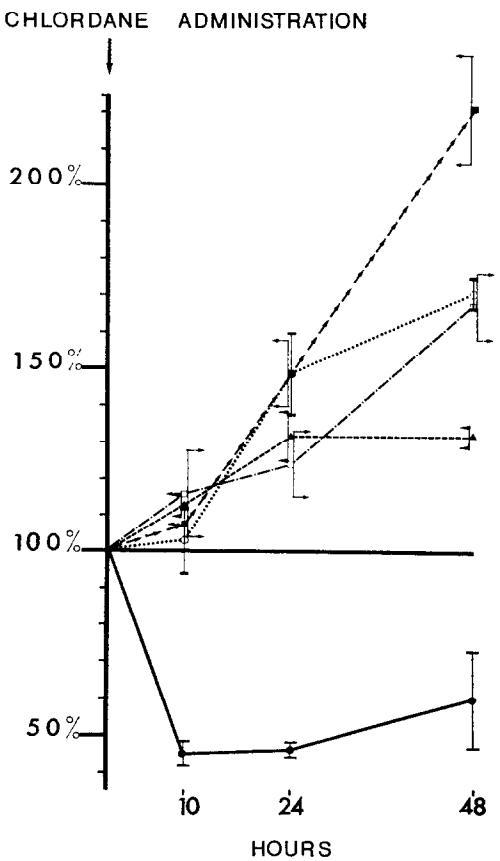

FIG. 2. Plot of the mean percentual values ± S.D. obtained in chlordane treated rats,——●—●—— RNase; – –▲– –▲– –▲– –▲– – liver weight; →■→→■→→■→→ cytochrome P-450 levels; ·····○·····○·····○·· aminopyrine demethylation; – · –□– · – · –□– · – · –□· – aniline hydroxylation.

Control values were: cytochrome P-450, 0·84 ± 0·03 mμmoles; RNase, 0·146 ± 0·023 units; aminopyrine demethylation, 3·27 ± 0·04 mμmoles HCHO; aniline hydroxylation, 0·159 ± 0·015 mμmoles *p*-hydroxyaniline, all expressed per minute per miligram microsomal protein; liver weight 2·40 ± 0·13 g per 100 g body weight.

Each point represents the mean of four experiments.

Ten hours after a single administration of chlorophenothane, a sharp decay in RNase was also produced, and undernormal values maintained along the investigated period. Liver weight, cytochrome P-450 and mixed-function-oxidase activities

determined were significantly elevated 24 and 48 hr after the administration of the drug, as it is shown in Fig. 3.

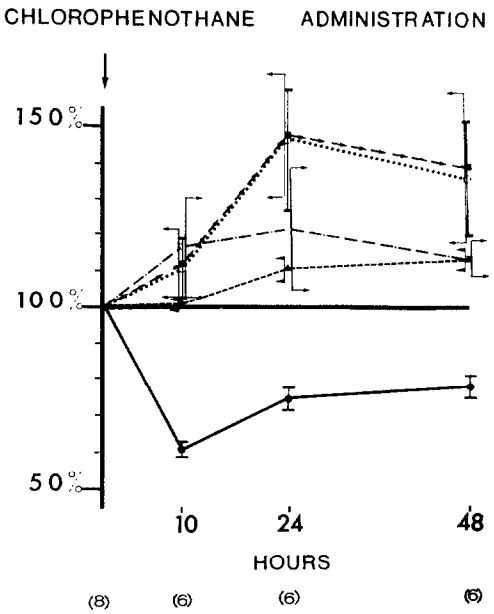

FIG. 3. Plot of the mean percentual values ± S.D. obtained in DDT treated rats,—●—●—●—●— RNase; - -▲- - -▲- - -▲- - -▲- - - liver weight; →→■→→→→■→→→→■→→→ cytochrome P-450 levels; · · ·○· · ·○· · · aminopyrine demethylation; — ·□·— · — ·□— aniline hydroxylation.
Control values were: cytochrome P-450 0·87 ± 0·05 mμmoles; RNase, 0·197 ± 0·046 units; aminopyrine demethylation, 3·40 ± 0·49 mμmoles HCHO liberated; aniline hydroxylation, 0·207 ± 0·016 mμmoles *p*-hydroxyaniline formed, per min per mg microsomal protein. Liver weight 2·61 ± 0·13 g/100 g body weight.
Figures in brackets represent the number of experiments for each point.

As the experiments were performed along a period of several months, the percentage values were determined in relation to controls included in each experiment, in order to prevent the inconvenience of the long-term variations observed in RNase and in oxidative drug metabolism, these recently described by Beuthin and Bousquet.[23]

DISCUSSION

Although the physiological role of RNases is poorly understood, it is known that they are able to hydrolyse cellular RNA's both *in vitro* and *in vivo* mainly the sterically exposed interribosomal segments of messenger RNA, easily available for attack[24] as well as part of the RNA of the larger ribosomal sub-units[25] both involved in binding the ribosomes together in active polysomes.

It is known that in mammalian cells stabilities of polysomes and RNA appear to be at least partly controlled by the activities of RNase[26,27] and it has been shown that RNase is implicated in the regulation of growth. Polysomes prepared from regenerating liver have a lower RNase activity[28] are larger than normal, and more active in

supporting protein synthesis *in vitro*.[29] Liver RNase activity also varies with age, being higher in adult than in immature rats according to Arora and de Lamirande,[30] and our own observations (results not included in this publication). Brewer *et al.*[31] have found that the RNase levels of liver postmitochondrial supernatant fractions from hypophysectomized rats were reduced to normal levels by administration of bovine growth hormone when protein-synthesizing capacity of polysomes was restored, and postulated that RNase plays a possible role in the regulation of protein synthesis in rats. Sarkar[14] has found during the action of synthetic corticosteroids an inverse relationship between RNase activity and synthesis of gluconeogenic enzymes.

From the results obtained in the present investigation it can be concluded that:

(1) With all the three agents used, (phenobarbital, chlordane and DDT) the decrease in the specific RNase activity was a constant and early phenomenon, preceding clearly the rise of cytochrome P-450 and activities of the mixed-function-oxidases that were measured.

(2) During phenobarbital chronic administration, when an increased steady state was established, concerning the values of P-450 hemoprotein and related enzyme activities, a constant low level of RNase was maintained.

(3) When prolonged phenobarbital treatment was suspended RNase activity rose sharply reaching values near 100 per cent 48 hr after the last administration, (and rising even slightly until 72 hr), much before normal values of cytochrome P-450, aniline hydroxylase and aminopyrine oxidative demethylase were completely attained.

Our results corroborate those presented by Louis-Ferdinand and Fuller[16] as regards the inhibition of RNase following prolonged administration of phenobarbital. However, while these authors got a complete suppression of RNase activity in rats treated with repeated administration of 100 mg/kg phenobarbital, we have not found such an important reduction with 80 mg/kg even for longer periods. Besides, 3 days after the last administration we have found RNase activities surpassing slightly the normal values, whereas in the mentioned study the activities were still near zero; at this same period aminopyrine demethylase activities were not statistically different from those found in rats killed under treatment while our animals showed a decreased demethylating activity. These divergences may perhaps be explained by the different strain of animals used.

The fact that modifications of RNase activities were inversely related to variations in liver weight, mixed-function-oxidase activities and cytochrome P-450, preceding both, their rise and decay, and reaching stationary levels before induced or normal stable biochemical conditions were established, suggests that RNase may possibly be considered as one of the conditioning factors of the steady states.

The results obtained in the present investigation, although not providing direct evidences, may be taken as additional arguments supporting the idea that RNase plays a role in the regulation of protein synthesis in mammalian cells,[32,33] hypothesis recently reformulated, based on studies of isolated cells and phytohaemagglutinin transformed lymphocytes,[15] and could be responsible for the accumulation of cellular RNA, mainly due to a decreased rate of degradation,[12] and consequently for the enhancement of protein synthesis produced by phenobarbital and other inducing agents of the same group.

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