STIMULATION OF NUCLEAR PROTEIN SYNTHESIS IN RAT LIVER AFTER PHENOBARBITAL ADMINISTRATION

Raymond W. Ruddon* and Cynthia H. Rainey
Departments of Pharmacology and Oral Biology
University of Michigan Medical and Dental Schools
Ann Arbor, Michigan 48104

Received May 27, 1970

SUMMARY

When a 40 min pulse of leucine-^{3}H was administered to rats 2 hrs after 75 mg/kg of phenobarbital (Pb), there was a 70% increase above control in labeling of microsomal protein and of a nuclear protein fraction extracted with 1 M NaCl. When a 40 min pulse of labeled leucine was given 24 hrs after a single injection of Pb (75 mg/kg), there was a 42% stimulation in the specific activity of microsomal protein but an 87% enhancement, compared to control, in the nuclear protein fraction. Separation of the nuclear fraction on DEAE-cellulose columns indicated that there was a 5-fold stimulation in the specific activity of one of the protein peaks from the nuclear protein fraction of the Pb-treated rats. These data indicate that Pb administration enhances the synthesis of various protein fractions in hepatic cells and suggest that these events may be involved in the enzyme induction produced by this drug.

Alteration of nuclear proteins has been implicated as a mechanism for the elevation of genetic activity produced by various chemical stimuli in mammalian cells. For instance, interaction of hormones with nuclear protein "receptors" may be involved in the induction of protein synthetic events in target tissues (1,2). It has been suggested that histones could serve as repressors of genetic expression and that modification of histones could be a crucial event in the gene activation process in mammalian cells (3,4). Recently, effects on the synthesis of non-histone protein fractions have been reported to occur after the administration of agents which are capable of producing enzyme induction in animal cells. For example, Bresnick (5) has reported that administration of 3-methylcholanthrene to rats produced an increase in the relative quantity of a species of hepatic nuclear protein extracted with 2 M NaCl. In addition, Stein and Baserga (6) have presented evidence that incorporation of

*Research supported by NIH-DE-02731 and a grant from the American Medical Assoc. Education and Research Foundation.

^{3}H-leucine into "acidic nuclear protein fractions" was elevated in mouse salivary glands 2 hrs after isoproterenol administration. The results of the present study indicate that phenobarbital administration leads to increased synthesis of a species of salt-extractable nuclear protein from rat liver.

MATERIALS AND METHODS

Male Sprague-Dawley rats (Spartan Farms, Lansing, Michigan), weighing 80-100 grams were used in the present study. Animals were injected with phenobarbital (75 mg/kg) or saline intraperitoneally prior to pulse-labeling i.p. with radioactive amino acid (leucine-^{3}H, 58.2 Ci/mmole; arginine-^{14}C, 237 mCi/mmole; or arginine-^{3}H, 881 mCi/mmole; obtained from New England Nuclear Corp.). After sacrifice, the livers were removed and homogenized in 4 volumes of ice-cold 0.44 M sucrose in a Dounce homogenizer (4 strokes with loose and 3 with tight-fitting pestle). Nuclei were isolated according to the Chauveau (7) procedure and washed three times with 10 volumes of 0.005 M $MgCl_2$ - 0.05 M Tris-HCl, pH 7.6 (TM buffer) prior to extraction. Histones were extracted from the nuclear fraction with 0.2 N HCl by the method of Pogo _et al._ (8). A non-histone fraction ("acidic nuclear proteins") was extracted by placing the nuclear pellet (about 0.8 gm nuclei pooled from 7 animals) in 50 ml of 1 M NaCl in a Waring blender and mixing for 1 min. The mixture was then stirred in the cold for 1 hr and centrifuged at 25,000 _g_ for 30 min in a Sorvall refrigerated centrifuge. The nuclear pellet was re-extracted with 50 ml 1 M NaCl as above for 1 hr, followed by stirring overnight in an additional 50 ml of 1 M NaCl. The supernatant fractions from the 3 extractions were pooled and then dialyzed against 6 volumes of distilled water to a molarity of 0.14 to precipitate nucleohistone. The mixture was centrifuged at 25,000 _g_ for 30 min. The supernatant fraction was subsequently concentrated to 10 ml in an Amicon ultrafiltration cell. Mitochondria were prepared by centrifugation of the post-nuclear (1000 _g_) supernatant at 12,500 _g_ for 20 min. The pellets were washed by resuspension in TM buffer and re-centrifugation as above. Microsomes were prepared by centrifugation of the post-mitochondrial supernatant at

105,000 g in a Spinco Type 40 rotor for 1 hr, followed by resuspension in TM buffer, and recentrifugation. The various protein fractions were solubilized in 10 ml of Scintisol-toluene phosphor mixture (1:5) and counted in a Packard Tri-Carb liquid scintillation spectrometer. Protein was determined by the method of Lowry et al. (9). Data were calculated as counts/min/mg protein. All assays were done in triplicate.

Separation of the acidic nuclear proteins was achieved by adsorbing the concentrated fraction (after dialysis for 12 hrs in a buffer containing 0.05 M Tris-HCl, pH 8.5, 1 mM dithiothreitol, and 10 mM EDTA) on a 2 cm x 30 cm DEAE-cellulose column. Elution of the protein fractions was carried out by washing the column with 600 ml of the Tris-EDTA buffer, followed by 600 ml of a linear gradient of NaCl in the same buffer. Fractions of 5.6 ml were collected and analyzed for absorbancy at 280 mμ. Aliquots (1 ml) of each fraction were counted in 10 ml of Scintisol-toluene phosphor as above.

TABLE 1

Effect of Phenobarbital (75 mg/kg) on Incorporation of Labeled Arginine into the Histone Fraction of Rat Liver*

Treatment	Duration of pulse	Incorporation (cpm/mg protein)
Experiment 1**		
Control	40 min	428
Phenobarbital for 2 hrs and 40 min	"	443
Experiment 2†		
Control	11 hrs	1,060
Phenobarbital for 12 hrs	"	1,460

* In each experiment, the histone fractions were prepared and pooled from 7 control and 7 treated animals.
**Arginine-^{14}C (5 μCi per animal)
† Arginine-^{3}H (62.5 μCi per animal)

RESULTS AND DISCUSSION

Table 1 illustrates the effects of phenobarbital (Pb) administration on incorporation of labeled arginine into the "histone" (i.e. acid-extractable) fraction of nuclear proteins from rat liver. When a 40 min pulse of arginine-^{14}C was given 2 hrs after Pb, there was no difference between the specific activities of the total histone fraction in the 2 groups of animals. However, when an 11 hr pulse was administered 1 hr after Pb injection, there was a 38% increase in the specific activity of the histone fraction from the Pb-treated animals. Separation of these fractions on polyacrylamide gels showed identical absorbancy tracings by densitometer analysis, and there was not a detectable, discreet increase in specific activity in a particular protein band. Since there is evidence indicating that histone synthesis occurs in conjunction with DNA synthesis in mammalian cells (10), this increase in histone labeling may reflect an increased cell proliferation in the liver which has been reported to occur after Pb administration (11,12).

The data in Table 2 indicates the specific activities of various subcellular protein fractions with a 40 min pulse of leucine-^{3}H administered 2 hrs after Pb. Significant increases in specific activity are seen in the micro-

TABLE 2

Incorporation of Leucine-^{3}H (200 μCi) into Various Cellular Proteins of Rat Liver 2 Hours after Phenobarbital (75 mg/kg)*

Treatment	Protein Fraction			
	Mitochondrial	Microsomal	Acidic Nuclear	TCA soluble supernatant**
		cpm/mg protein		
Control	9,448	8,276	3,816	6,828
Phenobarbital	10,791	14,078 (+71%)	6,484 (+70%)	5,380

* Fractions were prepared from 7 control and 7 treated animals. Pulse of leucine-^{3}H was for 40 min.
**cpm/ml of TCA soluble supernatant fraction.

somal and "acidic nuclear" (i.e. 1 M NaCl extractable and not re-associated with DNA at 0.14 M NaCl) protein fractions. The magnitude of the increased labeling is identical in the two fractions. This may indicate that events leading to the stimulation of incorporation of labeled amino acids into protein occur in both nucleus and microsomes or that the acidic nuclear protein fraction is largely synthesized in the microsomal fraction and then transported to the nucleus, in which case the increased label in the acidic nuclear species would merely reflect a general increase in the capacity of the microsomes to synthesize protein. This increase cannot be explained by an alteration of amino acid uptake or change in the specific activity of the soluble amino acid pool since the specific activity of the TCA-soluble pool was lower in the Pb-treated rats.

A similar experiment was performed 24 hrs after a single injection of Pb (Table 3). In this case, the magnitude of the elevation in specific activity of the microsomal fraction was less than it was with a 40 min pulse 2 hrs after Pb and about 1/2 the increase seen in the acidic nuclear proteins 24 hrs after Pb. This may indicate that 24 hrs after Pb there is a continued,

TABLE 3

Incorporation of Leucine-^{3}H (200 μCi) into Various Cellular Proteins of Rat Liver 24 hrs after Phenobarbital (75 mg/kg)*

Treatment	Protein Fraction			
	Mitochondrial	Microsomal	Acidic Nuclear	TCA soluble supernatant**
		cpm/mg protein		
Control	10,012	12,434	3,748	7,416
Phenobarbital	12,479	17,638 (+42%)	6,996 (+87%)	7,731

* Fractions were prepared from 7 control and 7 treated animals. Pulse of leucine-^{3}H was for 40 min.
**cpm/ml of TCA soluble supernatant fraction

preferential stimulation of the synthesis of nuclear protein species, or that the breakdown of a nuclear species which normally has a very short half-life (i.e. less than 40 minutes) is inhibited by Pb. In view of the short pulse times employed, the former explanation would seem the most likely. Apparently, the processes involved in the drug-induced enhancement of protein fabrication by microsomes are returning toward the control state 24 hrs after Pb, as the magnitude of the elevation in the specific activity of microsomal protein was considerably less than it was 2 hrs after drug.

Attempts to separate the total acidic nuclear protein fraction by polyacrylamide gel electrophoresis and to determine the specific activity of the large number of protein bands in the gels were inconclusive. Partial separation of the protein fractions was achieved on DEAE-cellulose columns. Figure

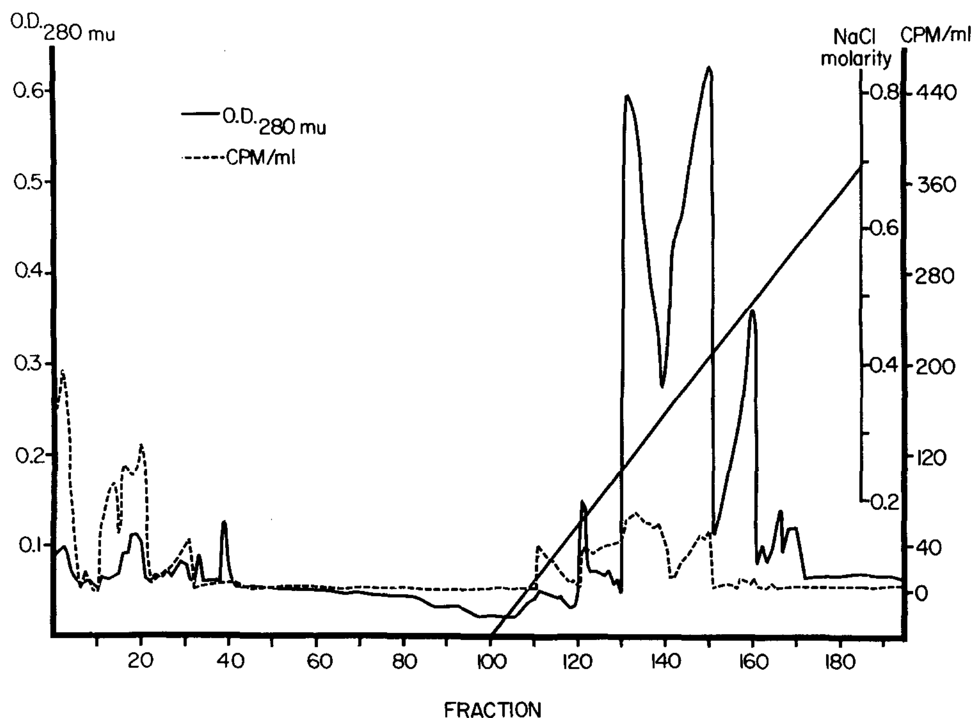

Figure 1. Fractionation of acidic nuclear proteins from the livers of saline-treated rats by DEAE-cellulose chromatography. Nuclear protein samples from 21 rats were pooled after pulse-labeling for 40 min with 200 μCi of leucine-^{3}H. Experimental details are given in Materials and Methods.

1 shows the pattern for the extracts from control animals. As can be seen, there were a number of small peaks that were eluted with the Tris-EDTA buffer prior to the addition of the NaCl gradient. The major peaks were eluted after the addition of the gradient. However, the specific activity of the early peaks was markedly higher than that of the major protein peaks. For example, the specific activity of the second peak (which appears as doublet on the cpm/ml profile) was 4970 cpm/mg protein whereas the specific activity of the first large peak eluted in NaCl was 1550 cpm/mg protein. This suggests that the peaks eluted in salt-free buffer have a higher rate of turnover or are more actively synthesized than the proteins eluted with the salt gradient. In addition, the fact that these more active proteins were not well adsorbed to DEAE-cellulose suggests that they are not very "acidic." A striking difference between the control pattern and that of the Pb-treated group was evident as illustrated in Figure 2. There was a marked increase in labeling of the

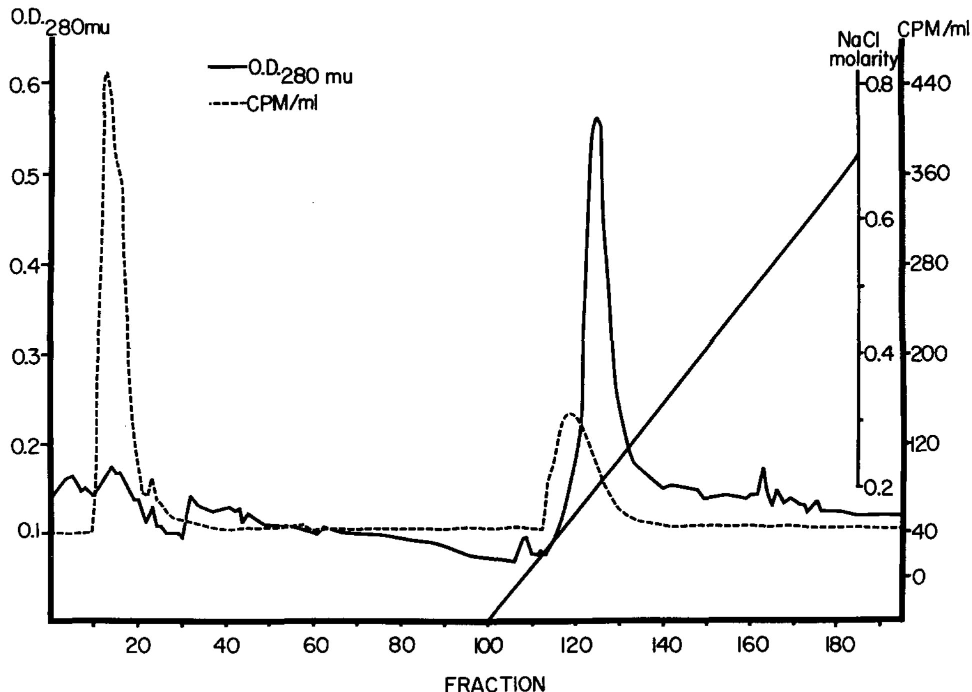

Figure 2. Fractionation of acidic nuclear proteins from the livers of phenobarbital-treated rats by DEAE-cellulose chromatography. Other parameters are as listed for Fig. 1.

second protein peak in the fraction from the Pb-treated animals; the specific activity of this peak was approximately 5-fold that of the corresponding peak in the control fraction. In addition, only one major peak was eluted with the NaCl gradient separation of the Pb fraction. These data suggest that 1) the synthesis of a specific species of hepatic nuclear protein is stimulated by Pb administration, and 2) Pb treatment markedly alters the extractability of nuclear proteins or their ability to reassociate with chromatin when the nuclear extracts are dialyzed to 0.14 M NaCl, since DEAE-cellulose fractionation indicated that certain protein species were apparently missing in the nuclear extracts from Pb-treated rats. A number of "acidic nuclear proteins" appear to reassociate with DNA (after 1 M NaCl extraction) when dilution below 0.4 M NaCl is carried out (13), and it is possible that Pb treatment enhances the reassociation of certain protein species with DNA. The significance of these findings is not yet clear, but the alterations in nuclear protein synthesis and properties seen after Pb administration may play a role in the enhancement of enzyme activity observed in liver after treatment with the drug. Additional studies are being carried out to further characterize these changes and to ascertain their relationship to enzyme induction.

REFERENCES

1. Jensen, E. V., Suzuki, T., Kawashima, T., Stumpf, W. E., Jungblut, P. W. and DeSombre, E. R. Proc. Natl. Acad. Sci. U.S., 59, 632 (1968).

2. Shyamala, G. and Gorski, J., J. Biol. Chem., 244, 1097 (1969).

3. Allfrey, V. G., Faulkner, R., and Mirsky, A. E., Proc. Natl. Acad. Sci. U.S., 51, 786 (1964).

4. Kleinsmith, L. J., Allfrey, V. G. and Mirsky, A. E., Proc. Natl. Acad. Sci. U.S., 55, 1182 (1966).

5. Bresnick, E., Fed. Proc., 29, 2778 (1970).

6. Stein, G. and Baserga, R., Fed. Proc. 29, 3207 (1970).

7. Chauveau, J., Moule, Y. and Rouller, C., Exptl. Cell Res., 11, 317 (1956).

8. Pogo, B. G. T., Allfrey, V. G. and Mirsky, A. E., Proc. Natl. Acad. Sci. U.S., 55, 805 (1966).

9. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., J. Biol. Chem., 193, 265 (1951).

10. Robbins, E. and Borun, T. W., Proc. Natl. Acad. Sci. U.S., 57, 409 (1967).

11. Burger, P. C. and Herdson, P. B., Amer. J. Pathol., 48, 793 (1966).

12. Conney, A. H. and Gilman, A. G., J. Biol. Chem., 238, 3682 (1963).

13. Kleinsmith, L. J., Personal communication.