MOLECULAR PHARMACOLOGY, 6, 247-258

Effects of Phenobarbital and 3-Methylcholanthrene on Amino Acid Incorporation into Rat Liver Chromosomal Proteins

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(Received November 13, 1972)

SUMMARY

BLANKENSHIP, JIM, AND KUEHL, LEROY: Effects of phenobarbital and 3-methylcholanthrene on amino acid incorporation into rat liver chromosomal proteins. *Mol. Pharmacol.* **9**, 247-258 (1973).

The effects of phenobarbital and 3-methylcholanthrene on amino acid incorporation into rat liver chromosomal proteins *in vivo* were studied. Although both drugs stimulated incorporation into histones and non-histone chromosomal proteins, the results of dual-isotope experiments revealed that the increases were not selective for any of the major non-histone chromosomal protein fractions. Rather, incorporation into all these fractions was apparently stimulated uniformly. This nonselective stimulation of incorporation may reflect drug-induced increases in amino acid incorporation preceding mitotic activity and liver growth.

INTRODUCTION

Induction of hepatic microsomal enzymes by drugs such as phenobarbital and 3-methylcholanthrene apparently involves activation of a portion of the genome. Administration of these drugs leads to increases in amino acid incorporation into the microsomal fraction (1, 2) and in RNA polymerase activity (3, 4). The induction can be blocked by inhibitors of protein or RNA synthesis (5, 6). Chromatins isolated from the livers of rats treated with phenobarbital or 3-methylcholanthrene have an enhanced ability to serve as templates for RNA synthesis (7, 8). The chromosomal proteins are apparently responsible for this alteration, because the template activities of chromatins from drug-treated and control animals are quite similar after extraction of the histones and some non-histone proteins with 2 M NaCl (8, 9).

Several groups of workers have studied the effects of phenobarbital or 3-methylcholanthrene on the metabolism of chromosomal proteins. Although Byvoet (10) found that phenobarbital had no effect on the half-lives of rat liver histones, Ruddon and Rainey (11) did observe an enhancement of amino acid incorporation into the histones as well as into the acidic nuclear proteins of rat liver after phenobarbital treatment. Yee and Bresnick (9) found that 3-methylcholanthrene also stimulated amino acid incorporation into a chromosomal protein

These studies were supported by United States Public Health Service Grants GM00153, GM13864, and FR05428.

[1] This is an essential portion of a thesis submitted to the Department of Pharmacology, University of Utah, in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Present address, Department of Cell and Molecular Biology, Medical College of Georgia, Augusta, Georgia 30902.

fraction (probably histones) and altered the electrophoretic profile of the histones.

It is well established that chromosomal proteins influence transcription. Histones inhibit DNA-directed RNA synthesis (12, 13) but apparently lack the heterogeneity and tissue specificity necessary for complete control of transcription [for review, see Stellwagen and Cole (14)]. Non-histone chromosomal proteins are much more heterogenous (15, 16), are tissue-specific (16, 17), are altered during differentiation (18), and can modify transcription in isolated chromatin (19–21). These characteristics suggest a role in the control of genetic expression.

If the non-histone chromosomal proteins are involved in genetic control, modifications in structure or metabolism of the proteins might be expected to occur during gene activation or repression. In fact, two of the early effects of many hormones on target organs are stimulation of RNA synthesis and a concomitant increase in amino acid incorporation into non-histone chromosomal proteins. Insulin (22), cortisol (22), somatotropic hormone (23), testosterone (23), 3,5,3′-triiodo-L-thyronine (23), and 17β-estradiol (24) have all been found to enhance incorporation into these proteins. The stimulation produced by cortisol and 17β-estradiol has been found to be selective, occurring in specific non-histone protein fractions. Acidic nuclear proteins from the livers of cortisol-treated and control rats were subjected simultaneously to electrophoresis on polyacrylamide gels by Shelton and Allfrey (25). All rats were adrenalectomized. The cortisol-treated rats received [^{3}H]leucine; the control rats, [^{14}C]leucine. The ratio of the two isotopes in each fraction indicated the relative rates of incorporation into proteins from treated and control animals. The rate of incorporation into one specific protein fraction increased within 2–3 hr after cortisol treatment and exceeded the control level by 200% within 7–8 hr. Teng and Hamilton (24) and Barker (26) found that 17β-estradiol produced a similar stimulation of amino acid incorporation into a single acidic nuclear protein fraction from the uterus of ovariectomized rats.

In the present study the effects of phenobarbital and 3-methylcholanthrene on amino acid incorporation into chromosomal proteins were investigated. Rates of amino acid incorporation into the histone and non-histone chromosomal protein fractions were determined at various times after drug treatment, and a dual-isotope technique similar to that of Shelton and Allfrey (25) was used to compare the relative rates of incorporation into individual non-histone chromosomal proteins.

METHODS

Animals. Male albino rats, 100–150 g in weight, were obtained from the Holtzman Rat Company, Madison, Wis. The rats were allowed free access to food and water until 24 hr before death, at which time they were deprived of food. Animals were maintained on an alternating light (14-hr) and dark (10-hr) schedule and were always killed about 2 hr after the beginning of the light period.

Drug treatments and protein labeling. The drug-treated animals received intraperitoneal injections of 3-methylcholanthrene (Sigma), 20 mg/kg of body weight, or phenobarbital (Merck), 75 mg/kg of body weight. Control rats were treated similarly with corn oil or 0.15 M NaCl, the vehicles for 3-methylcholanthrene and phenobarbital, respectively. At various times after treatment, the animals received intraperitoneal injections of [4,5-^{3}H]L-leucine (38.6 Ci/mmole, New England Nuclear) or of uniformly labeled [^{14}C]L-leucine (331 mCi/mmole, Amersham/Searle), both in 0.15 M NaCl. One hour later the animals were stunned by a blow on the head and decapitated; the livers were quickly removed and placed in ice-cold 0.25 M sucrose–25 mM KCl–5 mM $MgCl_2$–50 mM Tris-HCl buffer, pH 7.5.

Isolation of chromatin. The procedures for the isolation of chromatin and chromosomal proteins were performed at 4°. The livers were homogenized and the nuclei isolated by the method of Blobel and Potter (27), modified as follows: (a) the homogeni-

zer was run at 350 rpm instead of 1700 rpm, and (b) 2.1 M rather than 2.3 M sucrose served as the lower layer during centrifugation. The RNA:DNA ratios (0.112 ± 0.18, mean ± SD) and the protein to DNA ratios (2.45 ± 0.14) of nuclei isolated by this modified procedure were not significantly different from ratios obtained in this laboratory for nuclei isolated by the original method of Blobel and Potter (27). Chromatin was prepared from the isolated nuclei as described by Shaw and Huang (28), except that the final four Tris buffer washes were eliminated. The washes used in preparing chromatin did not remove appreciable amounts of protein from the nuclei, but served primarily to change the physical state of the chromatin.

Effects of drugs on metabolism of histones and non-histone chromosomal proteins. At various times after drug treatment animals received 100 μCi of [^{3}H]leucine; 1 hr later the chromatin was isolated as described above. The histone fraction was extracted from the chromatin with two 10-ml portions of 0.25 N H_2SO_4. The insoluble material which remained after the acid extractions (residual fraction) was stirred overnight in 10 ml of 0.01 M Tris-HCl buffer, pH 8.0, and resuspended by homogenization. In addition, a 2-ml portion of the original homogenate was centrifuged at 9000 × *g* for 10 min, and the supernatant cytoplasmic fraction was saved. The specific activities of the proteins in these three fractions were determined as described previously (29). A *t*-test for unpaired samples was used to determine whether the specific activities of the proteins from drug-treated animals were significantly different from those of control animals.

Isolation of dual-labeled chromosomal proteins for gel electrophoresis. Each experiment consisted of two drug-treated animals and two control animals. One control and one drug-treated animal received 100 μCi of [^{14}C]leucine; the other two animals received 500 μCi of [^{3}H]leucine. The liver from each animal was minced and divided into two equal portions. These portions were mixed as shown in Fig. 1. Two preparations were analyzed for each treatment time; the drug-treated animal received [^{14}C]leucine in one case and [^{3}H]leucine in the other.

The chromosomal proteins were extracted from chromatin by the method of Shaw and Huang (28). This consisted of extraction with 7.0 M urea–3.0 M NaCl, centrifugation to remove DNA, dialysis to remove the urea and NaCl, and lyophilization.

Gel electrophoresis. The procedure of Panyim and Chalkley (30) was followed. The gels were 15% acrylamide in 2.5 M urea and 0.9 M acetic acid (pH 2.8). Ethylene diacrylate was used in place of *N*,*N*′-methylenebisacrylamide in order to obtain gels which could be dissolved in alkali (31). The gels were electrophoresed before use for 2 hr at 2 mamp/tube.

The lyophilized chromosomal proteins

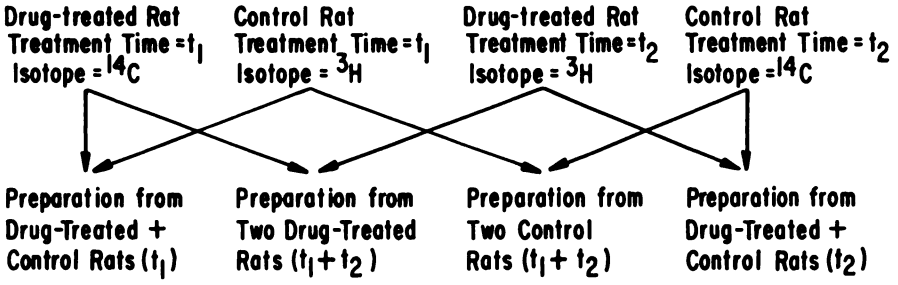

FIG. 1. *Procedure followed in dual-isotope experiments*

In each experiment two control and two drug-treated rats received an injection of labeled leucine. Following incorporation, the liver from each animal was divided into two equal parts. The liver halves were then mixed together as indicated. The resulting mixtures were further processed as described in the text. The treatment time for control animals refers to time of treatment with vehicle (0.15 M NaCl or corn oil).

were dissolved in 9.5 M urea–0.9 M acetic acid–0.5 M β-mercaptoethanol and incubated at 37° for 2 hr. An aliquot (0.15–0.30 ml) of this solution, containing 200–250 μg of protein, was applied to each gel. Electrophoresis was conducted at 175 V for 4.5 hr.

The gels were stained by soaking overnight in 1.0% Amido black–20% ethanol–7% acetic acid and destained electrically in 20% ethanol–7% acetic acid. Densitometric tracings were prepared in a Gilford model 2000 spectrophotometer with a linear transport attachment.

Three treated gels were used for each analysis. Each gel was sliced into approximately 50 segments, each 2.5 mm thick. The corresponding segments from the three gels were placed in single counting vials, and 1.2 ml of "NCS" solubilizer (a toluene-soluble, quaternary ammonium base supplied by Amersham/Searle) were added. The vials were capped and heated overnight at 60° to partially dissolve the gel segments. The resulting solution was diluted with 20 ml of scintillation fluid (29), and the vials were shaken for several days to dissolve the gels completely.

Counting procedures. Counting was done in a Packard Tri-Carb liquid scintillation spectrometer with automatic external standardization. The counts were converted to disintegrations by a computer programmed to use external standardization data in conjunction with quench correction curves.

Analytical measurements. Protein was determined by the method of Lowry *et al.* (32) as described previously (29). The RNA and DNA concentrations were determined by the methods of Mejbaum (33) and Burton (34), respectively.

Enzyme assays. The rates of *N*-demethylation of ethylmorphine and *p*-chloro-*N*-methylaniline were determined by the method of Anders and Mannering (35), except that a 9000 × *g* supernatant fraction of rat liver was assayed in place of the microsomal enzyme preparation. The reaction mixtures contained either 10 μmoles of ethylmorphine or 5 μmoles of *p*-chloro-*N*-methylaniline, and formaldehyde production was measured with the use of Nash's reagent (36).

RESULTS

Induction of drug-metabolizing enzymes. Two hepatic enzyme activities were measured at various times after drug treatment to verify that induction did occur in these experiments. The rates of metabolism of ethylmorphine and *p*-chloro-*N*-methylaniline were used as measures of the enzyme induction produced by phenobarbital and 3-methylcholanthrene, respectively. Between 8 and 12 hr after phenobarbital treatment there was an increase in the rate of ethylmorphine metabolism, and at 36 hr the rate was more than twice the control value. An increase in the rate of *p*-chloro-*N*-methylaniline metabolism occurred within 6 hr after 3-methylcholanthrene treatment, and a maximum rate nearly twice that of the control was reached at 12 hr. Remmer (37) found that about 12 hr after the administration of phenobarbital the rate of demethylation of methylaminoantipyrine increased in rat hepatic microsomes, and a maximum rate was reached after about 48 hr. Orrenius (38) detected an increase in microsomal enzyme activity within 6 hr after phenobarbital treatment. Other workers (39, 40) have reported increases in rat liver enzyme activities within 6 hr after the administration of 3-methylcholanthrene. Maximum activities occurred within 12–48 hr after drug treatment. These results are generally in agreement with those of the present study, which indicate that the onset of enzyme induction produced by either drug occurs within 6–12 hr after treatment, and that enzyme activity continues to increase for at least 12–48 hr. The times chosen for the study of drug effects on amino acid incorporation into chromosomal proteins were selected to span the initial period of induction (the first 24 hr following drug treatment), during which regulatory functions should be operative.

Electrophoretic profiles of chromosomal proteins. A typical electrophoretic pattern of the chromosomal proteins of rat liver is shown in Fig. 2A. The patterns generally

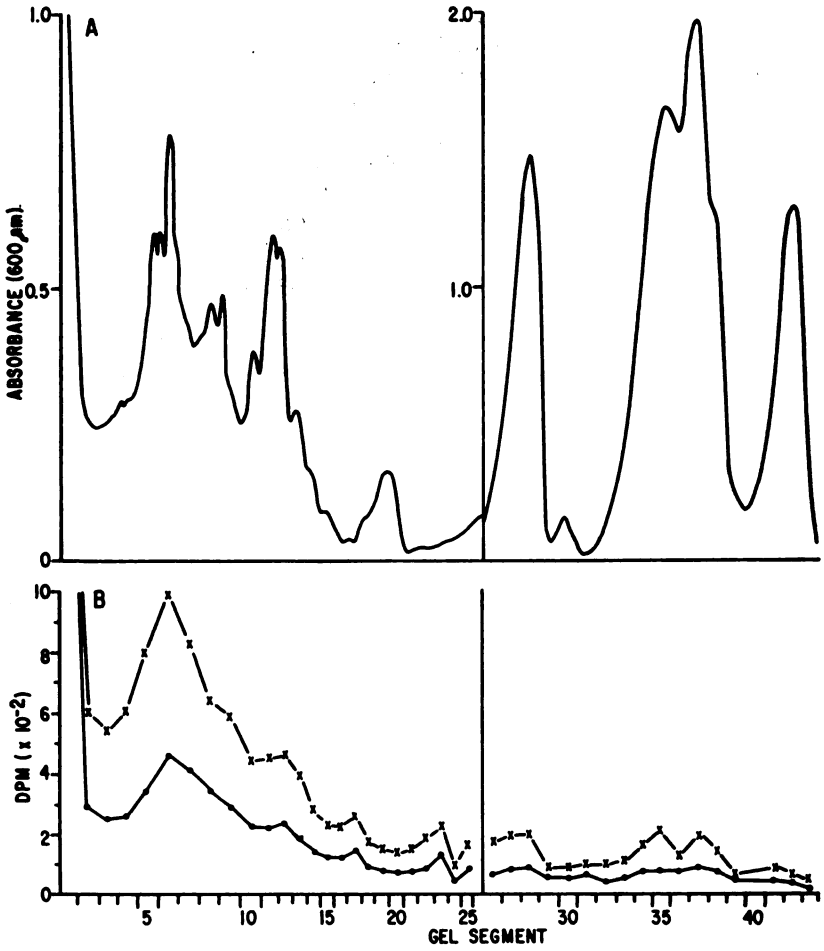

FIG. 2. *Electrophoretic profiles of chromosomal proteins from rat liver*

A. Densitometric tracing of electrophoretogram of chromosomal proteins. Proteins were stained with Amido black. The direction of migration is from left to right.

B. Radioactivity profiles from the same gel as in Fig. 2A. The ^{3}H-labeled proteins (×——×) were obtained from a rat given an injection of corn oil 12 hr before death, and the ^{14}C-labeled proteins (●——●) were derived from a rat that received 3-methylcholanthrene 12 hr before death. The higher ^{3}H activity compared to ^{14}C activity reflects, in part, the different amounts of the two isotopes injected

contained at least 20 distinct bands and were quite similar for each preparation analyzed. Shaw and Huang (28) characterized the slowly migrating components (segments 1–25) as non-histone proteins, and the more rapidly migrating components (segments 26–43) as histones.

No discernible changes in the electrophoretic profiles of these chromosomal proteins were observed within 24 hr after treatment with either phenobarbital or 3-methylcholanthrene. Yee and Bresnick (9) found that 3-methylcholanthrene alters the electrophoretic profile of the 2 M NaCl-extractable proteins of rat liver chromatin. At 12–48 hr after drug treatment there was a decrease in the very lysine-rich histone fraction and an increase in the more slowly migrating protein components. The procedures for extraction and electrophoresis of these proteins were different from those of the present study and may account for the difference.

Effects of drugs on metabolism of histones

and non-histone chromosomal proteins. Proteins of rat liver chromatin were fractionated into acid-extractable proteins (histones) and residual proteins (non-histone proteins). For each gram of liver fractionated, an average of 1.3 mg of histones and 1.8 mg of non-histone proteins was obtained. The proportion of non-histone proteins in the presently described preparations was higher than has been reported by some workers for rat liver chromatin (13), probably because of the less rigorous procedure for the purification of chromatin used in our studies. This purification procedure was used to retain chromosomal proteins for the dual-labeling analyses, which might have been extracted by a more rigorous procedure.

The acid-extractable proteins and the residual proteins were subjected to electrophoresis separately. As evidenced by the electrophoretic patterns, there was only a small amount of cross-contamination between the histone and non-histone protein fractions.

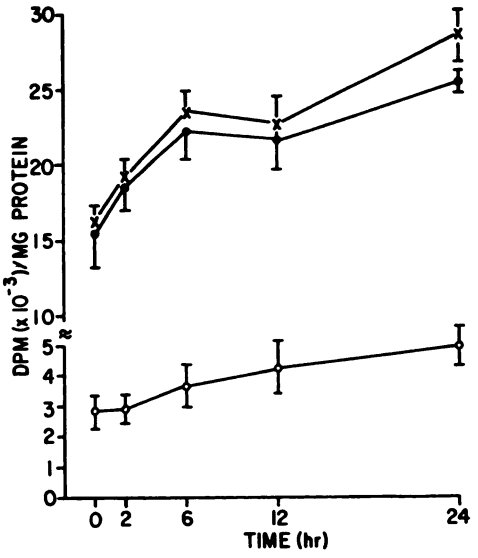

FIG. 3. *Effect of phenobarbital on [³H]leucine incorporation into rat liver cytoplasmic and chromosomal proteins*

The effects of phenobarbital treatment on the specific activities of the 9000 × *g* supernatant fraction (●——●), on the histone fraction (○——○), and on the non-histone chromosomal protein fraction (×——×) were determined at the times indicated. Rats in the zero time group received 0.15 M NaCl 12 hr before death. Values are the means ± standard errors from at least six animals.

Phenobarbital produced parallel increases in leucine incorporation into the residual chromosomal and cytoplasmic proteins (Fig. 3). The specific activities were significantly different ($p < 0.01$) from the controls as early as 6 hr after drug treatment. There was also an increase in the specific activity of the acid-extractable proteins; however, the specific activity did not reach a level significantly different ($p < 0.05$) from the controls until 24 hr after drug treatment. The highest specific activities detected for the three fractions occurred 24 hr after drug treatment (the longest treatment time investigated) and represented increases of 65–75% over control levels.

Ruddon and Rainey (11) also found that treatment of rats with phenobarbital increases the rate of amino acid incorporation into chromosomal and cytoplasmic proteins. Two hours after drug treatment the specific activities of the acidic nuclear and microsomal protein fractions were 70% greater than control levels. The rates of incorporation into both fractions were still elevated 24 hr after drug treatment. A small increase in incorporation into the acid-extractable nuclear proteins was observed 12 hr after drug treatment. Although the protein fractions are not identical, the results of Ruddon and Rainey are similar to those of the present study.

At 24 hr after the administration of 3-methylcholanthrene there were significant ($p < 0.02$) increases in the rate of leucine incorporation into the two chromosomal protein fractions (Fig. 4). The specific activities were 30–40% above control levels. No significant changes were detected in the cytoplasmic fractions.

Yee and Bresnick (9) observed that 3-methylcholanthrene enhanced the incorporation of [^{14}C]lysine into the 2 M NaCl-extractable proteins of rat liver. An increase in incorporation was observed 3 hr after drug treatment, and a maximum specific activity twice that of the controls was reached between 6 and 24 hr. Yee and

Bresnick did not detect any effect on tryptophan incorporation into these proteins, and speculated that the increase in incorporation may occur in the histone fraction, since histones do not contain tryptophan. These results are not in agreement with those of the present study; however, a direct comparison is probably inappropriate because different extraction procedures were used in the two studies.

Effects of drugs on metabolism of individual non-histone chromosomal protein fractions. Electrophoretic profiles of radioactivity incorporated into chromosomal proteins of a drug-treated (3-methylcholanthrene, 12 hr) and a control animal are represented in Fig. 2B. The proteins from a control animal (labeled with ^{3}H) and a drug-treated animal (labeled with ^{14}C) were subjected to simultaneous electrophoresis as described under METHODS. In all preparations analyzed, much more activity was found in the non-histone proteins than in the histones, probably because of the very slow turnover of the latter. The histones stained very darkly; consequently, samples taken from this region for counting were highly quenched. This, combined with the low levels of radioactivity in the histones, resulted in highly variable results for these proteins. Therefore only the results for the non-histone proteins will be reported.

To detect drug-induced changes in the rate of metabolism of individual non-histone chromosomal proteins, the ratio of the isotope from the drug-treated animal to that from the control animal was determined for each segment of the electrophoretogram. Segments with unusually high or low ratios were presumed to contain proteins whose metabolism had been altered by the drug. The ratios varied greatly from one preparation to another. The comparison of different preparations was simplified by dividing the ratio for each gel segment by the average ratio for that electrophoretogram. As a result of this normalization, a value of 1.20 indicates a 20% greater incorporation than the average for the whole preparation (1.00).

Two preparations were analyzed for each treatment time. To eliminate possible isotope effects, the isotopes given to the drug-treated and control rats were reversed for the two preparations.

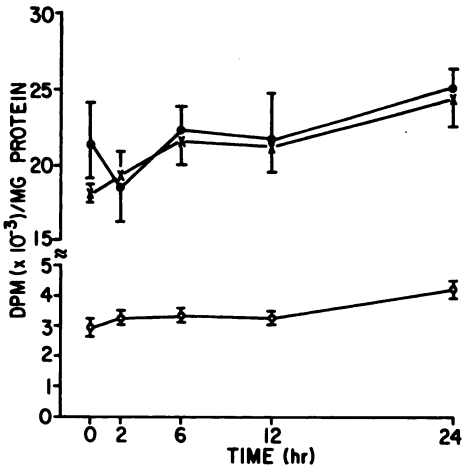

FIG. 4. *Effect of 3-methylcholanthrene on [^{3}H] leucine incorporation into rat liver chromosomal and cytoplasmic proteins*

Details were as described for Fig. 3, except that the zero time group received corn oil instead of 0.15 M NaCl.

The variability due to experimental error and differences between animals was determined by analyzing several control preparations (Fig. 5). From the results of these control preparations, 95% tolerance limits were calculated at a confidence level of 95%.

The ratios for the first gel segment in each preparation are not shown. Because of a high concentration of stain, these samples were highly quenched and the values obtained were probably inaccurate. More accurate ratios for several control preparations and most of the drug treatment times were obtained from unstained gels. These ratios were within the range set by the other gel segments.

The normalized isotope ratios obtained for preparations from phenobarbital- and 3-methylcholanthrene-treated animals are shown in Figs. 6 and 7. Fewer than 5% of the normalized ratios from these preparations lay outside the 95% tolerance limits calculated from the control preparations, and none of the pairs of preparations run for each treatment time contained corre-

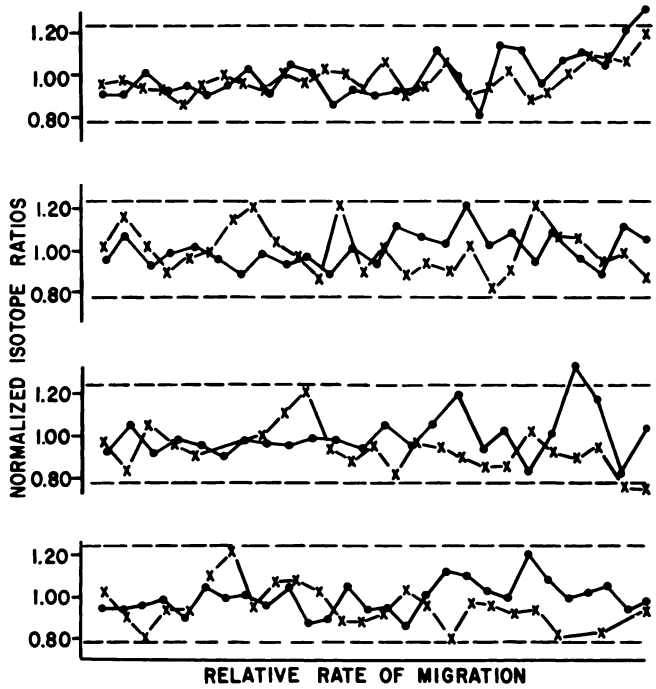

FIG. 5. *Relative rates of leucine incorporation into non-histone chromosomal proteins of paired control animals*

Normalized isotope ratios are plotted for each gel segment for the region of the gel containing the non-histone chromosomal proteins. Data from four pairs of animals are plotted at $^{3}H:^{14}C$ ratios (×——×); the other data are plotted as $^{14}C:^{3}H$ ratios (●——●). The dashed lines represent the 95% tolerance limits at $p = 0.05$, calculated from the pooled results of the control preparations.

sponding points which lay outside these limits. Consequently these ratios are best attributed to random fluctuation, and no selective drug effect was detected.

Ruddon and Rainey (41) used a similar technique to determine the effects of phenobarbital treatment on individual acidic nuclear protein fractions from rat liver. The ratios of activities in proteins from a control animal and an animal treated 2 hr earlier with phenobarbital revealed an apparent difference in the rate of labeling of proteins in one region of the gel. Their data, however, may not indicate a drug effect, as will be discussed below.

DISCUSSION

The dual-isotope technique used in this study is the method of choice for detecting alterations in the metabolism of individual components in a mixture of proteins. Because tissues from control and from treated animals are mixed prior to fractionation, several major sources of experimental variation are eliminated, namely, those associated with fractionation of the protein components and with preparation of individual protein fractions for counting. In addition, the extent of experimental variability resulting from other factors may easily be assessed by comparing proteins from two animals which have been treated in the same manner (e.g., two control animals). In the present study such comparisons were extremely useful in interpreting the data from treated animals.

Radioactivity profiles obtained by comparing the non-histone chromosomal proteins of control animals with those of phenobarbital-treated (Fig. 6) and 3-methylcholanthrene-treated (Fig. 7) animals are quite similar to those obtained by comparison of two control animals (Fig. 5). These results, therefore, fail to provide evi-

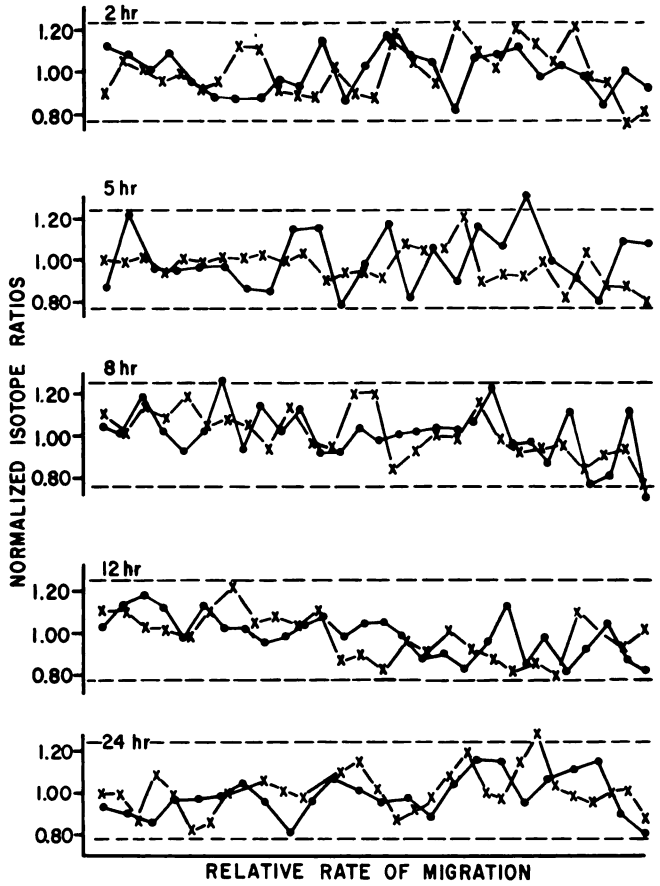

FIG. 6. *Effects of phenobarbital on relative rates of leucine incorporation into non-histone chromosomal proteins*

Details were as described for Fig. 5, except that data are presented as the ratio of activity from the drug-treated animal to that from the control. The time of death after drug treatment is indicated above each graph.

dence, under the experimental conditions employed, that induction by phenobarbital or by 3-methylcholanthrene is preceded or accompanied by alterations in the metabolism of specific non-histone chromosomal proteins. Of course, alterations might not be detected if they were small or if they occurred in minor protein species.

Phenobarbital was selected for study because of its dramatic effects on the liver. This drug, if given over a period of several days, greatly increases mitotic activity of liver cells (42), increases liver weight by 40–50% (43), increases strikingly the relative amount of smooth endoplasmic reticulum in hepatic cells (5), and induces synthesis of drug-metabolizing enzymes (44). If alterations in the metabolism of non-histone chromosomal proteins are generally involved in induction, such changes would certainly be expected to occur in the liver cells of phenobarbital-treated rats. Our failure to find changes in either phenobarbital- or 3-methylcholanthrene-treated animals suggests that drug-induced stimulation of protein synthesis is not necessarily mediated by alterations in the rates of synthesis or degradation of specific non-histone chromosomal proteins.

Two groups of workers, using methods similar to those employed in the present study, have reported alterations in the

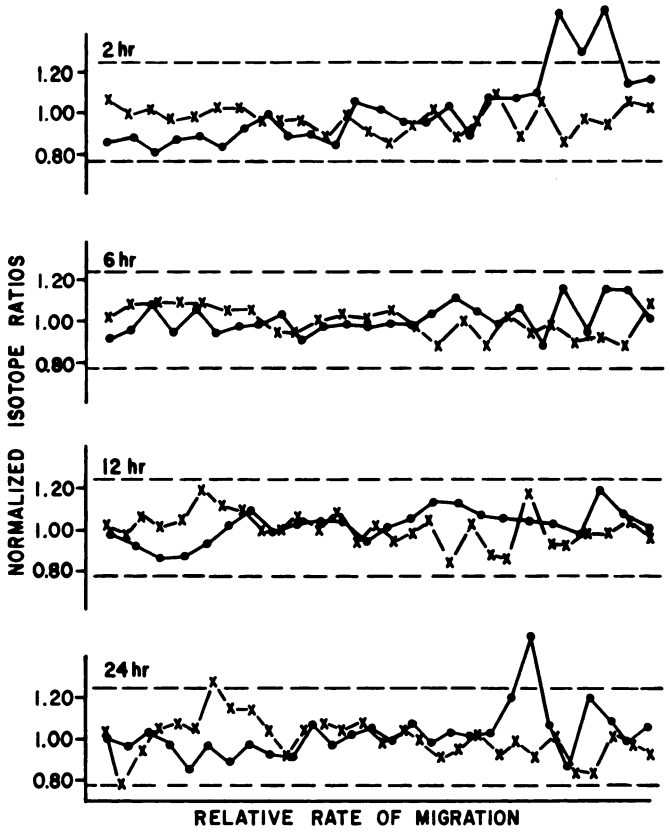

FIG. 7. *Effects of 3-methylcholanthrene on relative rates of leucine incorporation into non-histone chromosomal proteins*

Details were as described for Fig. 6.

metabolism of specific non-histone chromosomal proteins in animals treated with drugs known to be enzyme inducers. Shelton and Allfrey (25) found such an effect in the livers of cortisol-treated rats, and Ruddon and Rainey (41) reported a change in the rate of labeling of specific proteins following phenobarbital and nicotine administration. Neither group of workers assessed the variability inherent in their experimental procedure by comparing proteins from two animals treated in the same manner, as was done in the present study. In the work of Shelton and Allfrey the isotope ratio was so much different in one region of the gel from the remainder of the gel that there can be little doubt, even in the absence of such controls, that the observed effect was real. In the study of Ruddon and Rainey, on the other hand, this was not the case; in the absence of appropriate controls to assess the variability of their experimental system, the reported differences are questionable.

Obviously, the demonstration that a drug alters the rate of incorporation of an amino acid into specific non-histone chromosomal proteins does not justify the conclusion that the observed alteration is related to the induction of the synthesis of other proteins by the drug. This conclusion would require that regulation of the synthesis of one protein or class of proteins occur via an alteration in the rate of synthesis or degradation of a second protein. In fact, the results of Shelton and Allfrey (25) and others (24, 26, 39) do not necessarily indicate a change in chromosomal protein synthesis or degradation; rather, the results may reflect an alteration in binding of these

proteins to chromatin. Regulation of protein synthesis via modification of existing proteins, as suggested by Allfrey *et al.* (45) and Langan (46), seems plausible in view of the results reported above.

Although no stimulation of amino acid incorporation into specific non-histone chromosomal proteins as a result of phenobarbital or 3-methylcholanthrene treatment was observed, both drugs did cause a general increase in the rate of incorporation of amino acids into these proteins. This increase, however, paralleled increases in the histone and cytoplasmic fractions. Both phenobarbital (42) and 3-methylcholanthrene (47) stimulate rat liver growth and mitotic activity, and an increase in the synthesis of chromosomal proteins would be expected to accompany this stimulation. The effect of phenobarbital on liver growth is more pronounced than that of 3-methylcholanthrene (43), and a similar difference in the magnitude of drug effects on amino acid incorporation into chromosomal proteins was observed in the present study. Stimulation of amino acid incorporation into histones and non-histone chromosomal proteins has also been observed in mouse salivary glands treated with isoproterenol (an agent which stimulates mitotic activity in these glands) (48). Thus the increases in amino acid incorporation into the chromosomal proteins apparently reflect the initiation of drug-induced stimulation of liver growth and mitotic activity. Such generalized increases certainly lack the specificity which would presumably be required for induction of specific microsomal enzymes.

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