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Effect of Administration of 3-Methylcholanthrene on the Salt-Extractable Chromatin Proteins of Rat Liver

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SUMMARY

It had previously been demonstrated that an increase in the efficiency of the template function of liver chromatin occurs after the administration of 3-methylcholanthrene (3-MC) to rats. The difference between control and 3-MC systems is eliminated by extraction of the chromatin with 2 M NaCl, a procedure which removes many of the nuclear proteins.

The profile of 2 M NaCl-extractable protein components of liver chromatin differs after treatment of rats with 3-MC, as determined by either disc electrophoretic or gel filtration techniques. The earliest change was seen 3–6 hr after drug treatment. The kinetics of incorporation of [^{14}C] lysine into these components is also altered after drug treatment. After a 30-min pulse with the labeled precursor, a progressive increase in the specific activity of the 2 M NaCl-extractable proteins of liver chromatin was noted up to 24 hr after 3-MC treatment. At this time, a 100% increase over control levels was observed. Under the same conditions, the incorporation of [^{14}C] L-tryptophan into the 2 M NaCl-extractable components of liver was unaffectd by drug treatment. These changes may be related to the subsequent activation of the chromatin template for RNA synthesis.

INTRODUCTION

It has previously been shown that the injection of 3-methylcholanthrene into young rats is followed by a marked activation of the "aggregate" RNA polymerase system present in liver nuclei (1, 2). The increased activity resulted from an enhancement in the template efficiency of the deoxyribonucleoprotein, i.e., chromatin, as determined in a purified bacterial RNA polymerase system (3–5). Additional experiments indicated that the enhanced template efficiency, or genomic activation, was expressed qualitatively in the production of different types of RNA and quantitatively in the elaboration of more RNA chains (5, 6).

In eukaryotic organisms, the manner in which a region of the genome becomes activated for transcription has not been definitively ascertained. The mechanisms by which a genomic locus is rendered unavailable for transcription are likewise not known. These processes involve a sophisticated control system which is unique to higher organisms. Presently it is felt that the basic nuclear protein components play some role in this control mechanism.

Accordingly, we have studied the possible relationship of the 3-methylcholanthrene-induced genomic activation to alterations in the chromatin proteins. At this time, we report (a) a qualitative change in the composition of the latter constituents after 3-methylcholanthrene administration and (b) an alteration in the rate of incorporation of isotopic precursors into the chromatin proteins.

MATERIALS AND METHODS

Animals. Male albino rats, 50–70 g in weight, were obtained from the Cheek-

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Jones Company, Houston, and were allowed free access to both food and water unless otherwise stated. The rats were treated by intraperitoneal injection of 3-methylcholanthrene, 20 mg/kg of body weight, and were killed periodically thereafter by trans-section of the aorta. Control rats were treated similarly with intraperitoneal injections of corn oil.

For the preparation of labeled chromatin, corn oil- and 3-methylcholanthrene-treated rats received generally labeled [^{14}C] L-lysine (228 μCi/μmole), 10 μCi/100 g of body weight, via the tail vein. Each group consisted of three to five rats. The animals were killed 30 min later or as indicated in the accompanying tables.

In other studies, [4,5-^{3}H]L-lysine (28 Ci/mmole), 100 μCi/100 g of body weight, or generally labeled [^{14}C]L-tryptophan (54.5 mCi/mmole), 4–10 μCi/100 g of body weight, was injected via the tail vein.

Isolation of chromatin. The livers were removed from drug-treated and control rats, washed in cold 0.9% NaCl, minced, and homogenized in 2 volumes of 0.25 M sucrose–2.5 mM $MgCl_2$–10 mM Tris-HCl buffer, pH 7.4. Crude nuclei were isolated from the homogenate by centrifugation (7); the nuclear pellet was purified by centrifugation through 4 molal sucrose–50 mM KCl–25 mM $MgCl_2$–10 mM Tris-HCl buffer, pH 7.4, as described by Becker (7). Chromatin was extracted from the purified liver nuclear pellet by the method of Dingman and Sporn (8). The final chromatin preparation was dialyzed at 4° overnight against 0.01 M Tris-HCl buffer, pH 8.0. The pH given for the Tris buffers was measured at the temperature specified for the procedure. The RNA: DNA ratio of the chromatin was 0.01–0.02; the basic protein:DNA ratio was approximately 0.8–1.0. Prior treatment of the rats with 3-methylcholanthrene did not result in any change in these parameters.

Assay of template activity of liver chromatin. RNA polymerase from *Escherichia coli* (250 units/mg) was obtained from Miles Laboratories and was assayed essentially as described by Chamberlin and Berg (9). The details are given in the legend to Fig. 1. The precursor was [γ-^{32}P]ATP, purchased from International Chemical and Nuclear Corporation, with a specific activity of 800 μCi/μmole.

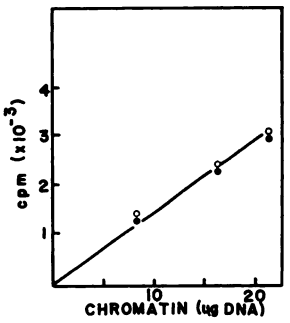

FIG. 1. *Effect of 2 M NaCl extraction on template efficiency of chromatin*

E. coli RNA polymerase (10 units) was incubated at 37° for 10 min with CTP, UTP, and GTP, 0.1 μmole each; [γ-^{32}P] ATP, 0.05 μmole (5 μCi); $MgCl_2$, 1.0 μmole; $MnCl_2$, 0.25 μmole; β-mercaptoethanol, 3.0 μmoles; Tris-HCl buffer, pH 8.0, 10 μmoles; and chromatin isolated after 18 hr from corn oil-treated rats (●——●) or from 3-methylcholanthrene-treated rats (○——○), as indicated, in a total volume of 0.25 ml. The incorporation is expressed on the ordinate as total counts per minute.

Extraction of chromatin proteins. The protein components were extracted from liver chromatin with 2 M NaCl essentially as described by Ohlenbusch *et al.* (10). For the gel filtration studies, the 2 M NaCl-extracted protein fraction was dialyzed overnight against 1 M acetic acid in the cold, lyophilized, and dissolved in 0.01 M HCl–8 M urea.

Sephadex chromatography of salt-extractable protein. Columns of either Sephadex G-100 or G-200, 0.9 × 220 cm, were prepared and equilibrated with 0.01 M HCl–8 M urea. The ultraviolet-absorbing material present in the concentrated urea solution had previously been removed by treatment with acid-washed Norit. The 2 M NaCl-extractable protein components of liver chromatin were placed on the Sephadex columns, and gel filtration was conducted with 0.01 M HCl–8 M urea. Fractions of approximately 1–1.5 ml were collected at a rate of 6 ml/hr and were monitored for protein at 230 nm. Aliquots were also taken for radioactivity measurements, using liquid scintillation techniques with Bray's scintillator mixture (11).

Analytical measurements. The DNA content of the chromatin was determined from the absorbance at 260 nm minus the absorbance at 320 nm by comparison with a standard curve which had been calibrated by the diphenylamine method (12). Under these conditions, a solution of DNA of 10 μg/ml gave an $A_{260} - A_{320}$ value of 0.450. In a number of determinations, good agreement, i.e., ±10%, was observed between this method and the diphenylamine procedure. The RNA and protein contents were ascertained by the orcinol procedure (13) and by the method of Lowry *et al.* (14), respectively.

Disc electrophoresis was conducted at room temperature on 15% acrylamide gels in 8 M urea at pH 4.2 or on 15% acrylamide–4 M urea gels at pH 2.8. The dimensions of these gels were 5 × 50 mm or 5 × 100 mm. The duration of the electrophoresis was 1.5 hr at 4 mamp/tube. The gels were stained with 0.1% Buffalo black in 7% acetic acid and destained with 7% acetic acid in 10% ethanol. Authentic samples of purified histone fractions were kindly supplied by Dr. Harris Busch and Mr. Charles Taylor of this department.

TABLE 1

Incorporation of [^{14}C] lysine into 2 M NaCl-extractable protein components of liver chromatin

Male rats weighing 50–70 g were treated with either corn oil or 3-methylcholanthrene in corn oil as indicated in the text. Each group consisted of three to five rats. At 24 hr after treatment, [^{14}C]-L-lysine, 10 μCi/100 g of body weight, was administered intravenously, and the rats were killed at the times indicated below. The experimental protocol for extraction of the salt-extractable protein fraction of liver chromatin is presented in the text.

Incorporation time	Control (A)	3-MC (B)	B:A
min	*dpm/mg protein*		
10	1190 ± 100 (3)[a]	990 ± 90 (3)	0.8
20	885 ± 150 (3)	1170 ± 100 (3)	1.3
30	820 ± 60 (8)	1530 ± 150 (5)	1.9
60	780 ± 100 (3)	1900 ± 250 (3)	2.4

[a] Average ± standard deviation (number of groups).

TABLE 2

Effect of 3-methylcholanthrene administration on specific activity of 2 M NaCl-extractable protein components of chromatin

Rats were treated with 3-methylcholanthrene and at the times indicated below received [^{14}C] lysine (10 μCi/100 g of body weight, intravenously). The rats were killed 30 min later, and the 2 M NaCl-extractable proteins were prepared as described in the text. Each group consisted of three to five rats. The zero time group included rats that had received corn oil at zero time and 3, 12, and 24 hr prior to the injection of the labeled amino acid. No difference was noted between the specific activities of these groups.

Time after 3-MC	No. of groups	Specific activity	Ratio of treated to control
hr		*dpm/mg protein*	
0	8	820 ± 60[a]	
3	3	1030 ± 100	1.3
6	3	1550 ± 150	1.9
12	4	1650 ± 150	2.0
24	5	1530 ± 150	1.9
48	3	1140 ± 130	1.4
72	3	870 ± 100	1.1

[a] Average ± standard deviation.

RESULTS

Effect of 2 M NaCl extraction on template efficiency of chromatin. As previously reported (6), the chromatin obtained from the livers of 3-methylcholanthrene-treated rats was more efficacious than similarly prepared control chromatin as a template for RNA synthesis with the *E. coli* RNA polymerase system. The contribution of chromatin proteins to this effect was ascertained by their extraction with 2 M NaCl (10) and subsequent reassay of the template activity. These results, which are presented in Fig. 1, show that the difference between the templates is eliminated by such treatment. These findings implicated the salt-extractable protein components of chromatin in the activation phenomenon reported previously (1–5), and suggested a more detailed study of these proteins.

First, the incorporation of lysine into the proteins was studied. The kinetics of lysine incorporation into the 2 M NaCl-extractable protein components of liver chromatin was

TABLE 3

Incorporation of [^{14}C] *lysine into liver supernatant fraction proteins*

[^{14}C] L-Lysine (228 μCi/μmole), 10 μCi/100 g of body weight, was injected into the tail vein of rats that had been treated with corn oil (for various times) or with 3-MC, 20 mg/kg, for the times indicated below. Each group consisted of three rats. The rats were killed 30 min later, the livers were removed and homogenized in 0.25 M sucrose, and the homogenates were centrifuged at $10^5 \times g$ for 60 min. The specific activity of the $10^5 \times g$ supernatant fraction was determined.

Treatment	No. of groups	Specific activity
		dpm/mg protein
Corn oil	5	820 ± 60[a]
3-MC, 3 hr	2	940; 875
3-MC, 6 hr	2	880; 1000
3-MC, 24 hr	2	730; 850
3-MC, 48 hr	2	745; 895

[a] Mean ± standard deviation.

significantly altered 24 hr after administration of 3-MC[1] to rats (Table 1). Little change in lysine incorporation was observed after a 10-min incorporation period. However, after 20 and 30 min, the ratio of the specific activity of the liver salt-extractable protein components from drug-treated rats to controls increased to 1.3 and 1.9, respectively.

The enhanced incorporation was noted as early as 3 hr after administration of the polycyclic hydrocarbon (Table 2). The maximum effect took place between 6 and 24 hr, at which time the specific activity of the salt-extractable protein fraction from the livers of drug-treated rats was twice the value observed with corn oil-treated animals. By 48 hr after 3-methylcholanthrene treatment, the ratio of the drug-treated to control specific activity was 1.4. Subsequently the specific activity in the drug-treated system returned to control levels.

During this time, little change was noted in the incorporation of [^{14}C]lysine into the protein components of the 100,000 × g supernatant fraction from liver (Table 3). Apparently, the number of protein components in liver affected by 3-MC treatment is relatively small compared to the total number present in that tissue, and hence no profound effect is seen in the 100,000 × g fraction. To ascertain that alterations in the amino acid pools were not responsible for the changes cited above, the specific activity of the free lysine was determined in liver 24 hr after drug treatment. These data, presented in Table 4, indicate that the kinetics of labeling of the liver lysine pool was not significantly altered after drug treatment.

To establish whether the observed alterations in the 2 M NaCl-extractable protein components were due to effects on the basic nuclear proteins, advantage was taken of the absence of tryptophan in the latter constituents. In a series of experiments, the salt-extractable protein components were isolated from the livers of control and drug-treated rats (24 hr after 3-MC treatment) that had received [^{14}C]L-tryptophan (10 μCi/100 g of body weight) 30 min prior to death. The label was incorporated into the salt-extractable protein components, thus indicating the presence of other proteins in addition to histones. However, the specific activities were similar for both the control and experimental systems, i.e., between 300 and 500 dpm/mg of protein. These experi-

TABLE 4

Specific activity of free lysine pool in liver after administration of 3-methylcholanthrene

[^{3}H] Lysine (28 Ci/mmole), 100 μCi/100 g of body weight, was injected into the tail vein of rats that had been treated 24 hr previously with either corn oil (controls) or 3-MC. Each group consisted of three to five rats. The rats were killed after the injection of the labeled amino acid at the times indicated below, and picric acid extracts of a liver homogenate were prepared as described under MATERIALS AND METHODS. The specific activity of the free amino acid pool in liver was determined as described in the text (15, 16). The results of two experiments are given.

Time	Control	3-MC-treated
min	*dpm/$\mu\mu$mole*	
10	1.6; 1.6	1.4; 2.0
20	0.9; 0.7	0.9; 0.8
30	0.2; 0.2	0.2; 0.2

[1] The abbreviation used is: 3-MC, 3-methylcholanthrene.

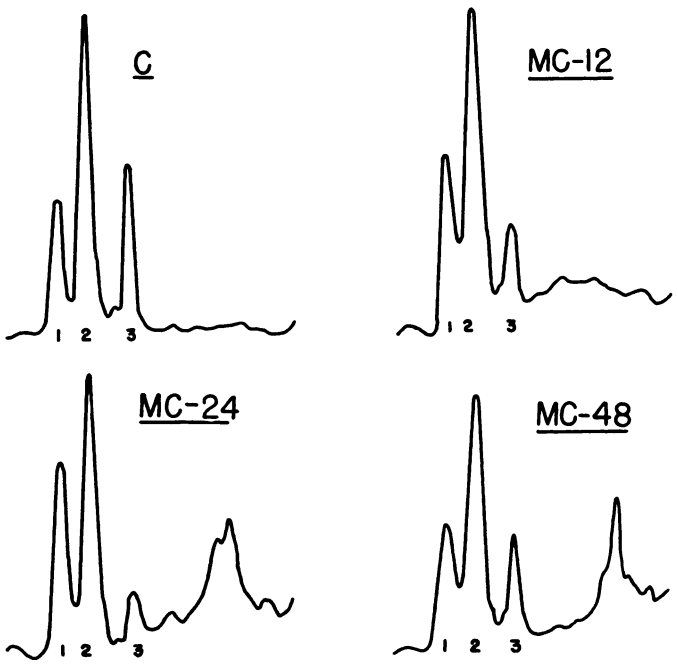

FIG. 2. *Disc electrophoresis of salt-extractable protein components*

The 2 M NaCl-extractable proteins were prepared as described in the text from the livers of corn oil-treated rats (upper left), 3-MC-treated rats after 12 hr (upper right), 3-MC-treated rats after 24 hr (lower left), and 3-MC-treated rats after 48 hr (lower right). The proteins were subjected to disc electrophoresis at pH 4.2, stained with 0.1% Buffalo black in 7% acetic acid, and destained with 10% ethanol–7% acetic acid. Densitometric tracings were prepared in a Joyce Loebl densitometer. The direction of the electrophoresis is from right to left. The positions of peaks 1, 2, and 3 are indicated by the Arabic numerals.

ments[2] suggest that the observed differences were due to protein components which did not contain tryptophan.

Disc electrophoretic mobility of nuclear proteins. The 2 M NaCl-extractable protein components of liver chromatin were subjected to disc electrophoresis at pH 4.2. Densitometric tracings of the stained gels are presented in Fig. 2. It is apparent that the profile of proteins is altered after administration of 3-methylcholanthrene. The more slowly moving components are increased, and a concomitant decrease in fraction 3 may be noted. Under the conditions of electrophoresis, authentic samples of the various histones traveled with the following mobilities: the GAR histone,[3] fraction 1; the AL, AR-5, and SLR histones, fraction 2; the VLR histone, fraction 3; aggregates of histones, especially the AR fraction, with the more slowly moving components.

Sephadex chromatography of salt-extractable protein. The 2 M NaCl-extractable protein components of liver chromatin were analyzed by Sephadex G-200 gel filtration chromatography (Fig. 3). At 24 hr after treatment with 3-methylcholanthrene, the elution pattern was markedly different from that of controls. The initial breakthrough peak, fraction 1 of Fig. 3, was increased, while fraction 4 was decreased after drug treatment. To examine this finding more closely, the salt-extractable protein components were prepared from

[2] We are particularly grateful to the referees of this manuscript, who reminded us of the utility of tryptophan in this regard.

[3] The following nomenclature for histones has been employed: the glycine- and arginine-rich fraction, GAR (or f2a1 or IV); arginine- and lysine-rich, AL (or f2a2); arginine-rich, AR-5 (or f3 or III); slightly lysine-rich, SLR (or f2b); very lysine-rich, VLR (or f1).

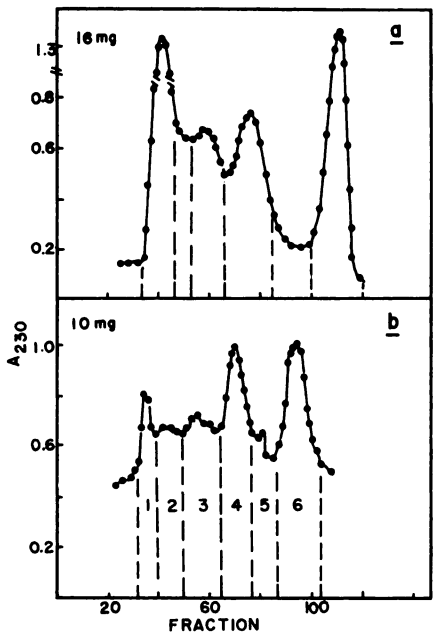

FIG. 3. *Sephadex G-200 chromatography of 2 M NaCl-extractable protein components*

The details of the chromatographic analysis are given under MATERIALS AND METHODS. The protein components isolated from control livers (b) and from the livers of 3-MC-treated rats after 24 hr (a) were placed on a column of Sephadex G-200, 0.9 × 200 cm, and eluted with 0.01 M HCl–8 M urea. The amount of protein placed on the column is indicated at the upper left of each graph. Fractions of approximately 1.0 ml were collected every 45 min and monitored at 230 nm for protein. The fractions were collected into pools indicated by the dashed lines.

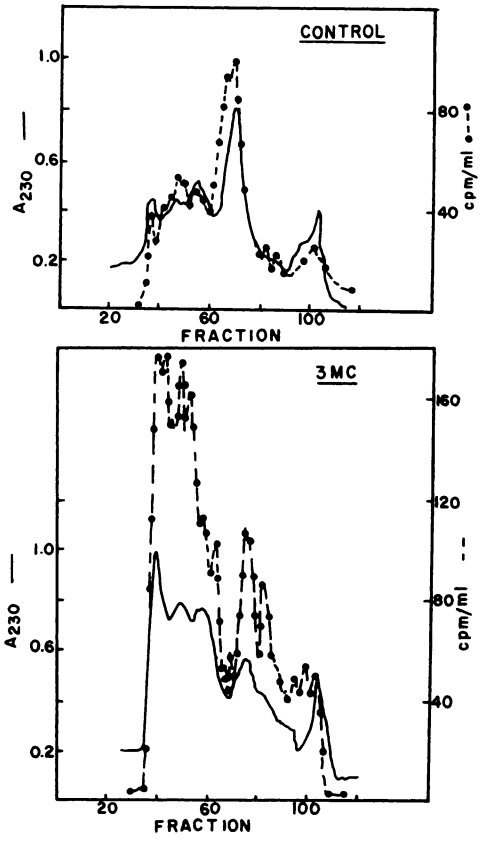

FIG. 4. *Sephadex chromatography after incorporation of [^{14}C] lysine*

Details are given under MATERIALS AND METHODS and in the legend to Fig. 3. The protein components were obtained from the livers of corn oil-treated (upper) and 3-MC-treated rats (lower) after 24 hr. The radioactivity of each fraction was determined.

control and drug-treated rats which had received [^{14}C]lysine 30 min earlier. The labeled chromatin proteins were separated on Sephadex G-200, and the specific activity of each fraction was determined. These results are presented in Fig. 4 and Table 5. Prior treatment of the rats with the polycyclic hydrocarbon resulted in the appearance of a greater amount of radioactivity in each fraction of the salt-extractable protein components (Fig. 4). The specific activity of each fraction (as defined in Fig. 3) is presented in Table 5. The specific activity was increased by 40–290%; the increase was greatest for fraction 6 and least for fraction 4.

DISCUSSION

The administration of 3-methylcholanthrene to rats not only results in elevation of the activities of some of the microsomal drug-metabolizing enzymes (reviewed in refs. 17–19) but also leads to changes in RNA metabolism (20) and in the capacity for protein synthesis (21, 22).

In this regard, Nebert and Gelboin (23) have carefully studied, by the use of inhibitors, the interrelationship of the transcription and translation processes in the benzanthracene-induced cell culture system. They have reported the requirement of two

TABLE 5

Incorporation of [^{14}C] lysine into 2 M NaCl-extractable protein components

Male rats, 80–100 g in weight, were treated with either corn oil or 3-methylcholanthrene 24 hr before receiving [^{14}C] lysine (10 μCi/100 g of body weight) via the tail vein. The rats were killed 30 min later, and the 2 M NaCl-extractable components were prepared from liver chromatin and analyzed by Sephadex G-200 chromatography. The column (0.9 × 200 cm) was eluted with 0.01 N HCl–8 M urea. The fractions are designated as in Fig. 3. Each value represents the average of three experiments. Each group consisted of three to five rats.

Fraction	Control (A)	3-MC (B)	B:A
	dpm/mg protein		
1	714	1640	2.3
2	840	1820	2.2
3	470	1430	3.1
4	770	1050	1.4
5	827	1370	1.7
6	63	243	3.9

phases in the induction phenomenon, an initial period, during which a specific RNA is produced, and a later phase, at which time the RNA is translated.

The effects on nucleic acid synthesis are preceded by an augmentation in the activity of the liver "aggregate" RNA polymerase system (1, 2). The cause of the latter is related to the arrangement of the proteins in the chromatin. The basicity of the chromatin proteins is at least partly responsible for their ability to form complexes with polyanionic DNA in the structure of the chromosome, and presumably it is this interaction which plays a regulatory role in the transcription process.

In this paper we have reported the qualitative shifts in the patterns of the 2 M NaCl-extractable protein components from liver after treatment of rats with 3-methylcholanthrene. The shift was demonstrable by both disc electrophoretic and chromatographic techniques.

In addition, the rate of incorporation of an isotopic precursor, [^{14}C]lysine, into the salt-extractable protein components was markedly altered following drug treatment. The latter effect was not caused by any disturbance in the free lysine pool of liver, nor was it the result of a generalized increase in protein synthesis, since no alterations were seen in the over-all incorporation of [^{14}C]lysine into the liver 100,000 × *g* supernatant fraction. Furthermore, tryptophan incorporation into these components was unaltered by drug treatment, suggesting an effect on the histones.

The kinetics of lysine incorporation into the salt-extractable protein components of chromatin from control liver show a rapid decay in specific activity. After treatment of rats with the polycyclic hydrocarbon, however, the kinetics indicates an active rate of synthesis.

In attempting to explain how genetic regions may be activated, i.e., how protein components may be removed, Allfrey *et al.* (24) suggested that modification of the inhibitory action of the basic proteins could be achieved by substitution of specific chemical groups at certain parts of these molecules. Such a modification of the latter would result in an alteration of their binding to DNA and ultimately lead to displacement from the gene. Considerable information has accrued during the last few years relating to this possible regulatory mechanism; these studies have been reviewed by Georgiev (25). Thus, a number of investigators (see ref. 25) have noted the alterations in the rate of methylation, acetylation, or phosphorylation of the basic nuclear components of chromatin during various developmental or growth-stimulatory processes. For instance, Libby (26) has suggested a relationship between the acetylation of histones and the growth phenomenon induced in the immature uterus by estrogens. A marked increase in the acetylation of both the arginine-rich and lysine-rich histones occurred upon the addition of small amounts of 17β-estradiol to uteri *in vitro*. These events would precede the effects of the hormone upon RNA and protein synthesis.

The changes in the quantitative aspects of the 2 M NaCl-extractable components and in the kinetics of lysine incorporation reported here may have taken place as a result

of group substitution of the basic chromatin proteins after prior treatment of rats with 3-methylcholanthrene. Furthermore, the various chromatin proteins may have been affected differently. This possibility is presently being studied in this laboratory.

REFERENCES

1. E. Bresnick, *Mol. Pharmacol.* **2**, 406 (1966).
2. H. V. Gelboin, J. S. Wortham and R. G. Wilson, *Nature* **214**, 281 (1967).
3. J. C. Madix and E. Bresnick, *Biochem. Biophys. Res. Commun.* **28**, 445 (1967).
4. W. N. Piper and W. F. Bousquet, *Biochem. Biophys. Res. Commun.* **33**, 602 (1968).
5. E. Bresnick and H. Mossé, *Mol. Pharmacol.* **5**, 219 (1969).
6. E. Bresnick, *Biochim. Biophys. Acta.* In press.
7. F. F. Becker, *Arch. Biochem. Biophys.* **126**, 380 (1968).
8. C. W. Dingman and M. B. Sporn, *J. Biol. Chem.* **239**, 3483 (1964).
9. M. Chamberlin and P. Berg, *Proc. Nat. Acad. Sci. U. S. A.* **48**, 81 (1962).
10. H. Ohlenbusch, B. Olivera, D. Tuan and N. Davidson, *J. Mol. Biol.* **25**, 299 (1967).
11. G. A. Bray, *Anal. Biochem.* **1**, 279 (1960).
12. K. Burton, *Biochem. J.* **62**, 315 (1956).
13. J. F. Drury, *Arch. Biochem. Biophys.* **19**, 455 (1948).
14. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
15. H. H. Tallan, S. Moore and W. H. Stein, *J. Biol. Chem.* **211**, 927 (1954).
16. H. H. Tallan, S. Moore and W. H. Stein, *J. Biol. Chem.* **219**, 257 (1956).
17. A. H. Conney, *Pharmacol. Rev.* **19**, 317 (1967).
18. J. R. Gillette, *Advan. Pharmacol.* **4**, 219 (1967).
19. H. V. Gelboin, *Advan. Cancer Res.* **10**, 1 (1967).
20. L. A. Loeb and H. V. Gelboin, *Proc. Nat. Acad. Sci. U. S. A.* **52**, 1219 (1964).
21. H. V. Gelboin, *Biochim. Biophys. Acta* **91**, 130 (1964).
22. W. R. Jondorf, D. C. Simon and M. Avnimelech, *Biochem. Biophys. Res. Commun.* **22**, 644 (1966).
23. D. W. Nebert and H. V. Gelboin, *J. Biol. Chem.* **245**, 160 (1970).
24. V. G. Allfrey, R. Faulkner and A. E. Mirsky, *Proc. Nat. Acad. Sci. U. S. A.* **51**, 786 (1964).
25. G. P. Georgiev, *Annu. Rev. Biochem.* **38**, 155 (1969).
26. P. R. Libby, *Biochem. Biophys. Res. Commun.* **31**, 59 (1968).