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Separation of Newly Synthesized Nucleohistone by Equilibrium Centrifugation in Cesium Chloride

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ABSTRACT: Nucleohistone, fixed by formaldehyde using previous methods, does not permit a separation of density-labeled, newly synthesized nucleoprotein. The reasons for this behavior lie in inter- or intrastrand cross-linking when fixation is performed at intermediate ionic strength (0.01–0.2) and also involve nucleoprotein aggregation in solutions containing elevated concentrations of CsCl. If fixation is performed at low ionic strength, and the equilibrium density centrifugation is performed in the presence of denaturing solvents, discrete separation of newly synthesized, density-labeled nucleoprotein is possible. Techniques for an effective reversal of nucleoprotein fixation are described. Histones can be recovered unchanged.

A great deal of valuable information has been obtained concerning DNA replication by exploiting the incorporation of a density label and performing a subsequent analysis by buoyant density equilibrium centrifugation in cesium chloride solutions. This approach has been particularly fruitful in work with prokaryotes. The more complex eukaryotic chromosome has been studied, on occasion, in a similar way, though an early step in such an analysis is to remove the proteins which constitute more than 50% of the chromosome material (Kidwell and Mueller, 1969).

A better understanding of chromosome synthesis in higher organisms requires that in addition to studying the mode of DNA replication, we also learn how and when the proteins become associated with DNA during replication. In principle much can be learned by utilizing the techniques of density labeling; however, the elevated ionic strengths required for density separation are such that almost all of the chromosomal proteins are dissociated by the conditions required for the analysis of the experiments (Ohlenbush *et al.*, 1967).

It is possible to avoid the problem of dissociation if the proteins are chemically bound to the RNA or DNA using formaldehyde (Brutlag *et al.*, 1969; Spirin *et al.*, 1965). However, we will show that if the chromosomal material is labeled by a short *in vivo* pulse of a density label, bromodeoxyuridine, then analysis of the chemically fixed nucleoprotein on a CsCl density gradient reveals that the density label is uniformly distributed throughout the nucleoprotein peak rather than asymmetrically organized on the denser side. Since the incorporation of such a density label has been used to identify and separate newly synthesized DNA, the system as presently used does not permit an identification and separation of newly synthesized nucleoprotein.

The reasons for the failure to separate density-labeled nucleoproteins have been studied. They lie in cross-linking during fixing and in the additional short range interactions among the material itself. Procedures are described which avoid these problems and lead to a partially successful resolution of newly synthesized, density-labeled chromosomal nucleoproteins.

Materials and Methods

Density Labeling. Hepatoma tissue culture (HTC)[1] cells were grown in Swims 77 medium supplemented with 5% calf and 5% fetal calf serum. Exponentially growing cells (200 ml, 5×10^5/ml) were labeled with 100 μCi [^{3}H]thymidine (Nuclear Dynamics, Inc., specific activity 24 Ci/mmol) and 1.0×10^{-5} M bromodeoxyuridine (Sigma Chemical) for 2 hr at 37°. The cells were collected by centrifugation at 2000*g* for 5 min, frozen, and homogenized directly in 25 ml of 10 mM $MgCl_2$, 10 mM Tris, 50 mM $NaHSO_3$, 0.25 M sucrose, and 1% Triton X-100 (pH 7.0) using a Potter-Elvejheim homogenizer. Nuclei were isolated by centrifugation at 1000*g* for 10 min and the pellet was washed three times by successive suspension and centrifugation from the above buffer. The nuclei were washed once with 10 mM Tris-HCl–12.5 mM EDTA (pH 8.0) and twice with glass-distilled water to generate the typical chromatin gel. The gel was vigorously sheared (VirTis Model 45 homogenizer) at 80 V in two bursts of 45 sec. The resulting solution which has lost the gel-like nature of chromatin is operationally defined as nucleohistone though it is known to contain contaminating membrane fragments. A large fraction of the membrane was removed from the nucleohistone by centrifugation at 27,000*g* for 30 min. The supernatant contains the nucleohistone used for the following experiments. For some of the experiments to be described Ehrlich ascites tumor cells were used as the source of nucleohistone.

Fixation of Nucleohistone and Centrifugation in CsCl. Nucleohistone was treated with formaldehyde of required concentration as defined in the text. The formaldehyde (Fisher Scien-

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[1] Abbreviations used are: HTC cells, hepatoma tissue culture cells; brUdRib, bromodeoxyuridine.

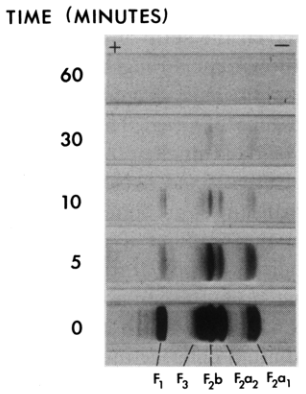

FIGURE 1: Fixation of nucleohistone by formaldehyde: 15% polyacrylamide gels of the histone released from DNA by acid treatment after fixation. Ehrlich ascites tumor nucleohistone was fixed by formaldehyde in 0.01 M triethanolamine (pH 7.0).

tific) was stored as a 37% solution in water containing methanol (15%) as a preservative. The formaldehyde solution was neutralized prior to addition. After fixation the nucleohistone solution was dialyzed exhaustively for 24 hr (4°) against a solution of identical ionic strength (see text) to remove the excess formaldehyde. Guanidinium chloride (2.102 g) (Heico, Inc., Delaware Water Gap, Pa.) and cesium chloride (1.600 g) (Fisher Scientific) were dissolved in the dialyzed nucleohistone solution and the solution was adjusted to a final volume of 5.5 ml (η = 1.4217) in 0.10 M Tris-HCl (pH 7.0). The solution was centrifuged at 42,000 rpm for 48 hr at 4° in a Beckman Spinco SW 50.1 rotor (160,000g). After centrifugation fractions were collected by puncturing the tubes and counting drops (20 drops/fraction). The fractions were assayed spectrophotometrically at 260 mμ and subsequently counted in Bray's solution (Bray, 1960) using a Nuclear Chicago Unilux III scintillation counter.

Analysis of Extent of Fixation. Nucleohistone, fixed with formaldehyde for a required time period, was adjusted to a final 0.4 N H_2SO_4, sonicated for 10 sec, and allowed to stand for 60 min. It was centrifuged at 27,000g for 20 min and the supernatant collected and analyzed for any histone which might have been extracted in acid. The solution was dialyzed against 95% ethanol (tenfold excess) for 24 hr at 4° and the insoluble histone collected by centrifugation. The histone was dissolved in 0.9 N acetic acid–20% sucrose and analyzed electrophoretically (Panyim and Chalkley, 1969).

Reversal of Fixation. Fixed nucleohistone was incubated at 37 or 60° (as described in the text) in a dialysis tube and subjected to at least six changes of buffer (1000-fold excess each time) over a period of 3 days. The release of histone from its cross-linking attachment to DNA was monitored by acid extraction as described above.

Results

Fixation of Chromosomal Nucleoprotein. We have studied fixation of nucleoprotein by formaldehyde at 4°, a temperature chosen primarily to minimize proteolytic degradation. Although 0.1% formaldehyde produces relatively little fixation (as judged by acid extractability of histone), the rate of fixation increases with formaldehyde concentration and is essentially complete after 30 min in the presence of 1% formaldehyde. Since chromosomal nucleoprotein consists primarily of the five major histone fractions in association with DNA we have examined those histone fractions which could be extracted with acid at intermediate stages during fixation to see if any one fraction were bound more rapidly to DNA than any other. The histones so extracted at various time intervals after initiating fixation are shown in Figure 1. There appears to be no difference in rate of fixation for the five histone fractions.

Sheared chromatin, the chromosomal nucleoprotein we have utilized for these studies, exhibits a considerable increase in viscosity at low ionic strengths. In general the lower viscosity observed at intermediate ionic strengths (0.01–0.1) is thought to be a result of the formation of a compact conformation by the nucleoprotein. It seemed likely that fixation of a compact molecular form might introduce covalent bonds where previously only short range van der Waals forces had been in operation. It is possible to test this idea by exploiting an earlier observation that the noncovalent bonds contributing to the compact form of nucleoprotein at an ionic strength of 0.01 can be abolished in the presence of urea. However, if as a result of formaldehyde treatment covalent bonds have been formed then the effect of urea on nucleoprotein structure should no longer be observed.

In Figure 2a the effect of treating nucleoprotein in 0.01 M Tris-HCl with 4 M urea is documented. The normally high s value of the nucleoprotein is reduced dramatically by urea, and upon removal of urea the rapidly sedimenting characteristic is regained. The nucleoprotein was then fixed with formaldehyde at a defined ionic strength ranging from 5×10^{-5} to 10^{-1} (in the absence of urea). The efficiency and rate of fixation appear to be independent of ionic strength as judged by acid extraction of histone (data not shown). The nucleoprotein preparations were then analyzed upon sucrose density gradients in urea solutions to test for the ability of urea to generate a low s value product. As shown in Figure 2b, nucleoprotein solutions fixed

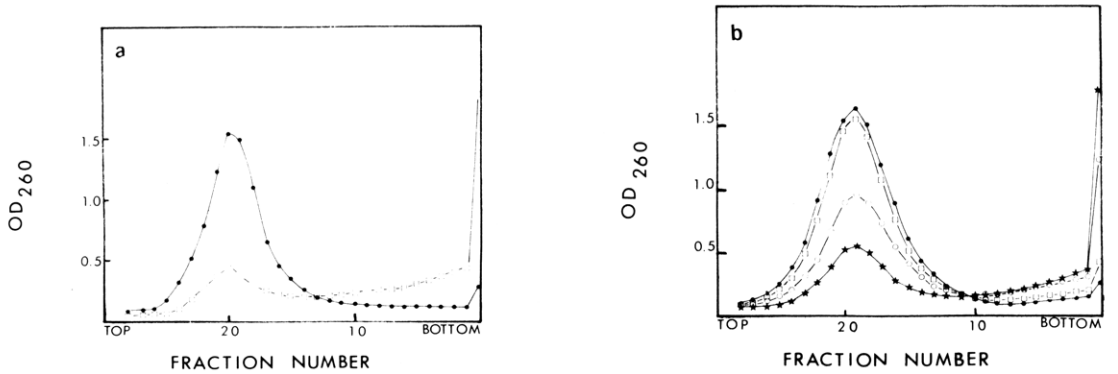

FIGURE 2: The cross-linking of nucleohistone by fixation with formaldehyde: (a) centrifugation of Ehrlich tumor nucleohistone through 30% sucrose–0.01 M Tris in the presence (●) or absence (□) of 4 M urea; (b) centrifugation of fixed Ehrlich tumor nucleohistone on 30% sucrose–0.01 M Tris–4 M urea (pH 7.0). Fixation was with 1% formaldehyde for 2 hr at 4° in ionic strengths of 5×10^{-5} M (●), 1×10^{-4} M (□), 1×10^{-2} M (○), and 5×10^{-2} M (★). Centrifugation was at 150,000g for 12 hr at 4°.

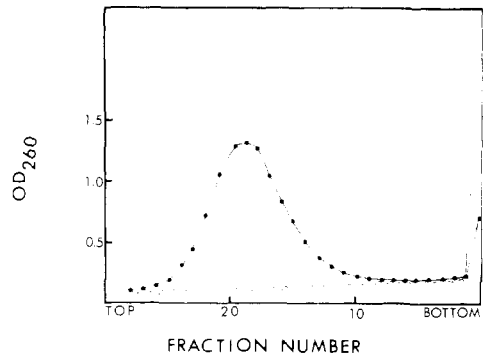

FIGURE 3: The aggregation of fixed nucleohistone by treatment with high ionic strength. Ehrlich tumor nucleohistone was fixed in 1.0% formaldehyde for 2 hr at 4° at 5×10^{-5} in ionic strength and then centrifuged in 30% sucrose–0.01 M Tris–2.0 M NaCl (pH 7.0) in the presence (●) or absence (□) of 3.0 M urea. Centrifugation was at 150,000*g* for 12 hr.

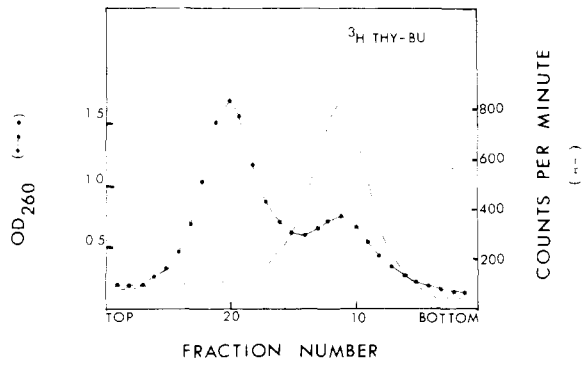

FIGURE 4: Equilibrium centrifugation of DNA containing bromodeoxyuridine. *HTC nucleohistone, isolated as described in* Materials and Methods, was treated with 6.808 g of CsCl to a final volume of 7.0 ml in 0.01 M Tris (pH 8.0). Centrifugation was in a Beckman 50 rotor at 40,000 rpm for 48 hr (100,000*g*) (Flamm *et al.*, 1966).

at very low ionic strengths give the slowly sedimenting form in urea; however, the higher *s* value form of chromosomal nucleoprotein is maintained in urea solutions if formaldehyde fixation is performed at the higher ionic strengths.

Formaldehyde fixation for the remainder of the studies to be described was performed at 1% formaldehyde in an ionic strength of 5×10^{-5}.

Aggregation of Fixed Nucleoprotein at High Ionic Strength. The primary aim of these studies was to obtain a monodisperse preparation of nucleoprotein molecules in a CsCl density gradient. We have assayed the state of aggregation of fixed nucleoprotein (fixed at low ionic strength) upon transferring the nucleoprotein to a high ionic strength environment both in the presence and absence of urea. The results of such a sucrose density analysis of fixed nucleoprotein are shown in Figure 3. In the absence of urea, fixed nucleoprotein in 2 M NaCl sediments totally during the same time period that 95% of the material remains in the gradient if urea (3 M) is also present. Clearly if monodisperse preparations are to be obtained in CsCl at high ionic strength then not only must fixation be performed at very low ionic strength but a denaturing agent such as urea or guanidinium chloride must be present with the CsCl during the equilibrium centrifugation analysis. Initial experiments utilized urea to inhibit aggregation, but additional studies revealed the even greater efficacy of guanidinium chloride which is currently the preferred denaturant and it was employed in most of the remaining work to be described.

Equilibrium Centrifugation of Density-Labeled Nucleoprotein. If monodisperse preparations of chromosomal nucleoprotein can be obtained, then it should be possible to incorporate a density label such as bromodeoxyuridine (brUdRib) into the DNA of the complex in a short pulse *in vivo* and subsequently to separate the brUdRib-containing chromosomal material from the material of normal density.

HTC cells in suspension culture were grown in the presence of brUdRib (1×10^{-5} M) and [^{3}H]thymidine for 2 hr. This represents approximately 12% of the generation time of these cells and we would expect that the same fraction of DNA should contain the density label if it is being incorporated normally into replicating DNA. That this is indeed the case is shown in Figure 4. The chromosomal nucleoprotein was applied directly to a CsCl gradient. Since the nucleoprotein had not been fixed, the proteins dissociate promptly and we see the buoyant density profile of normal density DNA (1.703) and brUdRib-containing DNA (1.738). All the [^{3}H]thymidine is associated with the brUdRib-containing DNA at 4°. Essentially none is in the lighter density peak indicating that the tritium label is monitoring newly synthesized DNA and is not measuring repair synthesis during the time course of this experiment.

A separate sample of this nucleoprotein was divided into three portions. One fraction was fixed in 1.0% formaldehyde 0.01 M triethanolamine (pH 7.0) (Brutlag *et al.*, 1969). The remaining sample was then fixed with 1.0% formaldehyde in 5×10^{-5} M triethanolamine buffer (pH 7.0). The nucleoproteins were then sedimented in cesium chloride in the presence of

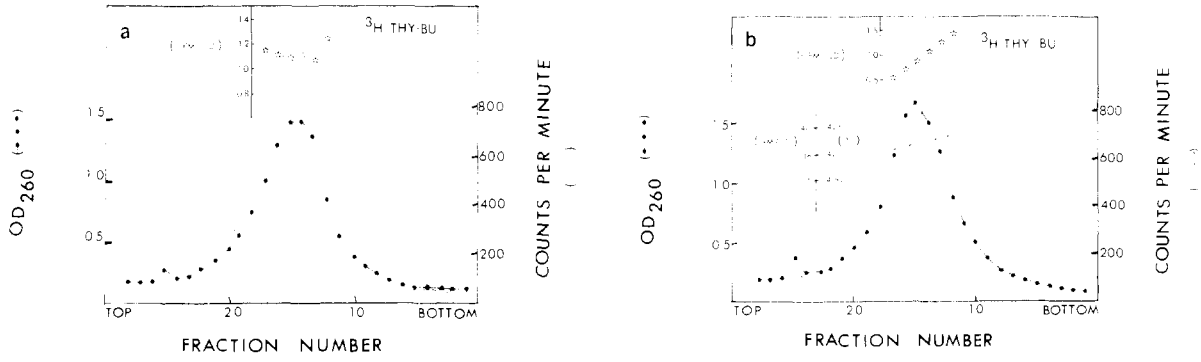

FIGURE 5: Equilibrium centrifugation of fixed nucleohistone containing bromodeoxyuridine. HTC cells were grown in the presence of bromodeoxyuridine (1.0×10^{-5} M) and [^{3}H]thymidine (100 μCi) for 2 hr. Nucleohistone was isolated in the usual manner. (a) HTC nucleohistone was fixed in 1% formaldehyde–0.01 M triethanolamine for 2 hr at 4°. (b) HTC nucleohistone was fixed in 1% formaldehyde–5×10^{-5} M triethanolamine for 2 hr at 4°. Centrifugation was in CsCl gradients containing guanidinium chloride (see Materials and Methods). The insert in the top center of the figure represents a quantitative indication of the degree of asymmetry of the distribution of cpm and OD 260 nm. The density (g/cm^3) at 4° and refractive index (η) are plotted in Figure 5b.

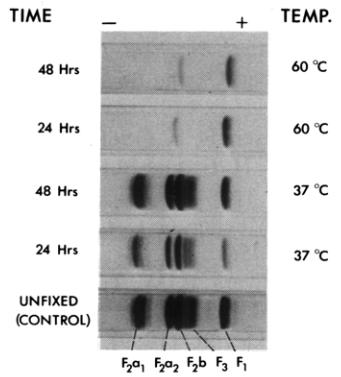

FIGURE 6: Conditions for reversal of histone fixation. Ehrlich tumor nucleohistone was fixed in 1% formaldehyde for 2 hr at 4° and after dialysis at 4° to remove excess formaldehyde, the nucleohistone was dialyzed exhaustively against 5×10^{-5} M triethanolamine at either 37 or 60°. The histone was then removed by extraction with acid and analyzed electrophoretically.

guanidinium chloride (3.0 M) at 4°. The low temperature is necessary to prevent reversal of fixation (see below). As shown in Figure 5a if the material was fixed at higher ionic strength the [^{3}H]thymidine radiolabel is uniformly distributed throughout the nucleoprotein peak in the CsCl density gradient and evidently no separation of light and dense nucleoprotein had occurred, presumably because of random aggregation. On the other hand, if the chromosomal material was fixed at low ionic strength and analyzed on a cesium chloride density gradient (Figure 5b), [^{3}H]thymidine is asymmetrically distributed toward the dense side of the nucleoprotein peak. The density shift from the peak of A_{260} (density = 1.386 g/cm^3) to the peak of [^{3}H]thymidine radioactivity (necessarily denoting the brUdRib density label) is ~0.005 g/cm^3. When nucleohistone fixed at low ionic strength is sedimented in a CsCl gradient without the denaturing agent (guanidinium chloride) then the results are similar to that seen in Figure 5a. Both the fixation at low ionic strength and sedimentation in CsCl density gradients containing guanidinium chloride are necessary for this separation of newly synthesized nucleohistone.

Reversal of Fixation. The merit of the observations described above lies chiefly in the ability to separate nucleoproteins on the basis of density, with an obvious application for the isolation of newly replicated chromosomal material. There are frequently circumstances in which one would like to isolate the histone associated with a specific fraction, for instance to ask whether freshly deposited histone were to be acetylated or phosphorylated. Accordingly, we have studied the conditions which lead to an efficient reversal of fixation without damaging the histones themselves. The reversal of fixation is facilitated by raising the temperature and by continuous removal of formaldehyde by exhaustive dialysis. The data of Figure 6 show that within 1 day a significant amount of all histone fractions can be extracted from previously fixed nucleoprotein if it is incubated at 37 or 60°. The higher temperature appears to selectively release only F_1 and we have restricted ourselves to a study of reversal at 37°. As shown in Figure 7 the reversal is complete in 48 hr at 37° and the histone electrophoretic pattern is identical with that of a standard histone pattern prepared directly without the intervention of fixation.

Discussion

The conditions for fixing of chromosomal proteins to DNA and for the reversal of this fixation have been reported. In order to separate and identify newly synthesized nucleohistone within the chromosomal material using a density label it is necessary to fix and to maintain the chromosomal material as a monodisperse system and to inhibit random aggregation and cross-linking between nucleoprotein molecules. This can be obtained, providing two key conditions are satisfied. (1) Fixation must be performed at very low ionic strength so that the nucleoprotein molecules are extended and have little interstrand interaction. If the fixation is performed at higher ionic strengths (≥0.01), then the formaldehyde introduces covalent bonds maintaining the compact conformation favored at these intermediate ionic strengths. (2) After low ionic strength fixation the subsequent density gradient analysis must be performed in the presence of urea, or preferably, guanidinium hydrochloride. The latter agent appears to maintain the solubility of the fixed nucleoprotein at higher concentrations within a density band. The advantageous effect of the denaturing solvents presumably lies in their ability to abolish short range van der Waals type forces generated among the fixed nucleoprotein molecules upon elevating the ionic strength. The ability of urea to disrupt such forces in unfixed chromatin at lower ionic strengths has been previously documented (Bartley and Chalkley, 1968). The effect of urea treatment of the rather complex chromatin structure was to produce a monodisperse system of nucleoprotein molecules each containing a single DNA molecule of molecular weight ~2×10^6 along with its attendant chromosomal proteins (Chalkley and Jensen, 1968).

The ability to generate covalent cross-links with formaldehyde in the more compact conformation of nucleohistone and not in the extended form indicates that histones on one part of the nucleoprotein are in close proximity to histones from other parts of the molecule in the more compact form of nucleohistone at intermediate (physiological) ionic strengths.

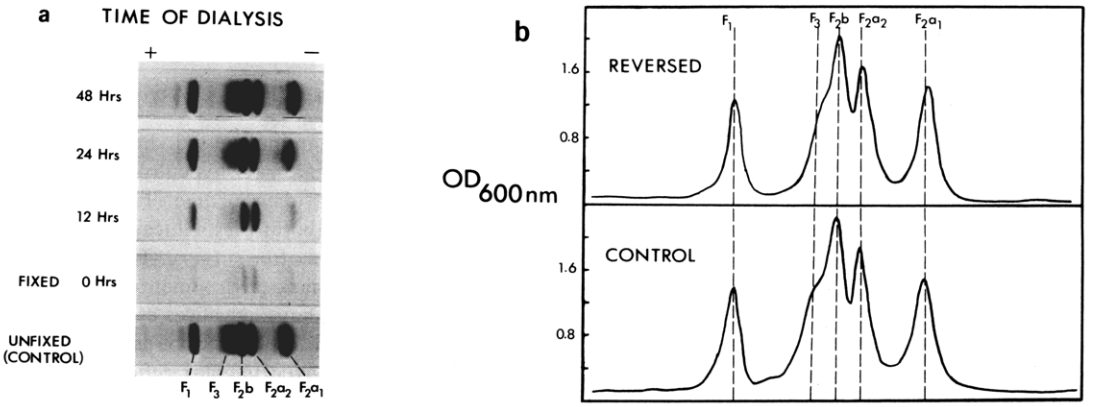

FIGURE 7: Quantitation of the reversal of histone fixation: (a) 15% polyacrylamide gels of the histone released by acid extraction; (b) densitometer scans of the polyacrylamide gels of histone before fixation (CONTROL) and after 48 hr reversal of fixation (REVERSED).

The ability to separate intact nucleoprotein and newly synthesized density-labeled nucleoprotein on the basis of density will be useful in studies of the heterogeneity of distribution of histone on DNA and also in terms of finding out more information about the sites of histone deposition on the replicating chromosome. Current experiments underway indicate that this latter process is highly organized and occurs in a somewhat unexpected way.

Acknowledgments

The critical advice of our colleagues in this laboratory is greatly appreciated.

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Gonadotropin Stimulation of Rat Testicular Protein Synthesis. Polysome Isolation and Activity in a Cell-Free System†

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ABSTRACT: Follicle-stimulating hormone plus luteinizing hormone were administered in combination by intratesticular injection to mature hypophysectomized rats 14–21 days postoperatively. Within 12 hr of administration of the gonadotropins, both testicular total RNA and polysomal RNA content were increased (33 and 35%, respectively). The use of an RNase inhibitor was found to be essential to the isolation of relatively undegraded polysomes from the testis. Sucrose gradient sedimentation analysis demonstrated that the polysomal preparations each had essentially the same A_{254} profiles regardless of the testis source; intact, hypophysectomized control, or experimental animals. These findings suggest that gonadotropins induced an increase in testicular polysomal content in the absence of a shift from monomeric to polysomal aggregates. Within 12 hr of hormone administration, free testicular polysomes exhibited a significant increase in protein synthesis *in vitro;* membrane-bound polysomes did not show this response. It is concluded that increased testicular protein synthesis following gonadotropin administration results from an increase in the number of ribosomes and from a selective stimulation of protein synthesis by free testicular polysomes.

Follicle-stimulating hormone[1] administered to either immature or mature hypophysectomized male rats was reported to stimulate testicular protein synthesis *in vitro* within 30 min (Means and Hall, 1967, 1968). Further investigation revealed that administration of FSH to either immature or mature hypophysectomized rats stimulated [^{14}C]amino acid incorporation into protein by testicular polysomes *in vitro* within 1 hr (Means and Hall, 1969, 1971). Means (1971) showed that FSH enhanced *in vivo* nRNA synthesis within 15 min in the immature rat testis thus demonstrating that the hormone affects both transcription and translation in its target tissue. The present investigation was undertaken to further elucidate the role of FSH and LH in the testis. The data will demonstrate that administration of FSH plus LH to mature hypophysectomized rats results in increases in testicular total RNA and polysomal RNA and selectively enhances the capacity of free polysomes to support *in vitro* [^{14}C]amino acid incorporation into protein.

Materials and Methods

Animals. The animals used in the investigation were Sprague-Dawley male rats hypophysectomized at the time of sexual maturity, 60 days of age. They were purchased from Hormone Assay Laboratories, Chicago, Ill., and shipped to this laboratory 2–3 days after surgery. The animals were kept in a 12-hr light–12-hr dark environment and fed Purina rat chow *ad libitum* and fresh orange slices every other day. In all studies reported here the animals were used 14–21 days postoperatively. Control or experimental refers to hypophysectomized rats which were injected with saline or hormones, respectively. Intact mature male rats were used on occasion for comparative purposes and are referred to as intact.

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[1] Abbreviations used are: FSH, follicle-stimulating hormone; LH, luteinizing hormone; DOC, deoxycholate, sodium salt.