distribution of label in the IEA patterns of the SON fractions was not affected by the addition of serum carrier, it seems unlikely that nonspecific binding of radioactive material to serum proteins occurs to any appreciable extent. There is the possibility that highly radioactive material in the SON fraction migrating with the same electrophoretic mobility is trapped and concentrated in the immune precipitate. This is unlikely, however, because of the dynamics of diffusion and precipitation in gels.

Since incubation mixtures contained cell sap fractions from normal liver only, the differences observed in the labeled proteins were due to the modified capacity of the microsomal fraction to direct the synthetic activity. The striking differences between the labeled IEA patterns of cell-free systems obtained from normal and staphylococcus-infected mice closely parallel the differences observed *in vivo*[5] and *in vitro*.[3] We conclude, therefore, that under suitable conditions, cell-free systems reflect the activity of intact tissues.

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*REGULATION OF RNA SYNTHESIS IN ISOLATED NUCLEOLI BY HISTONES AND NUCLEOLAR PROTEINS**

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Although nucleoli have long been recognized as major components of cells and nuclei and have been extensively studied,[1, 2] their precise biological role remains incompletely determined. Recent improvements in techniques for the characterization of nucleic acids and for the isolation of morphologically intact nucleoli from a wider range of animal[3–5] and plant[6] cells have helped provide strong evidence that the nucleoli function in the synthesis of ribosomes.[7–9] In addition, the methylation of RNA,[10, 11] and a synthesis of nuclear proteins[6] have been specifically attributed to nucleoli.

Further evidence that ribosomal RNA is synthesized in the nucleoli has been provided by work on the synthesis *in vitro* of RNA in intact, isolated nuclei[12] and nucleoli.[13, 14] Nucleoli were prepared from rat tumor and liver by procedures designed to permit retention of RNA-synthesizing ability. Incorporation of P^{32}-labeled ribonucleotides into nucleolar RNA was found to be dependent on the

presence of all four ribonucleoside triphosphates, to be inhibited by actinomycin D as well as by deoxyribonuclease and ribonuclease, and to produce an RNA resembling ribosomal RNA in nucleotide composition.

Isolated nucleoli represent the simplest subcellular system (of higher organisms) which is capable of the DNA-directed synthesis of RNA *in vitro* while retaining the influence of the original structural organization of the DNA, RNA polymerase, and associated proteins. The incubation conditions permit a degree of biochemical control of the *milieu* comparable to that obtained with soluble enzyme preparations. The isolated nucleolar system therefore provides a convenient and direct means for testing the effects of potential regulators on this part of the genetic mechanism.

It has been suggested[15] that histones function as gene suppressors; the possibility that they serve as regulators of chromosomal activity by interacting with specific segments of the DNA, thereby preventing the transcription of genetic messages from that locus, has attracted considerable interest. Work in several laboratories[16-21] has demonstrated that histones are indeed potent inhibitors of the DNA-dependent synthesis of RNA *in vitro*. In addition it was shown that removal of histones from chromatin by extraction with CsCl solution greatly increased the RNA-synthesizing activity,[16] and that the preincubation of calf thymus nuclei with trypsin to remove most of the histones caused the synthesis of RNA to be increased substantially.[22] These reports establish histones as general inhibitors of the RNA polymerase system, but do not in themselves establish that histones function in a selective way in regulating the type or message content of the RNA produced *in vivo*. We are reporting here that the addition or removal of histones also affects the amount of RNA synthesized by nucleoli and that, more importantly, the *composition* of the RNA produced is altered in a consistent way, approaching the composition of DNA in low-histone nucleoli and the composition of ribosomal RNA in high-histone nucleoli.

Materials and Methods.—Preparation and incubation of nucleoli: Nucleoli were prepared[14] from the Novikoff ascites rat tumor by procedures which were based partly on previous work with nuclei[12] and partly on the procedure of Desjardins *et al.*[5] The cells were centrifuged from the ascites fluid, then dispersed briefly in isotonic sucrose medium (0.25 *M* sucrose-5 mM $MgCl_2$–0.05 *M* potassium glycinate, pH 7.0) in a glass Potter-Elvehjem homogenizer with a tight-fitting Teflon pestle, and washed by centrifugation at 1200 rpm (350 × *g*) for 5 min in an International #269 head. The latter steps were repeated once. The cell sediment was suspended in three times its volume of hypotonic medium (10 mM $MgCl_2$–0.2 mM $CaCl_2$–0.05 *M* potassium glycinate, pH 7.0) and pressed without delay through a previously chilled French pressure cell (American Instrument Co.) at 3000–5000 psi. The pressed suspension was immediately mixed with an equal volume of sucrose medium (0.5 *M* sucrose-10 mM $MgCl_2$–0.2 mM $CaCl_2$–0.05 *M* potassium glycinate, pH 7.0) and centrifuged at 2000 rpm (900 × *g*) for 10 min in the #269 head. The sediment was suspended by use of a homogenizer in an amount of hypertonic sucrose medium (2.2 *M* sucrose-5 mM $MgCl_2$–0.05 *M* potassium glycinate, pH 7.0) 10 times the original cell volume. The suspension was centrifuged at 18,500 rpm (49,360 × *g*) for 45 min in the Spinco #21 rotor. The nucleolar pellet was a thin, colorless film on the bottom and side of the tube; the supernatant and white floating material were discarded. It was usually necessary to resuspend the pellet and centrifuge again in the hypertonic sucrose medium to remove contaminating particles. The final pellet was suspended in the isotonic sucrose medium. The entire preparation and storage of nucleoli was conducted at 0–4°. Purity and recovery were checked by microscopy using both phase contrast and staining with toluidine blue.[4] The nucleoli retained their morphological characteristics with no contamination by nuclei and with minimal contamination by amorphous or fibrous material. Recovery of nucleoli from the cells was 30–40%.

The standard components of the incubation medium, in a total volume of 0.5 ml, were: 1.0

μmole of ATP, 0.5 μmole each of CTP, GTP, and UTP, 2.5 μmoles of $MgCl_2$, 15 μmoles of K_2HPO_4, 5 μmoles of K-phosphoglycerate, 1 mg of rabbit muscle glycolytic enzymes,[12] 2.5 μmoles of mercaptoethanol, 125 μmoles of sucrose, 25 mg of dextran (mol. wt. 200,000–300,000) and 0.5 mg of bentonite. The amount of nucleoli used per incubation tube contained 0.15–0.3 mg of DNA. The incubation was conducted at 38° for 20 min and stopped by chilling the tubes in ice followed by addition of 0.5 ml of 0.1 *M* sodium pyrophosphate and 0.1 ml of 4.4 *N* perchloric acid. RNA synthesis was measured by the incorporation of P^{32} from α-P^{32}-UTP into cold perchloric acid-precipitable, hot perchloric acid-extractable RNA.[12] The specific activity of the UTP was 0.5–1.0 $\times$ 10^6 counts per minute per μmole (Nuclear-Chicago C115 B low background counter). Incorporation rates in the controls were about 5–7 mμmoles of UTP per mg of nucleolar DNA per 20 min. The inclusion of bentonite in the reaction mixture increased the apparent synthesis of RNA about 25% by decreasing the effects of a ribonuclease activity.[14]

For the RNA composition experiments, the components of the reaction mixture were increased tenfold in amount, and all four nucleoside triphosphates were labeled with P^{32} to the same specific activity, about 0.3 $\times$ 10^6 counts per minute per μmole. RNA was extracted by hot 10% NaCl and hydrolyzed with alkali. The distribution of P^{32} in the four 2′(3′)-nucleotides obtained by ion-exchange chromatography was regarded as the composition of the newly synthesized RNA.[12]

Histone fractions 1, 2a, 2b, and 3 were prepared from calf thymus nucleohistone by extraction with ethanolic HCl and with 0.2 *N* HCl.[23, 24] The arginine-rich fractions 2a and 3 were fractionated on Sephadex G75,[25] and the lysine-rich fractions 1 and 2b were fractionated by chromatography on carboxymethyl cellulose.[26] For incubation with nucleoli, the histones were freshly dissolved in water and were preincubated with the nucleolar pellet at 0° for 20 min before addition of the other components of the reaction mixture.

Analytical procedures: For the determination of HCl-soluble protein, an aliquot of the nucleolar suspension was centrifuged and the pellet was extracted with 0.2 *N* HCl; the protein content of the extract was determined by the method of Lowry *et al.*[27] The protein insoluble in perchloric acid was determined gravimetrically on a separate aliquot after washing in cold 0.4 *N* perchloric acid, digesting with 0.4 *N* perchloric acid at 90–93° for 15 min, washing with cold 5% trichloracetic acid, ethanol, 1:2 methanol–chloroform, and drying in a vacuum. Numbers of nucleoli were determined by dilution of aliquots with 10 vol of 0.01% toluidine blue in McIlvaine's buffer, pH 4, and counting nucleoli in the hemocytometer. Other analytical procedures were as previously described.[12]

Results.—Table 1 presents the RNA, DNA, HCl-soluble protein, and total perchloric acid-precipitable protein content of nucleoli prepared from Novikoff ascites tumor. The HCl-soluble proteins made up a large proportion of the total protein but did not, of course, represent solely histones or basic proteins. In other experiments the total proteins of nucleoli were separated and analyzed[24] and found to contain about 32 per cent histones (as determined by solubility, amino acid composition, and starch gel electrophoretic patterns) in addition to 22 per cent NaCl-soluble, 21 per cent NaOH-soluble, and 25 per cent residual proteins. Histones have also been found in pea nucleoli by Birnstiel *et al.*[28] although in smaller amounts than were reported here for the tumor nucleoli.

Figure 1 shows that the synthesis of RNA in isolated nucleoli was inhibited when calf thymus histones were added to the incubation medium. The degree of inhibition depended on the type and concentration of histone. The four main histone

TABLE 1

COMPOSITION OF NOVIKOFF TUMOR NUCLEOLI

DNA	RNA	Protein: Soluble in 0.2 *N* HCl	Protein: Insoluble in 0.4 *N* $HClO_4$
2.15*	2.30*	10.6*	13.7*

* Picograms per nucleolus.

Data are the average of six experiments. The protein soluble in 0.2 *N* HCl and insoluble in 0.4 *N* $HClO_4$ were determined on separate aliquots. Analytical details are in text.

fractions inhibited to different extents: fraction 1 (lysine-rich) was the most inhibitory at lower concentrations, although all fractions showed the same degree of inhibition at higher concentration. Whole histone was more inhibitory than any of the fractions, more so than would be expected from the sum of the inhibition by the individual fractions. It should be noted that Barr and Butler[20] reported whole histone to be less inhibitory than the fractions when tested with soluble RNA polymerase. Histone/DNA ratios of 2–3 were required for maximum inhibition of the intact nucleolar particles; these ratios were higher than observed by other authors using soluble RNA polymerase-DNA primer systems.[17, 19, 20]

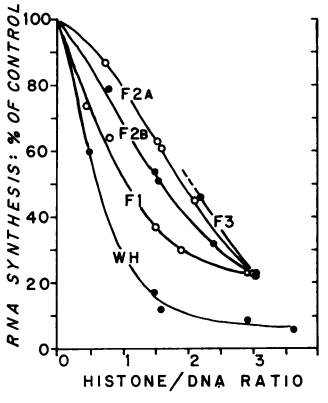

FIG. 1.—Inhibitory effect of calf thymus histone and fractions on RNA synthesis in isolated nucleoli. Histone or histone fractions were preincubated with nucleoli, and the incorporation of P^{32}-UTP into RNA was subsequently measured; details are in the text. The histone/DNA ratio refers to mg of histone added per tube and the mg of nucleolar DNA as determined by analysis. WH is whole calf thymus histone, and *F1*, *F2B*, *F2A*, and *F3* are the fractions in order of decreasing lysine content.

Figure 2 shows that the addition of histones to the nucleoli not only inhibited RNA synthesis but changed the nucleotide composition of the newly synthesized RNA. In these experiments, bentonite (which improves the recovery of total RNA by diminishing the effects of ribonuclease activity and which does not inhibit the synthesis of labeled RNA[14, 29]) was omitted from the incubation mixture to avoid possible interaction with the basic histones. It was subsequently observed that the RNA synthesized and less completely recovered in the absence of bentonite had a nucleotide composition altered in the direction of increasing C + G/A + U ratios; these data are shown as the "+bentonite" and "−bentonite" controls. While the "+bentonite" control represents the true synthetic capacity of the nucleoli much better than the "−bentonite" control, it could not be regarded as a control for the present data on the effects of histones, were it not for observations made in separate experiments.

Table 2 presents these observations, which indicate that whole histone and histone fractions have nearly as great an effect in improving the stabilization and total recovery of RNA as does bentonite. For this reason, it is believed that the "+bentonite" control provides the most valid data for comparison with the histone-treated nucleoli.

The composition of the RNA's synthesized in the presence of added histone fractions (Fig. 2) was distinctly different from the compositions in both controls. Whole histone and all the fractions except *F2A* caused a marked increase in the C + G/A + U ratio when compared with the "bentonite" control. The composition patterns caused by the individual fractions showed a definite tendency to differ from each other and from the pattern given by whole histone. The experiments must,

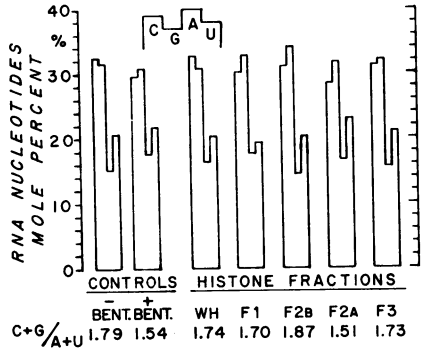

FIG. 2.—Effects of calf thymus histone and fractions on nucleotide composition of RNA newly synthesized in isolated nucleoli. Nucleoli were preincubated with the histones and incubated with all four ribonucleotides labeled with P^{32}. RNA was hydrolyzed and the 2′(3′)-nucleotides were separated by chromatography. The percentage distribution of P^{32} in the nucleotides is expressed as the composition of the *newly synthesized* RNA in mole per cent (for details see text). The composition of each RNA is plotted as bars in the order cytidylic, guanylic, adenylic, and uridylic acids (*CGAU*) and the ratio of these values, C + G/A + U, is shown below each composition. Bentonite was omitted in all incubations, except the one marked *CONTROLS + BENT.*, to avoid possible interaction with the histones; nevertheless, the *CONTROLS + BENT.* is regarded as the most suitable control for comparison with the histone-treated nucleoli (see text). Three separate complete experiments were run with the same histone preparations but different preparations of nucleoli. The amounts of histones used gave histone/DNA ratios of 1–2 for whole histones (one determination was lost) and 2–3 for the fractions. No definite correlation between nucleotide patterns and concentration of histone were noted, and the duplicate or triplicate values are presented as an average.

however, be repeated with other preparations of histones before specific effects of individual fractions, other than the general increase in the C + G content, can be regarded as established. Comparable results with a soluble bacterial RNA polymerase system were obtained by Hurwitz *et al.*,[30] who showed that calf thymus histone is more inhibitory in a soluble RNA polymerase system with A-T rich DNA as primer than with G-C rich DNA; in addition, the histone-complexed DNA directed incorporation of a smaller proportion of A and U nucleotides than uncomplexed DNA.

It was considered likely that the DNA-RNA polymerase system of the intact nucleoli was suppressed to some degree by the histone already present. Experiments were therefore done to test the effect of removal of histones by preincubation of nucleoli with trypsin, which is relatively selective for hydrolysis of histones due to their high arginine and lysine content.[22, 31]

The results in Table 3 indicate that HCl-soluble protein was removed by trypsin, as well as by the preincubation itself. Although release of suppression by removal of histone would be expected to cause an increase in RNA-synthesizing activity, a

TABLE 2

EFFECTS OF BENTONITE AND HISTONES ON STABILIZATION OF RNA DURING INCUBATION

Addition (mg/mg DNA)		Loss of RNA, %	Addition (mg/mg DNA)		Loss of RNA, %
None	—	19.3	F-1 Histone	1.5	8.6
				3.0	6.4
Bentonite	1.25	10.2	F-2b Histone	1.5	11.4
	2.10	6.1		3.0	10.6
Calf thymus histone	0.9	14.5	F-2a Histone	1.6	11.0
	1.5	9.3		2.9	10.7
	3.1	6.9	F-3 Histone	1.5	12.1
				2.9	11.4

Nucleoli (containing about 0.25 mg of DNA per tube) were incubated under standard conditions with the above additions. RNA and DNA were determined, and the percentage decrease in RNA/DNA ratio relative to nonincubated controls was calculated.

decrease was actually observed, quite possibly due to destruction of RNA polymerase by trypsin and proteolytic enzymes present in nucleoli. RNA and DNA were not removed by the treatment with trypsin nor were the nucleoli grossly altered in morphology. The composition of the RNA formed upon subsequent incubation of these nucleoli with the complete mixture was greatly altered, progressing toward lower C + G/A + U ratios as more HCl-soluble protein was removed.

TABLE 3

REMOVAL OF HCl-SOLUBLE PROTEIN BY TRYPSIN: EFFECT ON RNA SYNTHESIS AND NUCLEOTIDE RATIO

	1	2	3
Preincubation with:			
Trypsin, μg/ml	0	25	50
Trypsin inhibitor, μg/ml	0	20	40
HCl-soluble protein removed: percentage of nonincubated control	35%	42%	48%
RNA synthesis: percentage of preincubated control	(100%)	59%	54%
Composition of labeled RNA: C + G/A + U ratio	1.34	1.25	1.09

Nucleoli were preincubated in the isotonic sucrose medium with trypsin and trypsin inhibitor at 38° for 10 min as shown. Excess trypsin inhibitor was added, and the nucleoli were washed twice by centrifugation in the isotonic sucrose medium before addition of the incubation medium with P^{32}-UTP (including bentonite) for measurement of RNA synthesis. For compositions of RNA formed see Fig. 3. In other experiments, the loss of RNA-synthesizing activity due to the preincubation in the absence of trypsin was 20–30%.

In Figure 3 are shown the data on the changes in composition of newly synthesized RNA resulting from the removal of histones in the experiment of Table 3. Data from Figure 2 are also included, and the entire sequence is arranged in order of decreasing content of histones. Also included on the left are the compositions of Novikoff hepatoma ribosomal RNA[32] and nucleolar RNA, and on the right, tumor DNA (T substituted for U); these compositions were determined by chromatography and spectrophotometry.[12, 14] A clear trend is observed; the RNA progressed in composition from an RNA nearly resembling DNA, synthesized in nucleoli stripped of histones, toward an RNA resembling nucleolar and ribosomal RNA, synthesized in nucleoli inhibited by the addition of histones.

Discussion.—The work of Huang, Bonner, and Murray,[18] Hindley,[19] and Barr and Butler[20] with soluble RNA polymerase and of Allfrey and Mirsky[21] with calf thymus "aggregate" RNA polymerase has demonstrated that the ability of DNA to serve as primer for RNA polymerase reaction is greatly decreased by the formation of complexes with histones. It is clear that the four main histone fractions inhibit to different degrees although the results from the various laboratories differ widely as to the relative inhibitory activities, probably due to differences in the method of preparation of the histones and possibly due to alterations of the histone fractions from a true native state. Our results with the nucleolar particles, indicating the lysine-rich fraction 1 to be the most inhibitory, agree best with the work of Barr and Butler, and Huang *et al.* Barr and Butler also found polylysine to inhibit the RNA polymerase, and it is likely that any polycation capable of complexing the primer DNA would be inhibitory to the RNA polymerase system in a nonselective manner.

The question remains whether histones exert a *selective* regulatory action *in vivo* on the transcription of information ("readout") from DNA, affecting either the

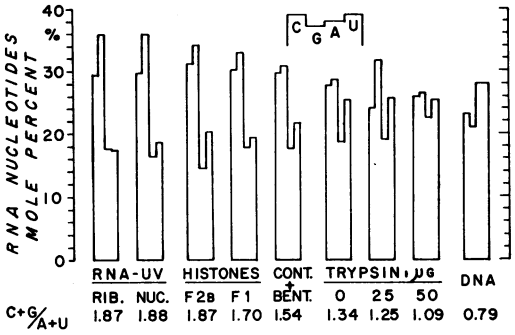

FIG. 3.—Effects of addition or removal of histones on the composition of RNA newly synthesized in isolated nucleoli. The data of Fig. 2 and Table 3 are summarized—in order of decreasing content of histones and basic proteins—and compared with the compositions of ribosomal and nucleolar RNA and of DNA. *TRYPSIN 0 µg*, *TRYPSIN 25 µg*, and *TRYPSIN 50 µg* are the additional data for columns 1, 2, and 3 of Table 3. *HISTONE F2B*, *HISTONE F1*, and *CONT. + BENT.* are from Fig. 2. *RNA-UV RIB.* and *RNA-UV NUC.* are the compositions of Novikoff tumor ribosomal RNA[32] and nucleolar RNA as determined by extraction with hot NaCl solution, alkaline hydrolysis, ion-exchange chromatography, and ultraviolet spectrophotometry.[12, 14] *DNA* is the composition of Novikoff tumor DNA as previously reported.[12]

relative amounts of the different types of RNA produced or the composition ("message content") of an RNA species. These results with the isolated nucleolar system, which represents an organizational level between the soluble enzyme systems and the intact cell, provide evidence that histones do indeed play a role *in vivo* in controlling the type of RNA produced in the nucleolus. We have previously noted[12] that the organized nucleus, both *in vivo* and *in vitro*, is able to synthesize large amounts of an RNA of high G + C content by use of a DNA template which in over-all composition is of high A + T content. Much of this high G + C RNA is produced in the region of the nucleoli[13, 14] and is presumably a precursor of ribosomal RNA.[7-9] The results presented here indicate that histones and nucleolar proteins are responsible for controlling the "readout" of the nucleolar associated DNA to produce the high G + C RNA characteristic of the ribosomes.

Summary.—Isolated nucleoli from the Novikoff ascites rat tumor were incubated with P^{32}-labeled nucleotides to determine the amount and composition of the RNA newly synthesized *in vitro*. Addition of calf thymus histone fractions inhibited the synthesis of RNA up to 90 per cent. The composition of the RNA in the presence of added histones was altered in the direction of higher cytidylic plus guanylic acid content. The nucleolar proteins were predominantly HCl-soluble and contained 32 per cent histones. When the nucleoli were pretreated with trypsin to remove these histones and other HCl-soluble proteins, the composition of the RNA synthesized was shifted in the direction of a lower cytidylic plus guanylic content. The RNA synthesized in the nucleoli stripped of basic proteins approached the composition of DNA, while the RNA synthesized in the presence of added histones approached the composition of ribosomal RNA. The evidence suggests that nucleolar histones are involved in regulation of the "readout" of DNA to produce the RNA characteristic of the ribosomes.

Abbreviations used are: ATP, CTP, GTP, and UTP for the triphosphates of adenosine, cytidine, guanosine, and uridine, respectively, and the letters C, G, A, U, and T for the nucleotide components of RNA and DNA (cytidylic, guanylic, adenylic, uridylic, and thymidylic acids, respectively).

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*TRANSIENT FREE RADICAL FORMS OF HORMONES: EPR SPECTRA FROM CATECHOLAMINES AND ADRENOCHROME**

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The initial mechanisms of action remain unknown for all hormones, despite the considerable effort that has been devoted to their study. It is not unlikely, however, that the chemical properties manifested *in vitro* differ little from at least some of the properties that characterize the initial steps of their actions *in vivo*.