as shown in Figure 7. The origin of these spectral changes is not certain, and further detailed investigations are required.

The authors are indebted to Professor M. Calvin for his constant interest and stimulating advice throughout this investigation. One of the authors (B.R.R.) is indebted to the Wellcome Trust for a travel grant.

The following abbreviations are used: Tris—Tris (hydroxymethyl) aminomethane; RuDP—Ribulose-1,5-diphosphate; DTNB—5,5'-Dithiobis-(2-nitrobenzoic acid).

* The work described in this paper was sponsored by the U.S. Atomic Energy Commission.

† On leave of absence from the Department of Biochemistry, University College, London.

[1] Rabin, B. R., and P. W. Trown, these PROCEEDINGS, **51**, 497 (1964).

[2] Ellman, G. L., *Arch. Biochem. Biophys.*, **82**, 70 (1959).

[3] Rabin, B. R., D. F. Shaw, N. G. Pon, J. M. Anderson, and M. Calvin, *J. Am. Chem. Soc.*, **80**, 2528 (1958).

[4] Yankeelov, J. A., *Anal. Biochem.*, **6**, 287 (1963).

[5] Hospelhorn, V. D., *Anal. Biochem.*, **2**, 180 (1961).

[6] Rohr, W., and A. L. Bassham, in preparation.

[7] Hirs, C. H. W., *J. Biol. Chem.*, **219**, 611 (1956).

[8] Crowley, G. T., V. Moses, and J. Ullrich, *J. Chromatog.*, **12**, 219 (1963).

[9] Trown, P. W., in preparation.

---

*ACTIVE AND INACTIVE REGIONS OF NUCLEAR CHROMATIN AS REVEALED BY ELECTRON MICROSCOPE AUTORADIOGRAPHY**

BY V. C. LITTAU, V. G. ALLFREY, J. H. FRENSTER,† AND A. E. MIRSKY

THE ROCKEFELLER INSTITUTE

Communicated May 26, 1964

Since RNA synthesis in the cell nucleus is a DNA-dependent process, an autoradiograph which shows the sites of RNA synthesis also indicates where the active DNA is located. The electron microscopic autoradiographs of thymus nuclei presented in this paper demonstrate that the DNA active in RNA synthesis is mainly in the diffuse, extended chromatin, rather than in the condensed, compact masses of chromatin. Since most of the DNA of thymus nuclei is present in the condensed masses of chromatin, these observations show that most of the DNA is inactive in promoting RNA synthesis.

Previous experiments on isolated thymus nuclei have shown that most of their DNA is inactive in RNA synthesis, for it was found that removing the DNA which is readily hydrolyzed by pancreatic DNAase (and this is 75–80% of the total DNA) does not diminish the rate of RNA synthesis. The remaining DNA, however, is essential for RNA synthesis, and the RNA being made in isolated thymus nuclei is largely of the "messenger" type.[1, 2] The earlier experiments on thymus nuclei and those reported in this paper are in line with the point of view, now widely accepted, that in differentiated cells of higher organisms many of the genes present are inactive.

The direct autoradiographic demonstration that RNA synthesis in intact nuclei is more active in diffuse than in condensed chromatin confirms the results of recent experiments[3] on isolated thymus nuclei in which chromatin from masses of nuclei

was separated into condensed and diffuse fractions. By labeling the nuclei with uridine-2-C^{14} before fractionation and then measuring the specific activity of the RNA in the components after fractionation, it was shown that RNA synthesis is more active in diffuse chromatin.[3] These observations on RNA synthesis in both intact nuclei and in nuclear subfractions make more definite and precise the long-held idea that condensed, pycnotic chromatin is relatively inactive.

Materials and Methods.—Incubation procedure: Nuclear suspensions prepared by the procedure referred to above[3] were incubated at 37°C for 30 min with shaking in a "tris"-buffered medium (pH 7.1) containing glucose, sucrose, sodium chloride, and calcium chloride, to which was added 0.5 mc of tritiated uridine (specific activity 3730 mc/mmole; 0.5 ml isotope solution per 5.0 ml nuclear suspension). The nuclei were washed once with cold incubation medium minus uridine, then three times with 0.01 *M* "tris" (HCl) buffer in 0.0033 *M* calcium chloride at pH 7.1. (This sucrose-free medium removes soluble proteins, much of the sRNA, and nuclear ribosomes.) The nuclei were divided into two parts, the first of which was resuspended in 0.01 *M* "tris" (HCl) buffer in 0.0033 *M* calcium chloride as before, the second in cation-free 0.25 *M* sucrose. (In cation-free medium the nuclei swell, and the condensed and diffuse regions of the chromatin are more easily distinguished.)

Electron microscopy and autoradiography: Fixation for electron microscopy was accomplished by the addition of 0.1 of the final volume of a 5% solution of OsO_4, at 0°C, to suspended nuclei in the last described media; they were centrifuged while fixing for 15 min, then dehydrated in an ethanol series and embedded in Epon 812. Autoradiographs of thin sections were prepared, using Ilford L4 emulsion, according to the method of Caro and van Tubergen,[4] and exposed from 53 to 59 days. Following staining with uranyl acetate, electron micrographs were made of the sections, without removing the gelatin of the emulsion. A Siemens Elmiskop I was used. (It was shown previously[5] that if calf thymus nuclei are incubated with randomly labeled uridine-C^{14} for 30 min, nearly all of the incorporated radioactivity is removed by ribonuclease, and over 95% of the total incorporated counts can be recovered as mononucleotides separable by chromatography from alkaline digests of the nuclear RNA. As it was therefore apparent that virtually all the uridine would have been incorporated into RNA, the electron microscopic preparations were not tested with ribonuclease to check that the radioactivity was in the RNA.) The count of background grains in this experiment was negligible.

Localization of DNA in electron micrographs: The use of nearly saturated solutions of uranyl acetate as a means of enhancing contrast in thin sections is widespread, and it surely has some specificity for nucleic acid.[6, 7] Even so, it was decided to demonstrate as directly as possible that the intensely staining nuclear material is the site of most of the DNA. The method of Feulgen staining and comparative light and electron microscopy of adjacent thick and thin sections was chosen for this purpose.

Serial thin and thick sections were cut from the same Epon-embedded block of nuclei used for the uridine-H^3 autoradiographs. The thin section cut for electron microscopy, about 0.07 μ thick, was mounted on a grid and stained in the usual way, i.e., with 1–2% aqueous uranyl acetate, pH 4.3, for 30 min. The immediately subsequent thick section, 0.2 μ thick, mounted on a glass slide coated with Mayer's albumin adhesive and then covered by a thin formvar film on top of the section, was stained by the Feulgen method for DNA. Some thicker sections, 0.5 and 2 μ, were also Feulgen-stained. Hydrolysis was for 20 min at 60°C in normal HCl, following the suggestion of Huxley and Zubay,[7] and staining was carried out for about 2 hr. Unhydrolyzed controls (20 min in normal HCl at room temperature) showed no staining, i.e., there was no red color in the nuclei. There was, however, a slight noticeable density (gray) in these controls, attributable to the osmium present in the material. This density also corresponded to the dark areas in phase contrast light micrographs.

Observations and Results.—DNA distribution within nuclei: Figure 1A is an electron micrograph of isolated calf thymus nuclei as they appeared after incubation and washing to remove ribosomes. (These sections are not covered with photographic emulsion, and were not treated to produce swelling.) Regions of

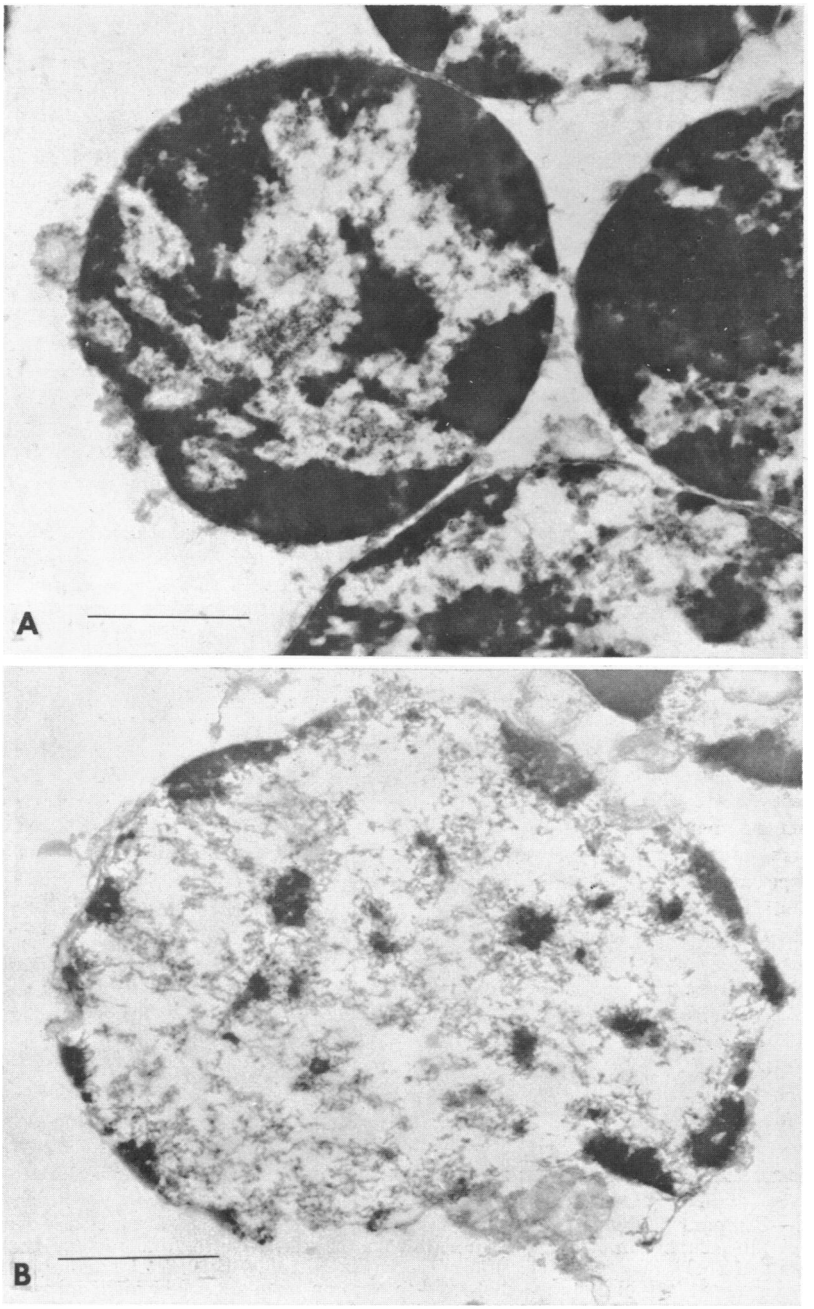

FIG. 1.—Electron micrograph of calf thymus nuclei isolated in isotonic sucrose solutions, incubated with uridine-H^3, and washed in "tris" buffer to extract ribosomes. (*A*) Nuclei at end of incubation. (*B*) Nuclei further treated with cation-free sucrose to cause swelling. Condensed and diffuse regions of the chromatin may be more sharply distinguished than in (*A*). × 21,000. The line in the lower left corner is one micron.

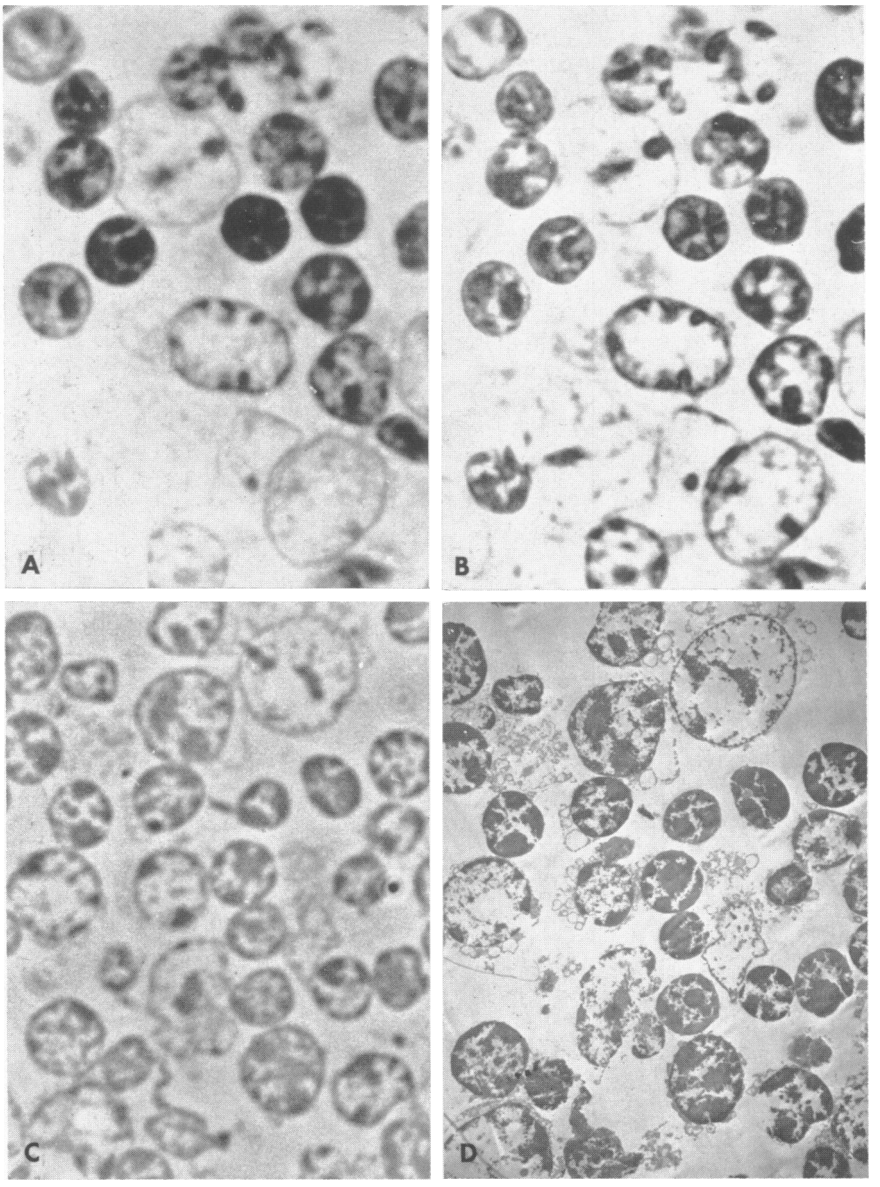

FIG. 2.—Comparative light and electron microscopy of thymus nuclear sections. These four pictures permit comparison of Feulgen-stained nuclei (as seen in the light microscope) with uranyl acetate-stained nuclei (as seen in electron micrographs). Adjacent thick and thin sections were cut from the same block of nuclear pellet fixed in osmium tetroxide. (*A*) Light micrograph of a thick (2 μ) section of a pellet of nuclei, stained by the Feulgen method. Photographic density is caused by the red color of the Feulgen-positive areas (DNA-containing areas) photographed through a green filter. (*B*) The same nuclei, photographed by phase contrast (no filter). The regions dark by phase contrast correspond to the Feulgen-positive regions. (*C*) Light micrograph of another thick section (0.2 μ) stained by the Feulgen method and photographed by phase contrast (no filter). (*D*) Electron micrograph of a thin section (0.07 μ) immediately adjacent to (*C*), stained by uranyl acetate. The condensed chromatin can be matched with corresponding phase contrast-dark regions in (*C*). With (*A*) and (*B*), this constitutes a demonstration that most of the DNA of these nuclei occurs in the condensed chromatin masses. All $\times$ 3,500.

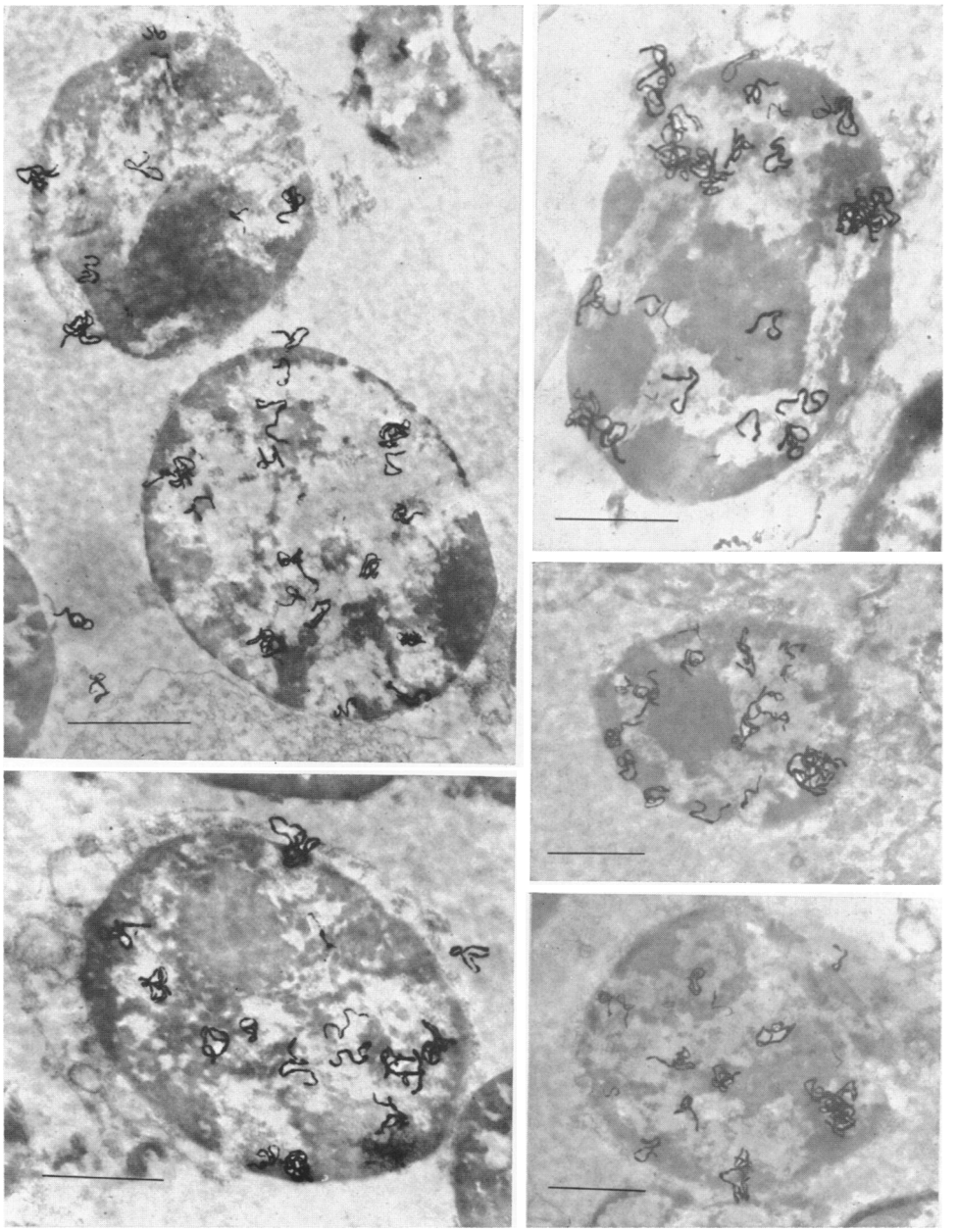

FIG. 3.—Electron microscope autoradiographs of isolated calf thymus nuclei, after incubation in uridine-H^3. Note location of silver grains chiefly over diffuse regions of the nuclear chromatin. × 21,000. The line in the lower left corner is one micron.

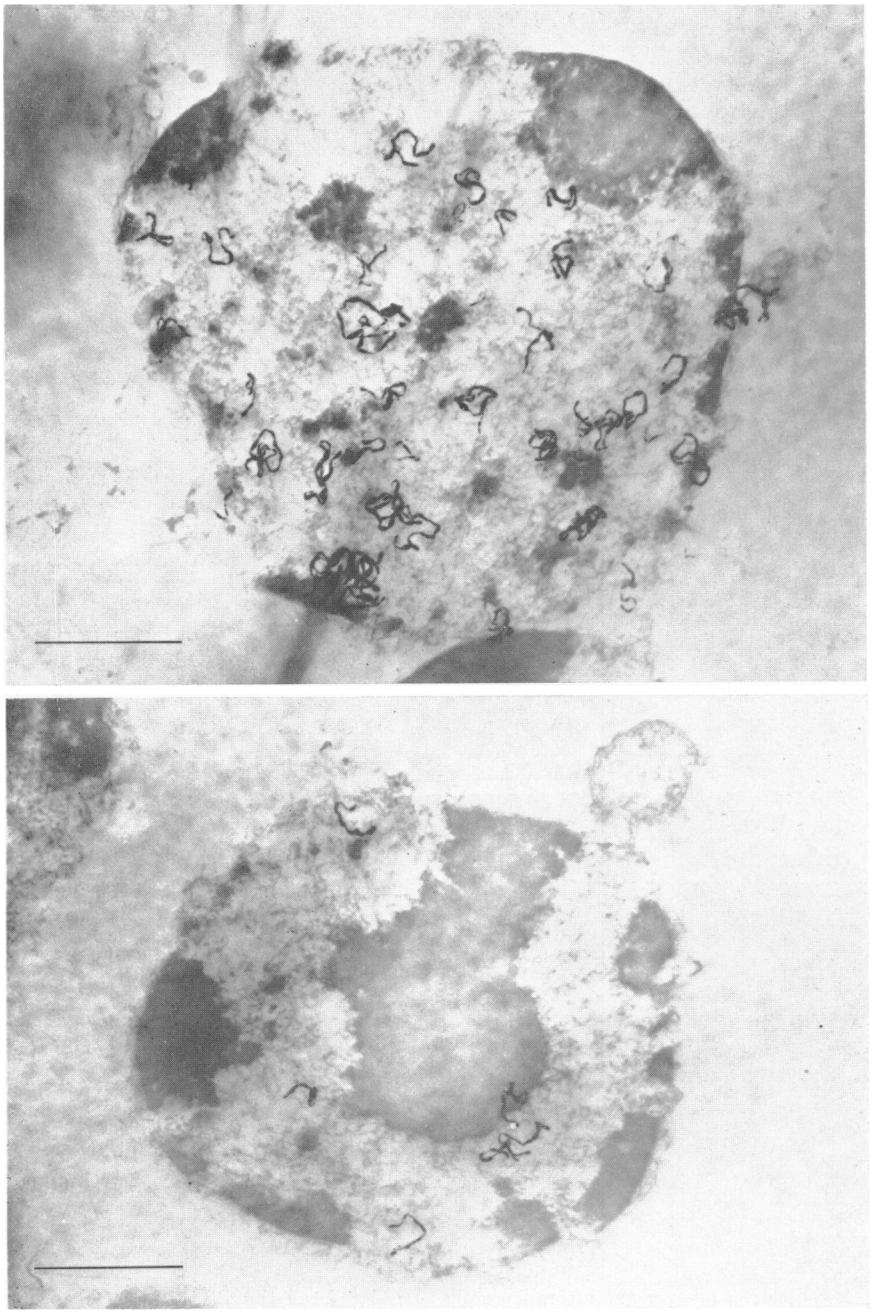

FIG. 4.—Electron microscope autoradiographs of thymus nuclei incubated with uridine-H^3 and then caused to swell in cation-free sucrose. As in Fig. 3, silver grains are predominantly over the diffuse chromatin. × 21,000. The line in the lower left corner is one micron.

dense chromatin, most frequently marginal, are especially prominent, while the remainder of the nucleus consists of a much looser array of filaments.

Figure 1*B* shows a typical nucleus after extraction of the ribosomes and subsequent swelling in cation-free medium: the condensed chromatin appears to be in smaller, perhaps more numerous, clumps, and the fibrils of diffuse chromatin are seen more clearly. Most of these fibrils range in size from 100 to 150 Å diameter, with an occasional one tapering down to approximately 50 Å.

Evidence for DNA localization in electron-dense chromatin clumps: The demonstration that the electron-dense material in uranyl acetate-stained electron micrographs is indeed DNA, as determined by the Feulgen reaction, was complicated by the fact that thick sections suitable for matching nuclei visible in the light microscope with the adjacent thin sections used for electron microscopy were nevertheless too thin (0.2 μ) for the red color of the Feulgen stain to be seen. It was easy to ascertain, however, that the dark areas seen in these sections by phase contrast microscopy were the same as the electron-dense areas in the electron micrographs. Then, using thicker sections (2 μ), it was also readily observed that Feulgen-positive areas in the nuclei were dark in phase contrast. From such comparisons (Fig. 2) one can establish that Feulgen-positive regions = phase contrast dark regions = electron-dense regions. Most of the DNA of these nuclei can therefore be considered to reside in the electron-dense areas of isolated nuclei (osmium-fixed, Epon-embedded, uranyl acetate-stained), which is to say, in the condensed chromatin. A few of the Feulgen-stained nuclei (Fig. 2*A*) show the two types of chromatin: between the condensed (darker) areas may be seen a less dark Feulgen-positive region, indicating that some DNA is present in the diffuse chromatin as well.

Intranuclear sites of RNA synthesis: Figures 3 and 4 are electron microscope autoradiographs showing incorporation of uridine-H^3 into isolated calf thymus nuclei. Figure 3 shows the grain distribution in incubated and washed nuclei; the nuclei in Figure 4 were caused to swell after the incorporation and washing had taken place. (It should be noted that these techniques do not remove more than a small fraction of the total, newly synthesized RNA.) The irregular black curly objects are developed silver grains, the result of the decay of tritium in the sections of nuclei immediately below. It might be added that not all of the nuclei showed incorporation of uridine, but the ones shown in electron micrographs are typical of those which did.

Two features of both sets of autoradiographs are striking: (1) most of the grains are over or near the areas of diffuse chromatin, and (2) although large areas of condensed chromatin are present (see especially Fig. 3), few grains occur over them. The conclusion is that not only most, but indeed almost all, of the synthesis of RNA in these nuclei occurred in the diffuse chromatin.

These observations on intranuclear RNA synthesis in thymus nuclei are consistent with the earlier report by T. C. Hsu[8] that chromocenters in mouse cells (strain H_{4C}) are relatively inert in the incorporation of H^3-uridine into RNA.

Summary.—Much of the DNA in the nucleus of a highly differentiated cell (the calf thymocyte) occurs in a condensed or compacted state, visible as electron-dense clumps in the electron microscope and as densely staining regions after Feulgen staining. A small part of the DNA occurs in a more diffuse, extended state.

When autoradiographs are prepared after labeling nuclear RNA with tritiated uridine, it is found that most of the radioactive RNA occurs in the diffuse chromatin areas, and that the condensed chromatin is virtually inactive in RNA synthesis. This is a direct demonstration of localized nuclear activity which verifies earlier conclusions that much of the DNA of the nucleus is repressed, as far as "messenger" RNA synthesis is concerned,[2] and that the repressed DNA occurs in isolable clumps of condensed chromatin.[3]

* Supported, in part, by a research grant (RG-4919) from the USPHS.
† Recipient of a research career development award (CA-17857) from the USPHS.
[1] Sibatani, A., S. R. de Kloet, V. G. Allfrey, and A. E. Mirsky, these PROCEEDINGS, **48**, 471 (1962).
[2] Allfrey, V. G., and A. E. Mirsky, these PROCEEDINGS, **48**, 1590 (1962).
[3] Frenster, J. H., V. G. Allfrey, and A. E. Mirsky, these PROCEEDINGS, **50**, 1026 (1963).
[4] Caro, L., and R. P. van Tubergen, *J. Cell Biol.*, **15**, 173 (1962).
[5] Allfrey, V. G., and A. E. Mirsky, these PROCEEDINGS, **45**, 1325 (1959).
[6] Watson, M., *J. Biophys. Biochem. Cytol.*, **4**, 475 (1958).
[7] Huxley, H. E., and G. Zubay, *J. Biophys. Biochem. Cytol.*, **11**, 273 (1961).
[8] Hsu, T. C., *Exptl. Cell Res.*, **27**, 332 (1962).

*HORMONAL CONTROL OF ENZYME SYNTHESIS IN BARLEY ENDOSPERM**

BY J. E. VARNER AND G. RAM CHANDRA

RIAS, BALTIMORE

Communicated by James Bonner, May 4, 1964

The activities of several enzymes of isolated barley endosperm increase markedly in response to added gibberellic acid.[1–6] In the normal, intact germinating seed, evocation of these same enzymatic activities in the endosperm is caused by the embryo which is known to produce gibberellic acid.[7, 8] We have, in the present case then, an example of hormonally regulated enzymatic activity and one with which it is particularly convenient to work since the principle enzyme involved is α-amylase. We shall show below that the gibberellic acid-dependent increase in α-amylase activity in barley endosperm is due to *de novo* synthesis of the enzyme. Thus, when isolated barley endosperm is treated with gibberellic acid in the presence of C^{14}-labeled amino acids and the α-amylase subsequently isolated, it is found to contain label.

We shall further show that the α-amylase produced in response to application of gibberellic acid is identical with that synthesized by the normally germinating seedling. Finally, we shall show that the gibberellic acid-induced synthesis of α-amylase is suppressed in the presence of actinomycin D, and that the effect of gibberellic acid is therefore upon the expression of the genetic information which controls α-amylase production.

Materials and Methods.—Dry barley seeds (*Hordeum vulgare*, var. Himalaya) were cut in half along their equatorial axes and the embryo halves discarded. The endosperm halves were soaked in 1% sodium hypochlorite for 15–20 min, rinsed in sterile distilled water, and transferred aseptically to sterile moist sand contained in Petri dishes. After incubation for 3 days at 17–23°, ten