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*THE ROLE OF HISTONES IN THE MAINTENANCE OF CHROMATIN STRUCTURE**

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In the nuclei of many interphase cells some of the chromatin is dense; the rest is diffuse. In a thymus lymphocyte, for example, the larger part of the chromatin is in dense masses and only a relatively small part is diffuse (Fig. 1). It is now known that RNA synthesis proceeds mainly in the diffuse chromatin[1] and not in the dense chromatin.[2] Since RNA synthesis is a DNA-dependent process, it is clear that in such a nucleus only a small part of the DNA (that which is in the diffuse chromatin) is active as a template in the RNA polymerase reaction.[3, 4]

What holds chromatin together in clumps? In this paper we present experiments which indicate that histones bind chromatin threads together into dense masses. The experimental procedure was to remove histones selectively from isolated thymus nuclei and after each extraction to examine the nuclei with the electron microscope. It was found that removing the lysine-rich histones (which comprise 20% of the total histone), and only these histones, loosened the structure of the dense chromatin. Removing arginine-rich histones did not have this effect.

The special role of lysine-rich histones in binding chromatin threads together was shown by restoring histones to histone-depleted nuclei, in which chromatin masses had been broken down into a loose fibrous network. Only the lysine-rich histones caused chromatin masses to reappear, although both arginine-rich and lysine-rich histones combined with such nuclei; indeed, if a mixture of the two histones was added to histone-depleted nuclei, presence of arginine-rich histones prevented the lysine-rich histones from clumping the chromatin threads.

In previous experiments on thymus nuclei which were digested with trypsin it was observed that, as a result of proteolytic activity, the dense masses of chromatin were converted into a loose fibrous network.[5] Since much histone was released by trypsin treatment, it seemed likely that the loss of histone was responsible for the change in structure of chromatin. Trypsin acts on histones but does not affect many native proteins until they have been denatured. Concerning the effect of trypsin on thymus nuclei, it could, therefore, be said that trypsin was acting on histones; it could not, however, be said that trypsin was not acting on any other protein although over 90 per cent of the nonhistone protein remained acid-precipitable after tryptic digestion of the nuclei.

Histones were differentially extracted by two procedures: (1) a stepwise extraction at 0° C with hydrochloric acid-ethanol mixtures in which the acid concentration is successively increased from 0.01 *N* to 0.02 *N*, 0.04 *N*, 0.06 *N*, 0.08 *N*, and finally to 0.18 *N*, keeping the ethanol concentration constant; and afterwards, while the acid is kept at 0.18 *N*, the ethanol concentration is dropped from 80 to 50 per cent and then to 10 per cent. This is a modification of a procedure due to Johns *et al.*[6] The thymus nuclei used in these experiments were isolated in a 0.25 *M* sucrose–0.003 *M* $CaCl_2$ medium[7] and were washed free of sucrose in 80 per cent ethanol. In each case extraction was for 1 hr with gentle stirring. After each step in the extraction, the suspension was centrifuged. Histones were precipitated by adding the clear supernates to 3.5 vol of acetone at room temperature. The proteins were then dried and weighed. (2) In the other procedure extraction was in two stages, first with 0.1 *M* citric acid–0.125 *M* NaCl (pH 2.0) and then with 0.2 *N* HCl, as described by Daly and Mirsky.[8] Histones were precipitated in acetone, those in the citric acid extract after first being dialyzed in the cold against 0.1 *N* HCl, to remove citric acid. The thymus nuclei used in these experiments were isolated in 0.01 *M* citric acid (pH 2.6).[8] We have recently found that formic acid can serve for the isolation of nuclei and subsequently for the extraction of lysine-rich histone, if the pH for each step is the same as when citric acid is used.

Properties of Extracted Histones.—When histones are extracted with HCl-ethanol mixtures, it was found by Johns *et al.*[6] that beginning with an ethanol concentration of 80 per cent, arginine-rich histones are extracted at first and when the ethanol concentration drops to 50 per cent, lysine-rich histones begin to be extracted. In our experiments, 62 per cent of the histone was extracted in 80 per cent alcohol and the rest in 50 per cent and 10 per cent alcohol, when all solutions are 0.18 *N* with respect to HCl. Although the ratio of lysine- to arginine-rich histone increased as the alcohol concentration dropped, there was no point that marked a critical increase in the amount of lysine-rich histone extracted. There was thus no point at which a change in the fine structure of chromatin could clearly be ascribed to removal of a particular fraction of histone.

The relative amounts of lysine- and arginine-rich histone in the HCl-ethanol extracts were determined by two procedures: (1) by electrophoresis on cellulose polyacetate (by a procedure which will be described in another paper); (2) by fractionation in 5 per cent perchloric acid,[9] which precipitates arginine-rich histone, leaving lysine-rich histone in solution. These fractions were dialyzed free of perchloric acid, precipitated by acetone and weighed. They were finally redissolved and examined electrophoretically. These histone fractions were sub-

sequently added to nuclei depleted of their lysine-rich histone by citric acid extraction to see which of them were able to cause clumping of chromatin fibrils.

Daly and Mirsky[8] found that 0.1 *M* citric acid–0.125 *M* NaCl extracted lysine-rich histone along with a small amount of arginine-rich histone, which could be removed by precipitation at pH 10.6. Subsequent extraction of the nuclear residue by 0.2 *N* HCl removed arginine-rich histone. The lysine-rich histone was found to be 20 per cent of the total mass of histone extracted. In the present series of experiments, electrophoresis on cellulose polyacetate showed that in the citric acid extraction a marked separation of histones had occurred. The lysine-rich fraction (extracted in citric acid) migrated more rapidly at pH 9 than did the arginine-rich fraction (extracted in 0.2 *N* HCl) and although the former had a faint band due to the arginine-rich fraction, the latter did not show a band due to the lysine-rich fraction.

When histones were added to histone-depleted nuclei, a known weight of the nuclei was suspended in a cold sucrose-phosphate buffer medium [1.0 ml of 0.25 *M* sucrose–3 mM $CaCl_2$, 0.5 ml of 0.1 *M* sodium phosphate·0.25 *M* sucrose buffer (pH 6.75), 0.4 ml of 0.1 *M* glucose solution containing 3.75 mg NaCl and 4.19 mg $MgCl_2 \cdot 4H_2O$ per ml, and 0.1 ml H_2O][7] of a composition favorable for experiments on the metabolic activity of isolated nuclei,[7] and to the nuclei was added a known weight of histone dissolved in the same medium. The mixture was stirred gently in the cold for 15 min and then centrifuged. The nuclei were finally resuspended in the sucrose medium before being fixed for electron microscopy.

Electron Microscopy—Procedure and Results.—The procedure is given in the legends for Figures 1 and 2.

Effects of Selective Histone Removal with Citric Acid.—Thymus nuclei isolated in dilute (0.01 *M*) citric acid (Figs. 2 and 6) look much like nuclei of intact cells (Fig. 1). When the citric acid concentration is raised to 0.10 *M*, all of the lysine-rich histone is extracted (about 20% of the total histone), along with a trace of arginine-rich histone, and now (Figs. 3 and 7) there appears a network of fibrils instead of clumps of chromatin. That this change is indeed due to removal of lysine-rich histone is shown by the reappearance of clumped chromatin when lysine-rich histone is restored (Figs. 4 and 8). If, however, arginine-rich histone is added to the histone-depleted nuclei, most of the fibrils remain (Fig. 5), although all of the histone combines with the chromatin.

Effects of Differential Histone Removal Using Ethanol-HCl Solutions.—When an entire series of HCl-ethanol extracted nuclei is fixed in OsO_4 and examined with the electron microscope, it is found that not until 62 per cent of the histone is extracted is the change in fine structure seen in all nuclei. At a concentration of 0.08 *N* HCl–80 per cent ethanol a few nuclei begin to show signs of a loosening in their chromatin; by 0.18 *N* HCl–80 per cent ethanol this is more marked; and in 0.18 *N* HCl–50 per cent ethanol the effect reaches its limit (i.e., the chromatin is in the form of a fibrillar network). The description just given holds for nuclei that are suspended in a sucrose-phosphate buffer medium after extraction of histone. (Similar preparations of control and extracted nuclei, fixed in 5% glutaraldehyde at pH 7.2 in phosphate buffer followed by 1% OsO_4 at pH 7.4 in acetate-veronal buffer, give the same result.) When the nuclei are suspended in 80 per cent ethanol after extraction, the first slight effect is noticed at 0.18 *N* HCl–80 per cent ethanol and the maxi-

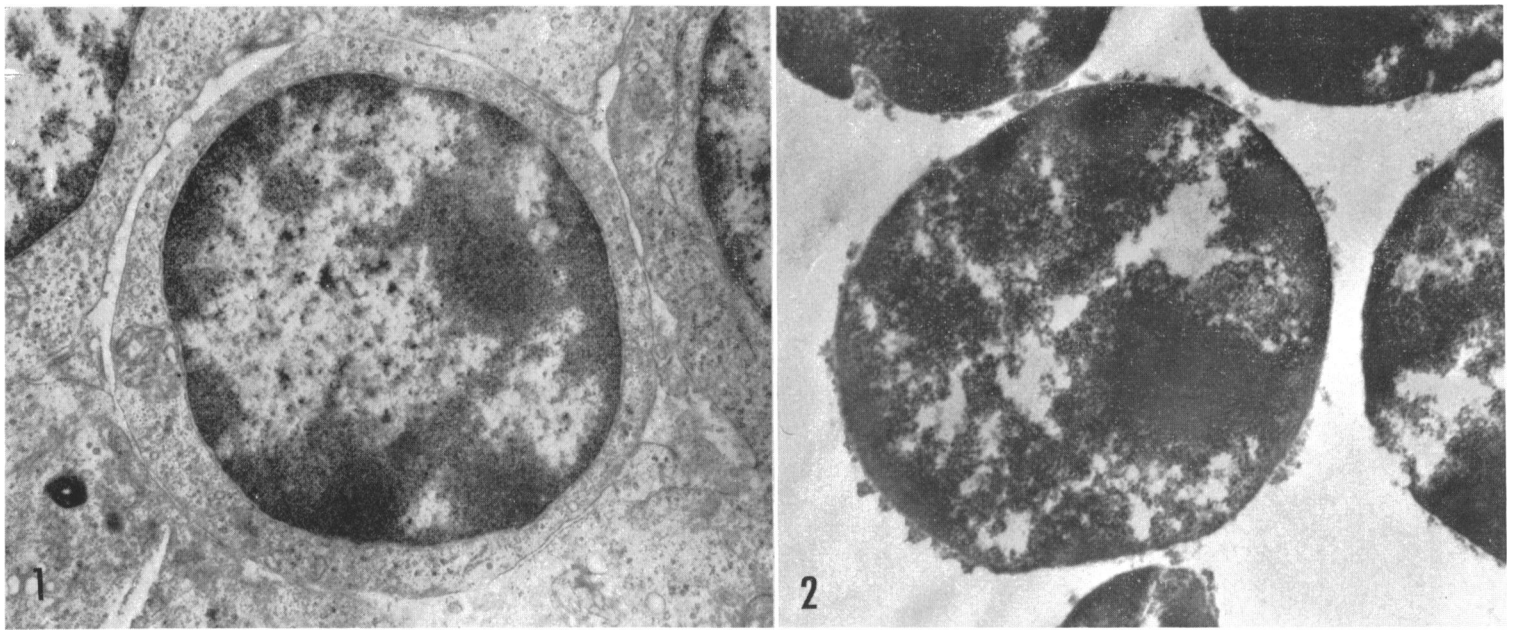

FIG. 1.—Calf thymus cell. Note that the chromatin in the nucleus occurs in both a dense and a diffuse condition. ×15,000.

FIG. 2.—Nucleus isolated in 0.01 *M* citric acid, histones unextracted. As in the thymocyte, Fig. 1, there are regions of intensely dense chromatin, stained heavily by uranyl acetate, as well as areas of diffuse lightly stained chromatin. The latter are not as well preserved here as in the intact cell. ×20,000.

Fig. 1 represents a thymocyte of the calf. Small pieces of calf thymus tissue were fixed, within minutes of the animal's death, at 0°C in 1% OsO_4 pH 7.0 in acetate-veronal buffer. Figs. 2–8 are of calf thymus nuclei isolated in citric acid and finally suspended in a sucrose-phosphate medium. Nuclei were prepared for electron microscopy by fixation with osmium tetroxide as follows: one tenth the final volume of a 5% aqueous solution of OsO_4 was added at 0°C to nuclei suspended in sucrose-phosphate medium. After stirring to ensure mixing, suspended nuclei were allowed to stand for 30–60 min at 0°C and finally centrifuged in a clinical centrifuge to form a pellet. Dehydration was in an ethanol series and final embedding in Epon 812 (a slightly modified version of Finck's[10] mixture B was employed). Sections were stained in 1% or 2% uranyl acetate for from 20 min to an hour. Electron micrographs were made on a Siemens-Elmiskop I, using the double condenser system and objective apertures of 50 microns at 80 kilovolts. The micrographs were taken at instrument magnifications of 10,000 and 33,000 times.

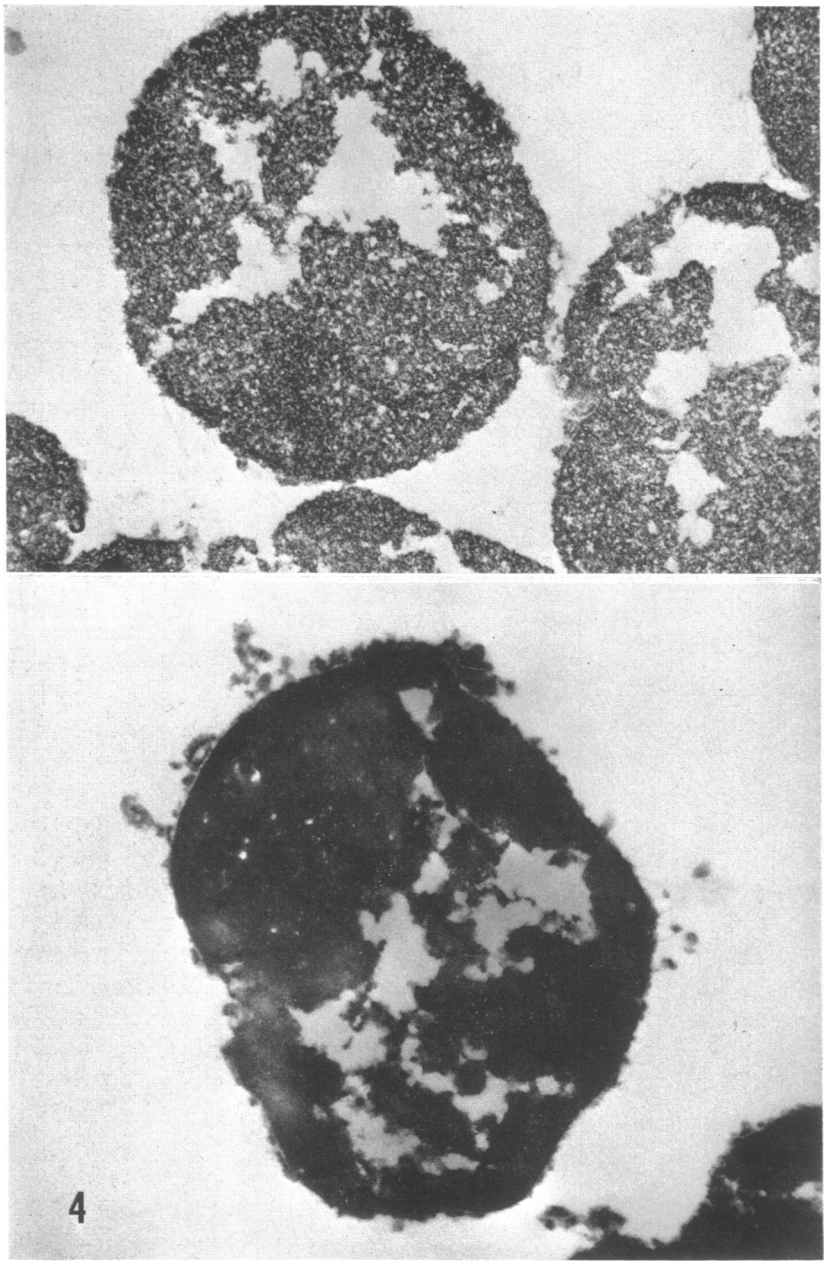

FIG. 3.—Nucleus from which lysine-rich histone has been extracted by 0.1 *M* citric acid–0.125 *M* NaCl. The chromatin now has the appearance of a network of fibrils. ×20,000.

FIG. 4.—Nucleus from which lysine-rich histone was extracted and twice the amount of lysine-rich fraction removed was added back (compare with Fig. 3). The chromatin is not only strongly condensed but is more darkly stained than in the control (Fig. 2). ×20,000.

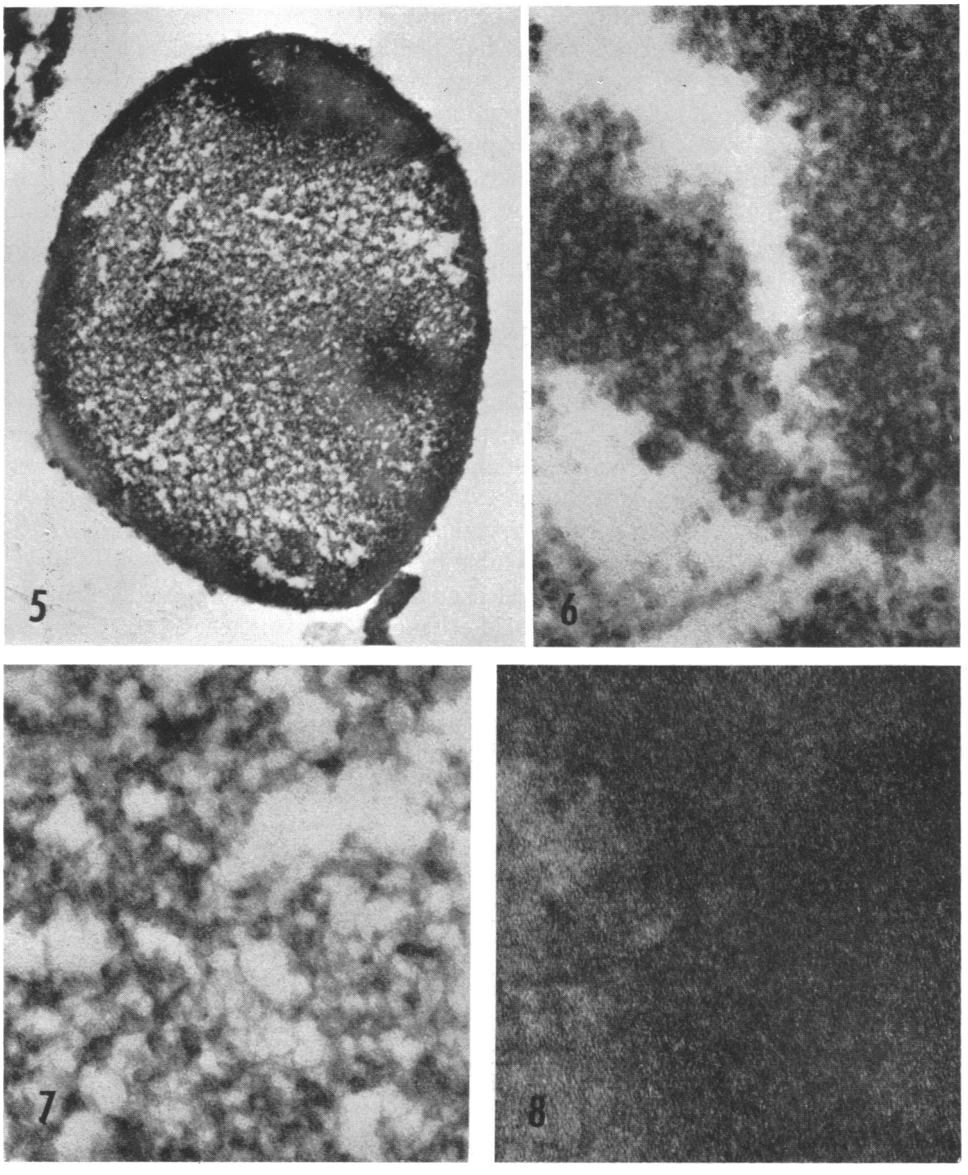

FIG. 5.—Nucleus from which lysine-rich histone was extracted and twice the amount of histone (as arginine-rich histone) removed was added back (compare with Figs. 3 and 4). Some of the chromatin has condensed, but most of it still retains the fibrillar network structure. ×20,000.

FIG. 6.—Higher magnification of chromatin of a control nucleus like the one in Fig. 2. Both dense and diffuse regions are shown, in neither of which can fibrils or other structures be distinguished clearly. ×100,000.

FIG. 7.—Higher magnification of chromatin of a nucleus from which lysine-rich histone has been extracted, like that shown in Fig. 3. The network is composed of fibrils of varying thickness, the lower limit of which is about 50 A. ×100,000.

FIG. 8.—Higher magnification of chromatin in a nucleus like that in Fig. 4, in which lysine-rich histone was added back to nuclei from which histone had been extracted. The chromatin now appears darker and denser than in the control (Fig. 6). ×100,000.

mum at 0.18 *N* HCl–10 per cent ethanol. In this series the fibrous network is not formed, but a marked "loosening" of the chromatin does occur.

Extraction with a graded series of HCl-ethanol shows that more than 50 per cent of the total histone can be extracted without any marked change in appearance of chromatin. This is almost entirely arginine-rich histone. In this method of extraction it cannot be said that when the point is reached at which a marked change in the appearance of chromatin does occur, the change is due to removal of a particular histone fraction. What can be done, however, is to test the various lysine- and arginine-rich histone preparations isolated from the extracts made by the graded series of HCl-ethanol mixtures for their ability to cause clumping of chromatin fibrils. When these histones are added to nuclei, such as those shown in Figure 3, it is found that each preparation of lysine-rich histone causes clumping and that no arginine-rich histone does.

Nuclei Treated with Picric Acid-Ethanol.—Since hydrochloric acid-ethanol mixtures are strong reagents cytologically, the possibility should be considered that the morphological change observed is due primarily to treatment with acid-ethanol, rather than to the extraction of histone. To test this point, nuclei were treated with picric acid-ethanol mixtures of the same pH and ethanol content as the HCl-ethanol mixtures. Histones are not soluble in picric acid-ethanol; nor does picric acid-ethanol produce the morphological change in chromatin observed when histones are extracted by hydrochloric acid-ethanol. It seems, therefore, that this change in chromatin is due to extraction of histone, and not simply to the effect of acid-ethanol.

Discussion.—Dense and diffuse chromatin have also been referred to as heterochromatin and euchromatin.[11–13] The latter terms were introduced in a notable paper by Heitz in 1928.[14] Heitz observed in the cells of certain mosses that even after telophase, some segments of chromosomes did not uncoil and therefore remained pycnotic. These chromosomal segments remained throughout interphase. Such pieces of chromatin were called *heterochromatin* by Heitz, and the rest of the chromatin, which did uncoil, he called *euchromatin.* In later work, partly by Heitz, and also in recent work on the mammalian X chromosome, it has been shown that heterochromatic segments of chromosomes are inert.

Since the dense chromatin of the thymus interphase nucleus resembles heterochromatin in being condensed and also in being less active than diffuse chromatin, there has been a tendency to refer to condensed and diffuse chromatin of such interphase nuclei as heterochromatin and euchromatin. It should be recognized, however, that in the thymus nucleus, and also in many other cases, it is not known whether or not the condensed chromatin consists of masses of chromosomal segments that did not uncoil at telophase. To call such condensed chromatin heterochromatic is to imply therefore that something is known about this chromatin that is in fact not known. Therefore, we consider that for the present, at least, the dense and diffuse chromatin of the thymus nucleus should *not* be referred to as heterochromatin and euchromatin.

It should indeed be noted that there are types of cells in which the dense chromatin definitely is not heterochromatic in Heitz's sense. In maturation of granulocytes, for example, the diffuse, loosely arranged chromatin becomes progressively more condensed.[15] This transformation occurs without an intervening mitosis so that appearance of the dense chromatin is surely not due to the failure of chromo-

somes to uncoil at telophase. In mature granulocytes the dense chromatin should, therefore, not be referred to as heterochromatin (and it is not by the authors of the paper on granulocytes).[15] In other cells, such as thymus lymphocytes, dense chromatin should be called heterochromatin only if it has been shown that such chromatin is a segment of a chromosome that did not uncoil at telophase.

In an interphase nucleus, such as that of a thymus lymphocyte, which is actively synthesizing RNA (an activity present only in the diffuse chromatin), it seems highly probable that there is a flux in the chromatin, so that intermittently most, if not all, of the chromatin is in the diffuse state. Experiments are now in progress to determine whether there is indeed a dynamic state of the chromatin. With respect to DNA synthesis there already is evidence along these lines. In a superb autoradiographic study of DNA synthesis in interphase nuclei of proliferating cells in the salamander, Hay and Revel[12] showed that the uptake of thymidine occurs rapidly only in the diffuse chromatin. Since interphase cells engaged in DNA synthesis do contain clumps of dense chromatin, and since all of the DNA in the nucleus is replicated, it seems clear that during the interphase period every part of the chromatin has its time in the diffuse state.

In the present investigation we have shown that removal of lysine-rich histone from condensed chromatin causes it to dissociate into a diffuse network of fibrils and subsequently restoring the lysine-rich histone produces clumps of condensed chromatin. Removal and addition of arginine-rich histone did not cause these changes. Although both histones are combined with the phosphoric acid groups of DNA, it is clear that there is a fundamental difference in the way they combine: lysine-rich histone molecules cross-link the fibrils, whereas arginine-rich histone molecules combine alongside the fibrils. The lower limit of thickness of the fibrils after removal of lysine-rich histone is about 50 A. Since a double helix of DNA is about 20 A thick and since the DNA is still combined with arginine-rich histone (and probably with other protein) after removal of the lysine-rich histone, the thinner chromatin fibrils probably contain one DNA double helix. Our experiments indicate that lysine-rich molecules combine with phosphoric acid groups of DNA to cross-link DNA double helices and that arginine-rich histone molecules combine with the phosphoric acid groups along a double helix. The possibility that histones may link adjacent DNA helices has been suggested by X-ray diffraction studies.[16]

Because of the difference in the way in which lysine- and arginine-rich histones combine with DNA, as shown by observations on the fine structure of chromatin, it is of interest to compare the ratios of arginine- to lysine-rich histone in dense and diffuse chromatin. Such a quantitative comparison is possible because a fractionation is feasible in isolated thymus nuclei.[11] Our preliminary experiments show that the ratios of arginine- to lysine-rich histone are approximately the same in dense and diffuse chromatin. Although the lysine-rich histone in diffuse chromatin does not extensively cross-link fibrils, it still is present and presumably combined with DNA.

In dense chromatin the cross-linking of fibrils produces a relatively impenetrable mass. This condition is sufficient to account for the inactivity of such chromatin. In diffuse chromatin, on the other hand, penetrability may not be a sufficient condition for activity.

Summary.—The dense, inactive chromatin of thymus lymphocyte nuclei is

formed by lysine-rich histone cross-linking the chromatin fibrils. Arginine-rich histone is bound to the chromatin fibrils but does not cross-link them.

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*THE SYNTHESIS OF α-KETOGLUTARATE FROM SUCCINATE AND CARBON DIOXIDE BY A SUBCELLULAR PREPARATION OF A PHOTOSYNTHETIC BACTERIUM**

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During the past two years evidence has been obtained for the role of ferredoxin in carbon assimilation by bacterial enzyme systems. The direct participation of ferredoxin in carbon fixation was found first in cell-free extracts of the anaerobic bacterium *Clostridium pasteurianum*,[1] and later in extracts of photosynthetic bacteria[2, 3] and other anaerobic bacteria.[4, 5] In these organisms ferredoxin is used in the reductive synthesis of pyruvate from acetyl coenzyme A and CO_2 [eq. (1)]:

$$\text{Acetyl-CoA} + CO_2 + \text{Ferredoxin}_{red} \rightarrow \text{Pyruvate} + \text{CoA} + \text{Ferredoxin}_{ox}. \quad (1)$$

This mechanism for the reductive assimilation of carbon dioxide and acetate[7] was tentatively designated the pyruvate synthase system.[2] In this reaction, the strong reducing potential of ferredoxin (−420 mv at pH 7) is used directly, without a drop to the level of pyridine nucleotides (−320 mv at pH 7). Pyruvate synthase has been partly purified from both anaerobic[5] and photosynthetic[3] cells.

Recently, the chlorophyll-containing subcellular particles (henceforth called particles) of the photosynthetic bacterium *Chlorobium thiosulfatophilum* were shown to use radiant energy for the reduction of ferredoxin and, in the presence of