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Thesis

Histone Modifications and Their Relationship to Constitutive Heterochromatin Content in Cultured *Peromyscus* Cells

MASTER

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Margaret Sprenkle Halleck

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HISTONE MODIFICATIONS AND THEIR RELATIONSHIP TO CONSTITUTIVE HETEROCHROMATIN CONTENT IN CULTURED *PEROMYSCUS* CELLS

by

Margaret Sprenkle Halleck

ABSTRACT

Biochemical research in recent years has provided evidence that control of genetic activity in eucaryotic cells may be accomplished by the modulation of chromatin structure caused by reversible modifications of chromatin proteins, in particular, the histones. In order to test the hypothesis that biochemical modifications of histone proteins are involved in condensation of chromatin (heterochromatization) histones were analyzed from three cultured cell lines from mice of the genus *Peromyscus*. These cell lines provide a unique, comparative system in which to study histone modifications associated with heterochromatin, since they all have the same diploid number and euchromatin content, yet, by flow cytometric and chromosome banding analysis, have been shown to differ in their genomic content of a specialized type of condensed chromatin, constitutive heterochromatin.

Radiolabeled histones extracted from these cell lines were analyzed using preparative polyacrylamide gel electrophoresis with non-ionic detergent present to facilitate separation of hydrophobic histone variants. It was found that *P. eremicus* cells, which contain 34% more heterochromatin than *P. boylii* cells, also contain a larger proportion of the more hydrophobic H2A variant (MHP-H2A). This MHP-H2A variant was also two times more phosphorylated than the other, less hydrophobic H2A

variant (LHP-H2A) in these cell lines. When unlabeled histones were extracted and subjected to analytical polyacrylamide gel electrophoresis it was found that these high heterochromatin P. eremicus cells also contained 28-35% more unacetylated H4, 22-29% more unacetylated H3, and 18-22% more unacetylated H2B than low heterochromatin cells (P. boylii, P. crinitus). Thus, histone acetylation was found to be inversely proportional to constitutive heterochromatin content in these cell lines. This relationship between histone acetylation and heterochromatin content was further explored by inducing hyperacetylation by treatment of cultures with 15 mM sodium butyrate for 24 hours. The percentage of unacetylated H4 remaining following treatment was found to be proportional to the amount of constitutive heterochromatin in the genome. Certain histone modifications were thus found to be related to constitutive heterochromatization in these cell lines.

The same relationships between modification of these histones and heterochromatin content found in the cell lines with differing amounts of constitutive heterochromatin were also confirmed using chromatin fractions containing different amounts of heterochromatin. These chromatin fractions were prepared from the high heterochromatin cell line, P. eremicus, by hydrodynamic shearing of nuclei and separation through steep sucrose gradients. The rapidly sedimenting (RS) chromatin fraction containing 42% heterochromatin, displayed a greater proportion of the more phosphorylated, MHP-H2A variant than the slowly sedimenting (SS) chromatin fraction, containing 27% heterochromatin. The heterochromatin-enriched RS chromatin fraction also contained 20% more unacetylated H3 and H4 than the SS chromatin fraction. Further, heterochromatin content of these chromatin fractions was theoretically

determined using the histone modification data as a basis for calculation. These calculated percentages agreed very closely with those estimated by satellite DNA content of the chromatin fractions, further supporting the concept that these histone modifications are involved in constitutive heterochromatization.

All of the observations presented in this work indicate that (1) heterochromatin contains a greater amount of the MHP-H2A variant than euchromatin, (2) this H2A variant is more phosphorylated than the LHP-H2A variant and, (3) heterochromatin contains a lesser amount of nucleosomal histone acetylation than euchromatin.

The implications of these results are that (1) the more phosphorylated MHP-H2A variant may contribute to higher order condensation of chromatin in constitutive heterochromatin and (2) lack of histone acetylation in constitutive heterochromatin is probably not due to acetylase inaccessibility of these histones, but is more likely due to deacetylation associated with reduced genetic activity.

PART I. INTRODUCTION

Since the early days of cytology, darkly-staining regions of chromatin have been noted in the interphase nuclei of many organisms. These condensed portions of chromatin, often referred to as "chromocenters" in classical cytology textbooks, were found to be related to whole, or sometimes portions of certain mitotic chromosomes which failed to uncoil at the end of mitosis. This differential degree of condensation, or heteropycnosis, of interphase chromatin was studied in developing spermatocytes by T. H. Montgomery (1904). He referred to entire chromosomes that remained condensed during interphase as "heterochromosomes". This concept was later expanded by Heitz (1928) to refer to the heteropycnotic behavior of portions of chromosomes that remained condensed throughout interphase. He introduced the term "heterochromatin" to refer to those specific chromosome regions remaining condensed in interphase, and "euchromatin" to refer to those portions that uncoil and become invisible following mitosis.

Heterochromatin was believed to be genetically inert, based on early observations of the sex chromatin in Drosophila (Stern, 1929; Muller and Painter, 1932; Heitz, 1933). Thus the process of heterochromatization came to be associated with chromatin inactivation. Recent biochemical studies of this process have focused attention on a particular class of chromatin proteins, the histones which have been found to be repressors of genetic activity. In particular, reversible modifications of these proteins through phosphorylation, acetylation, methylation and ADP-ribosylation appear to be important factors in the modulation of genetic activity and the structure of chromatin (Elgin and Weintraub, 1975).

In order to determine if a basis exists for the idea that histone modifications are integral in controlling the structure of condensed chromatin, I have examined the relationship between extent of histone modification and heterochromatization. Using cells unusually rich in constitutive heterochromatin (a specific type of condensed chromatin) I have investigated histones modified by phosphorylation, acetylation and variation in amino acid content and I have compared amounts of these modified histones in these cells with those measured in cells containing normal amounts of this condensed chromatin.

I.1. Chromatin Structure

Eucaryotic organisms possess an enormous amount of DNA which, if extended, would be on the order of a few meters in length. Somehow, this is compacted into a nucleus only a few microns in diameter, where it is packaged as the structure we call chromatin. This compaction is accomplished through the complexing of DNA with histone protein, which creates a condensation of the DNA at several levels of organization.

Chromatin is a complex of DNA, RNA, histone and nonhistone protein. Although the complement of nonhistone proteins, and the RNA content of chromatin tend to be quite variable, depending upon the source, the most consistent feature of chromatin composition is the stoichiometry of histones to DNA. The mass ratio of the histone to DNA in chromatin is approximately 1:1. This close association of DNA and histone forms the first level of DNA compaction in chromatin, the nucleosome.

<u>The nucleosome concept</u> -- A major breakthrough in the understanding of the structure of chromatin came about with the discovery that DNA is associated with repeating protein particles in structures now called

"nucleosomes" (Olins and Olins, 1974; Kornberg, 1974; VanHolde et al., 1974; Oudet et al., 1975). Very simply stated, nucleosomes are particles of histone protein with a strand of DNA wrapped around them (Kornberg, 1974; Oudet et al., 1975). These structures have been visualized in the electron microscope as 70-100Å spherical repeating particles, spaced along the DNA strand, resembling "beads on a string" (Woodcock, 1973; Olins and Olins, 1974). Nuclease digestion studies have indicated that nucleosomes are composed of histone core particles, made up of two each of the four histones H2A, H2B, H3 and H4, associated with DNA about 140 base pairs in length (Solner-Webb and Felsenfeld, 1975; Shaw et al., 1976). These histone octomers appear to be missing histone H1, however. Further, it has been proposed that the DNA is mainly localized on the outside of these particles, being coiled around the histone core (Noll, 1974; Pardon et al., 1975). Thus, the first level of DNA compaction in chromatin results from the coiling of DNA about a histone core.

The 100Å nucleofilament -- Electron microscopic evidence reveals that there are internucléosomal spacer regions of DNA between the nucleosome particles in physically disrupted chromatin or in chromatin depleted in histone H1. Although the length of DNA in this spacer region varies between organisms (Noll, 1976; Lohr et al., 1977), the association between this DNA spacer region and H1 in intact chromatin produces a further compaction of DNA, coiling the spacer DNA between two adjacent nucleosomes (Worcel, 1978). This results in a condensation from a loose "string of beads" to a closely packed 100Å thick, uniform fiber of nucleosomes without spacer regions called the "nucleofilament" (Finch and Klug, 1976). Histone H1 thus appears to be necessary for

this second level condensation of DNA. This results in a compaction of the original DNA length by a factor of about 7 (Bak et al., 1977).

The 200-300 Å "thick" fiber -- Metaphase and interphase chromatin prepared in the presence of divalent cations and viewed by election microscopy has been observed to be composed of fibers 200-300 Å thick (DuPraw, 1968; Ris, 1975). Representing a third level compaction of DNA in chromatin, this "thick" fiber configuration is thought to be the result of a supercoiling of the 100 Å nucleofilament. This conformation has been suggested to occur by a coiling of the nucleofilament into either a solenoid-like structure of closely-packed nucleosomes (Finch and Klug, 1976) or discrete, periodic, collective arrays of 6 to 10 nucleosomes (Marion and Roux, 1978; Strätling et al., 1978; Becak and Fukuda, 1979) called "superbeads" (Hozier et al., 1977). Others (Olins and Olins, 1979; Rattner and Hamkalo, 1979; Worcel, 1978) have suggested that there is no single mode of folding the 100 Å nucleofilament to form this higher-order structure. Instead, solenoids, discontinuous solenoids, superbeads, and other closely packed arrays of nucleosomes represent a variability in folding over short regions of the 200-300 Å fiber. The existence of this thick fiber is apparently dependent upon the presence of divalent cations and H1 histone, for it reverts to the 100 Å nucleofilament when H1 is partially removed or chromatin is treated with EDTA (Finch and Klug, 1976; Vengerov and Popenko, 1977; Worcel and Benyajati, 1977). Thus, again, H1 is probably involved in this higher order compaction of DNA. The total condensation of the original DNA length is thus increased to a factor of about 40 (Finch and Klug, 1976; Bak et al., 1977).

Higher-order folding -- Chromatin "loops" - A further condensation of DNA occurs in the formation of both interphase and mitotic chromatin, but the details of this process are still obscure. Electron microscopic evidence for ordered arrays of 200-300 Å fibers aligned next to the nuclear membrane and in association with nucleoli suggests that heterochromatization involves a higher order structuring of this thick chromatin fiber (Davies and Haynes, 1976). Some have suggested that a higher order arrangement of chromatin may be accomplished by attachments of chromatin, perhaps through nonhistone proteins, to a nuclear matrix or to the nuclear membrane (Hozier, 1979; Comings, 1968). Igo-Kemenes and Zachau (1978) proposed that chromatin strands in interphase nuclei are not freely mobile, but are topologically constrained by cross-linking to each other, or to a supporting nuclear structure. This results in loops, or "domains" of chromatin between attachment points. Thus, a further condensation of DNA into heterochromatin in interphase chromatin may be accomplished by loops, represented by flexible solenoids or chains of superbeads, which have been calculated to further reduce the original DNA length by a factor of 300-600 (Georgiev et al., 1978).

Higher order constraints placed on chromatin by anchorage points to a nonhistone protein scaffolding have also been suggested to contribute to the structure of mitotic chromosomes (Paulson and Laemmli, 1977). This organization, too, results in loops of chromatin between attachment points. Further, the highest order compaction of DNA in mitotic chromosomes might also be explained by this loop model. Tightly coiled metaphase chromosomes might be formed by a superhelical arrangement of chromatin loops attached to a cylindrically coiled axial structure or

matrix of the chromosome. DNA packing ratios of 10^3 or 10^4 can be obtained by this model, which are close to that calculated for the total length reduction of DNA during packaging in the mitotic chromosome (Georgiev et al., 1978).

Clearly, histones play a major role in the structure of chromatin. Changes in the higher order structure of chromatin may be caused by modifications at the nucleosomal level of chromatin organization. Alterations in nucleosome structure caused by modifying histones through acetylation and phosphorylation or by changing the nucleosome core composition with histones of varying amino acid composition are thought to be responsible for modulation of chromatin structure allowing for differential gene activation (Swift, 1974; Pederson, 1978; Worcel, 1978). Such modifications at the nucleosomal level could be expected to influence the histone-histone, histone-DNA, histone-nonhistone interactions responsible for higher order chromatin structure. Similar modifications of histone H1 may also be responsible for modifying the higher orders of chromatin structure allowing differential gene activity. As a result of these considerations a major objective of the work presented here was to investigate the possibility of a correlation between the higher order condensed structure of constitutive heterochromatin and modification of inner nucleosomal histones.

I.2. Heterochromatin

Heterochromatin is currently viewed as a higher order, condensed, structure of chromatin. In interphase cells, using electron microscopy,

heterochromatin is visualized as darkly staining masses of material aligned next to the nuclear membrane and often in association with nucleoli. As we have seen, Heitz (1928), using light microscopy, related interphase heterochromatin to portions of mitotic chromosomes that failed to uncoil in interphase. The definition of heterochromatin was further refined by Brown (1966) who proposed the term "constitutive heterochromatin" for those chromosome regions that are always condensed in both members of a homologous chromosome pair, and "facultative heterochromatin" to refer to this condensation when it occurs in only one homologue. Thus, constitutive heterochromatization involves the persistent condensation of chromatin in both maternal and paternal homologues. In facultative heterochromatization the two homologous chromosomes differ--one is heterochromatic, and the other is euchromatic, as in the inactivation of one X chromosome in female mammals (Lyon, 1961), or of the entire paternal chromosome set in the male mealy bug (Brown and Nur, 1964). In addition to their heteropycnotic appearance and genetic inertness, both types of heterochromatin have been found to replicate late in S phase, following the replication of euchromatin (Lima-de-Faria, 1969).

A third type of condensed chromatin in interphase nuclei is also recognized. This is non-specifically condensed chromatin associated with temporary and reversible inactivation of the genome. A typical example is that seen in the genetically inactive, condensed chromatin of mature lymphocytes, which can be decondensed upon the stimulation of gene activity with phytohemaglutinin. This type of inactive chromatin has been used in model studies by some (Frenster, 1963, 1974); yet it is <u>not</u> termed heterochromatin by others (Comings, 1967a; Ris and Korenberg,

1979) due to the reversibility of its condensed and inactive state. Thus, not all condensed chromatin in the interphase nucleus is heterochromatin as defined by Heitz (1928) and Brown (1966), although all heterochromatin is condensed.

Though usually ignored, these distinctions between the various forms of condensed chromatin seen in interphase cells become especially important in biochemical studies of the nature of heterochromatin. The DNA and protein components of chromatin which maintain the specific condensation of facultative or constitutive heterochromatin may be very different from those of the differentiation-associated condensed chromatin of lymphocytes or erythrocytes. In addition, however, there are certain attributes, such as lack of genetic activity that are common to all types of condensed chromatin, in mitosis or interphase. This suggests that there may be biochemical attributes common to them all.

Since it is generally accepted that heterochromatization and condensation of chromatin involves inactivation of the genome, the biochemical nature of this process has been a subject of interest for many years. Because the specific mechanism of heterochromatization is not known, investigative interest has centered on the histone proteins, which are known to act as non-specific repressors of genetic activity and, as we have seen, are also known to play a major role in the structure of chromatin. Experimentation in the 1960's suggested that histones function as inhibitors of gene transcription, but that no particular histone fraction was responsible for this repressive effect on RNA synthesis (Huang and Bonner, 1962; Allfrey and Mirsky, 1963). Since histones also affect the condensation of DNA in chromatin it was suggested that histones might repress genetic activity by producing

chromatin condensation (Johnson et al., 1974; D'Amato, 1977). Research in histone chemistry in recent years has provided evidence that modulation of chromatin structure leading to activation or repression of genetic activity may be accomplished by reversible modifications of these proteins (Elgin and Weintraub, 1975; D'Amato, 1977). For example, one type of histone modification, phosphorylation, has been found to be associated with chromatin condensation at many levels of chromatin organization (Gurley et al., 1978b). This includes histone phosphorylation during mitotic chromatin condensation (Gurley et al., 1978a), histone phosphorylation during submicroscopic changes in chromatin organization during interphase (Hildebrand et al., 1974, 1976), and histone phosphorylation associated with constitutive heterochromatin (Gurley et al., 1978c).

The purpose of the studies reported here was to investigate the relationship of modified histones (phosphorylated and acetylated) and variant histones to the structure of constitutive heterochromatin. This relationship was studied in two different ways. First, a comparative study of histone modification was made in cultured cell lines containing different amounts of constitutive heterochromatin. Secondly, the chromatin from one of these cell lines, containing a high proportion of constitutive heterochromatin, was then fractionated in order to further study the same histone modifications in heterochromatin-rich and euchromatin-rich fractions.

I.3. Constitutive Heterochromatin

The investigation of histone modifications presented in this dissertation was carried out using tissue culture cell lines known to

differ in their constitutive heterochromatin content. Constitutive heterochromatin has been found to have certain characteristics which separate it from all other types of condensed chromatin. Most constitutive heterochromatin appears to have the following general properties:

1. Remains condensed and intensely staining through the cell cycle. This is a persistent state of condensation which does not change with the cell proliferation cycle or a developmental cycle, nor is it associated with a particular tissue.

2. Genetic inertness. Regions now recognized as constitutive heterochromatin have been found to contain few mutatable genes. Thus heterochromatin can be lost or added to the genome with little recognizable genetic effect (Muller and Painter, 1932; Ris and Korenberg, 1979).

3. Specific location within the mitotic karyotype. Constitutive heterochromatin is usually located in the centromeric region of chromosomes, although it may also be telomeric, involving whole or parts of chromosome arms. This location is always on both homologues of a pair. Individuals within a species may be polymorphic for constitutive heterochromatin (Craig-Holmes et al., 1973).

4. Usually contains highly repetitious or satellite DNA sequences. After investigating several mammalian species with varying amounts of pericentromeric and telomeric constitutive heterochromatin, Yunis and Yasmineh (1971) concluded that constitutive heterochromatin was a special type of chromatin containing most of the satellite DNA or highly repetitious simple sequence DNA that is not transcribed. The development of the method of in situ hybridization of copies of satellite DNA

(or RNA) sequences to chromosome preparations (Pardue and Gall, 1970) has greatly aided this type of analysis. Using this method Pardue and Gall showed, that radioactive RNA copied from isolated mouse satellite DNA was located over centromeric constitutive heterochromatin, indicating the location of satellite sequences in this condensed chromatin. Yasmineh and Yunis (1970) utilized another method to show that constitutive heterochromatin is associated with satellite DNA in the mouse. After fractionating mouse chromatin into condensed and dispersed fractions they found the bulk of the satellite DNA sequences were located in the DNA isolated from the condensed fraction (presumably containing constitutive heterochromatin). There are, however, exceptions to the association of satellite DNA and constitutive heterochromatin. Some organisms shown to contain constitutive heterochromatin cannot be demonstrated to have a satellite. Such is the case with the Chinese hamster, which displays constitutive heterochromatin but no repetitive DNA sequences (Arrighi et al., 1974).

5. Stains by C-banding technique. While experimenting with the in situ hybridization technique to locate repetitious DNA on human chromosomes, Arrighi and Hsu (1971) noted that during the procedure chromosomes could be differentially stained with Giemsa. Since the facultative heterochromatin of the X chromosome could not be stained by this technique but centromeric heterochromatin with repetitious DNA was stained, heterochromatin stained by this technique was referred to as constitutive heterochromatin (Hsu, 1975). Similar conclusions about this type of staining were reached by Pardue and Gall (1970) and Yunis et al. (1971). This procedure, called the C-banding technique now has several variations, but the standard treatment involves four basic steps

before Giemsa staining: Fixation of the chromosomes, treatment with acid, treatment with base, and prolonged treatment with hot salt (Holmquist, 1979). Theories concerning the mechanism of C-banding vary, but it is now generally believed that the procedure induces a preferential loss of DNA from euchromatin, leaving the DNA in constitutive heterochromatin to be stained by Giemsa (Comings et al., 1973; Pathak and Arrighi, 1973; Holmquist, 1979). The reason why DNA should be preferentially lost from euchromatin during this procedure is not known. Some have suggested that the repetitive, simple sequence DNA in the constitutive heterochromatin reanneals during the hot salt treatment (Arrighi and Hsu, 1971; Yunis et al., 1971; Comings et al., 1973). Holmquist (1979) has suggested that DNA in heterochromatin does not reanneal but that differential rates of depurination may account for the observed selective solubilization of DNA from euchromatin.

6. Replicates late in S phase. As mentioned before, the DNA of heterochromatin tends to replicate late in S phase of the cell cycle (Lima-de-Faria, 1969). Facultative heterochromatin has been found in some cases to replicate later than constitutive heterochromatin (Pathak et al., 1973a, 1973b), but in general, both replicate later than euchromatin. Exceptions to this behavior have been reported, however. In some instances, constitutive heterochromatin, banding by the C-band technique (see above), has been shown to start replication early in S and finish before many other non-C-banding areas (Bostock and Christie, 1974, 1975; Hsu and Arrighi, 1971; Bostock and Prescott, 1971).

Thus it can be seen that constitutive heterochromatin is viewed as a specific type of condensed chromatin with certain characteristic properties. In general, however, it may be said that there are three definitions of constitutive heterochromatin:

1. Cytological -- Heteropycnotic portions of homologous chromosomes that remain condensed throughout the cell cycle and stain by the C-band technique.

2. Operational -- Condensed chromatin containing few structural genes and exhibiting a low amount of genetic activity.

3. Biochemical -- Chromatin often containing highly repetitious, simple sequence, satellite DNA, replicating late in S phase of the cell cycle.

Much of the confusion surrounding the literature on constitutive heterochromatin results from different viewpoints arising from these different definitions and thus, various experimental approaches.

I.4. The *Peromyscus* System

The investigation of histone modifications in constitutive heterochromatin requires a comparative cell system in which significant amounts of this type of condensed chromatin can be demonstrated. A unique, comparative system is available in cultured cell lines from deer mice of the genus *Peromyscus*. Hsu and Arrighi (1966) have demonstrated that, although all species of *Peromyscus* have a diploid chromosome number of 48, the number of chromosome arms in these species varies from 56 (*P. crinitus*) to 96 (*P. eremicus*, *P. collatus*). These differences were first thought to be due to pericentric inversions and translocations of genetic material until some of these species were re-examined

using the C-band technique (Arrighi and Hsu, 1971). Pathak *et al.* (1973a) found that when the karyotypes of *P. crinitus* and *P. eremicus* (which represent the two extremes of the distribution) were compared, all of the short arms were heterochromatic. *Peromyscus eremicus* had 40 more C-banding short arms than *P. crinitus*. Measurements of the length of the long chromosome arms in these species indicated that both species contained essentially the same amount of euchromatin. G-banded chromosome preparations (prepared by another banding method that produces distinctive patterns of deeply stained bands separated by lightly stained interbands when stained with Giemsa) demonstrated that the euchromatin of these species was essentially the same (Pathak *et al.*, 1973a). Thus, *P. eremicus* was found to have more chromosomal material than *P. crinitus*, present as short, constitutively heterochromatic short arms. This was later confirmed by Deaven *et al.* (1977) and Gurley *et al.* (1978) who showed by flow cytometric measurements that *P. eremicus* contained 36% more DNA than *P. crinitus*.

Not all cytological differences within the *Peromyscus* genus are due to changes in the number of heterochromatic short arms, however. Arrighi *et al.* (1976) compared the karyotypes of *P. crinitus* (56 chromosome arms) and *P. leucopus* (72 chromosome arms) and found that not all short arms in *P. leucopus* were heterochromatic. G-band analysis showed evidence for pericentric inversions in this species. Deaven *et al.* (1977) have also shown that another species with a number of short chromosome arms, *P. maniculatus*, demonstrates a similar condition. They found that not only were many of the short arms in this species euchromatic, but flow cytometric analysis indicated that cells from this species contained the same amount of DNA as *P. crinitus*, which has been

shown to have few short arms. Thus, not all species with many short arms in their karyotype contain elevated levels of heterochromatin or DNA. Therefore, C-banding and flow cytometric analysis have been found to be necessary tools for determining the heterochromatin content of the various karyotypes present in the Peromyscus system.

The large quantity of constitutive heterochromatin present in P. eremicus also demonstrates many of the characteristics generally attributed to this type of condensed chromatin. As mentioned above, it stains well by the C-band technique. Further, DNA from P. eremicus cells contains a GC-rich satellite DNA, banding to the heavy side of the main band in neutral CsCl density gradients (Clark et al., 1973; Mace et al., 1974). P. crinitus DNA does not contain such a satellite (Hazen, 1978). Hazen et al. (1977) demonstrated that radioactively labeled c-DNA (copy DNA) or RNA made from this P. eremicus GC-rich satellite can be hybridized in situ to the short arms of P. eremicus chromosomes. This suggests that the C-bandable heterochromatin in this species contains the satellite. The DNA in the short arms of P. eremicus has also been shown to replicate late in S-phase of the cell cycle (Pathak et al., 1973; Kuo, 1979). Furthermore, this constitutive heterochromatin was found to have a rather low level of genetic activity, as evidenced by the results of Hazen, et al. (1977), who demonstrated that labeled c-DNA made from cytoplasmic poly(A)RNA does not hybridize well to the short arms of P. eremicus chromosomes. These properties (satellite DNA, late-replicating DNA, and low genetic activity) may be used as an assay for constitutive heterochromatin in chromatin fractionation procedures.

Since many of the cytological and biochemical aspects of constitutive heterochromatin can be attributed to the heterochromatin of P. eremicus cells, this species is an ideal biological system with which to investigate classical constitutive heterochromatin. In addition, there are other species within the genus, differing essentially only in their content of this constitutive heterochromatin, that can be compared to this species. Cultured cell lines from these animals are also readily obtained by primary tissue culture techniques and easily maintained in the laboratory. Thus, cultured Peromyscus cells were selected for the investigation of histone modifications in constitutive heterochromatin presented in this work.

PART II. HISTONE MODIFICATIONS AND CONSTITUTIVE HETEROCHROMATIN CONTENT OF *PEROMYSCUS* CELL LINES

II.1. The Phosphorylation and Subfractionation of Histone H2A Variants Relative to Heterochromatin Content

II.1.1. Introduction

The phosphorylation of histone proteins and the possible involvement of this modification in changing the physical state of chromatin has been the subject of much interest. The heterogeneity introduced by these modifications into an otherwise relatively conserved protein suggests that phosphorylation may play a functional role in modulating the structure of chromatin, allowing for gene expression and replication during cell division (Elgin and Weintraub, 1975). However, recent work has also suggested that there may be some heterogeneity in chromatin structure introduced by primary structural variants of some histones. Subfractions of histones H2A, H2B, and H3, separable by their different hydrophobic properties during acid-urea polyacrylamide gel electrophoresis in the presence of nonionic detergents, have been resolved from a number of organisms, from a variety of tissues and also from cultured cells. In some cases, the primary structural differences of these subfractions have been determined (Franklin, and Zweidler, 1977; Blankstein *et al*, 1977; Cohen *et al*., 1975).

Recent work (Gurley *et al*., 1978c) has suggested that the extent of histone H2A phosphorylation may be quantitatively correlated with the amount of constitutive heterochromatin present in the genome. It was found that when two cell lines varying in their amount of constitutive heterochromatin were compared, the line with a greater amount of condensed chromatin contained larger amounts of phosphorylated H2A.

Comparable results in a very different cell system have been reported by Neumann et al. (1978). They found that when Friend erythroleukemia cells were stimulated to differentiate through the use of dimethylsulfoxide (DMSO), H2A phosphorylation was greatly increased. This increased H2A phosphorylation appears to correlate with the appearance of large quantities of coarse, densely condensed chromatin which forms in the nucleus following treatment with DMSO (Sato et al., 1971). Histone H2A primary structural variants have also been studied in the Friend erythroleukemia-DMSO system (Blankstein and Levy, 1976). Changes in the ratio of these subfractions were observed during cell line establishment and activation to DMSO sensitivity (Blankstein and Levy, 1976).

Another correlation between H2A phosphorylation and condensed chromatin is suggested by the work of Ruiz-Carrillo et al. (1976) on the maturation of avian erythroid cells. It was found that H2A is the only histone in avian erythrocytes that continues to be phosphorylated during the late stages of red cell maturation, which are characterized by nuclear inactivation and chromatin condensation.

These results led me to believe that if H2A phosphorylation really does play some role in the condensation of chromatin, then a comparative study of the subfractions of H2A and their phosphorylation in Peromyscus cell lines would provide a test of such a hypothesis. This study successfully demonstrates that consistent differences in the phosphorylation of H2A subfractions as well as differences in the relative amounts of the H2A subfractions exist in these cells, and that these differences can be correlated with heterochromatin content.

II.1.2 Materials and Methods

1. Cell Cultures

The primary cell cultures used in these experiments were from two sources. Ear fibroblast cultures of Peromyscus eremicus (strain 2352 ♀) and Peromyscus crinitus (strain 2350 ♀) were a generous gift of Dr. T. C. Hsu, Department of Biology, The University of Texas, M. D. Anderson Hospital and Tumor Institute, Houston, Texas.

The history and characterization of the P. eremicus and P. crinitus lines used in these experiments have been described previously (Pathak et al., 1973a). Ear fibroblast cultures of Peromyscus boylii were initiated in our laboratory from a female specimen which was trapped in Los Alamos County, New Mexico. Identification of the specimen as P. boylii was confirmed by Dr. James S. Findley, Department of Biology, University of New Mexico. The specimen is deposited in the Museum of Southwest Biology (MSB 37296) at that institution. These cells have been grown continuously in culture for six months. The karyotype of these cells has been determined to fall within the range of karyotypic variation shown by Schmidly and Shroeter (1974) for P. boylii.

Cells were grown as monolayers in Ham's F-10 medium (Ham, 1963) supplemented with 20% fetal calf serum, penicillin (100 units/mℓ) and streptomycin (100 µg/mℓ). Under these culture conditions all cell lines were found to exhibit an 11-12 hr generation time.

2. C-Banding

Cells were removed from monolayer culture by trypsinization, fixed in 3:1 methanol-acetic acid and mounted on slides as described by Deaven and Petersen (1974). Constitutive heterochromatin was then preferentially stained with Giemsa, using a modification of the C-band

technique (Deaven and Petersen, 1974; Hsu and Arrighi, 1971) as described previously (Gurley et al., 1978c). Constitutive heterochromatin was observed as darkly staining regions of chromatin in both interphase nuclei and metaphase chromosomes.

3. Growth Kinetics

Monitoring of the growth kinetics of the cultures was carried out using a series of small replica cultures which were initiated at the same time as the experimental cultures. At various times during the course of the experiment, the cells in one of these replica cultures were removed by trypsinization and made into a monocellular suspension (Deaven and Petersen, 1974). The cell density was then determined using an electronic particle counter. By following the growth of each cell type in this manner it was possible to calculate generation times and to expose the cells to [^{3}H]-lysine for exactly the same number of generations.

4. Isotope Incorporation for Labeling of Histones

Each cell type used for the isolation of histones was grown in eight Blake bottles, each containing an initial concentration of 5-7 x 10^6 cells in 100 mℓ of growth medium. The cultures were allowed to grow exponentially to 1.1 x 10^7 cells, at which time 0.05 μCi/mℓ [^{3}H]-lysine (spec. act. 8 Ci/mMol) were added. The cultures were then allowed to grow to 3.1 x 10^7 cells (1.42 generations in [^{3}H]-lysine). At this point, the 100 mℓ of growth medium in each Blake bottle were replaced with 100 mℓ of the same medium containing 20 μCi/mℓ of carrier-free $H_3{}^{32}PO_4$ and the cells were allowed to grow for 2 hr. The cultures were harvested at 3.5 x 10^7 cells.

5. Cell Harvest

Due to the sensitivity of histone proteins to the proteolytic activity of trypsin (Gurley et al., 1978c), use of this enzyme was avoided during cell harvest. Following the 2 hr $H_3{}^{32}PO_4$ labeling period, the growth medium on each Blake bottle was poured off and replaced with 25 mℓ ice cold Saline G (Puck et al., 1958) containing 0.05 M sodium bisulfite. Sodium bisulfite was used to inhibit phosphatases and proteases during harvest (Gurley et al., 1975; Panyim and Chalkley, 1969). The bottles were immediately placed on ice, after which the rest of the harvest was carried out at 4°C. The cells were scraped from the glass surface with a rubber policeman and decanted. Each bottle was rinsed with 50 mℓ Saline G containing 0.05 M sodium bisulfite which was added to the cell suspension. In order to prevent the cells from sticking to the walls of centrifuge tubes during centrifugation, the cell suspension was then made 20% with respect to calf serum (Gurley et al., 1978c). The cells were then removed from suspension by centrifugation for 10 minutes at 1000 X g.

6. Flow Microfluorometry (FMF)

1. DNA Content--To determine the relative DNA content of the two cell lines, confluent cell cultures (which contained cell cycle distributions rich in G_1 cells) were removed from monolayer as previously described (Kraemer et al., 1971), fixed in 70% ethanol, and treated with the fluorescent DNA stain mithramycin (Pfizer Diagnostics Division, Pfizer, Inc., 100 µg mithramycin/mℓ of physiological saline containing 15 mM $MgCl_2$) (Crissman et al., 1977). Samples from each cell line were mixed and then subjected to flow microfluorometry (FMF) as described previously (Kraemer et al., 1972). In this manner, the excess

DNA content of P. eremicus cells over the DNA content of P. boylii cells could be measured as the increased fluorescence of the G_1 peak of P. eremicus as compared to the G_1 peak of P. boylii.

2. Cell Cycle Analysis--Non-labeled cultures of the two cell lines were initiated at the same density as the labeled cultures used for the preparations of histones. These were grown to the same density as the ^{32}P-labeled cultures and then fixed and stained as described above for FMF analysis. Cell cycle analysis of the DNA histograms resulting from FMF analysis was carried out using a PDP 11/40 computer as previously described by Dean and Jett (1974).

7. Histone Isolation

Histones were extracted from 2.8×10^8 cells by the first method of Johns (1964), as previously described for cultured cells by Gurley and Hardin (1968) using the following modifications described by Gurley et al. (1975): (1) Sodium bisulfite (0.05 M) was present in both the 0.15 M NaCl used to wash the chromatin and in the 5% $HClO_4$ used to extract histone H1 (Panyim and Chalkley, 1969), and (2) 0.14 M 2-mercaptoethanol was present in all solutions used to extract and recover arginine-rich histones in order to prevent dimerization of histone H3 (Smith et al., 1970). The histones are separated by this method into three classes: (1) The very lysine-rich histone H1, (2) the histone mixture containing histones H2A, H3 and H4, and (3) the lysine-rich histone H2B. The histones were dissolved in water and lyophilized to dryness before being subjected to preparative electrophoresis.

8. Histone Purification and Fractionation by Preparative Electrophoresis

The lysine-rich histones H1 and H2B were mixed together and subjected to preparative polyacrylamide gel electrophoresis without detergent as previously described (Gurley and Walters, 1971) using the urea-acetic acid method of Panyim and Chalkley (1969) adapted for use with a Canalco Prep-Disc apparatus (Canalco, Inc., Rockville, MD).

Detergent-urea-acetic acid polyacrylamide gel preparative electrophoresis of the histone mixture containing H2A, H3 and H4 was carried out in the presence of 0.38% of the non-ionic detergent Triton DF-16 (Rohm and Haas) according to the procedure of Alfageme et al. (1974) using a 4.5 cm 12% polyacrylamide, 6 M urea, 0.9 N acetic acid gel cast in the Canalco Prep-Disc apparatus. Before application of the sample, free radicals were scavenged from the detergent gel by electrophoresis of 0.3 mℓ 1 M cysteamine in 0.9 N acetic acid at 190 V for 40 min (Alfageme et al., 1974). In both of these electrophoresis systems purified histone fractions were removed from the bottom of the gel by a cross-flow of 0.9 N acetic acid buffer collecting 2 mℓ fractions every 2 min for liquid scintillation counting (Gurley and Walters, 1971). Individual histones were located by their [^{3}H]-lysine incorporation profiles. The incorporated $^{32}PO_4$ was determined from the ^{32}P associated with each fraction. The amount of [^{3}H]-lysine in each histone peak has previously been shown to be proportional to the amount of protein in the peak (Gurley & Walters, 1971); thus the relative phosphate incorporation into each histone fraction can be determined from the $^{32}P/^{3}H$ ratio in each peak.

9. Determination of Cellular ATP

Cells used for ATP analysis were grown in four Blake bottles per cell line. Each cell line was grown to the same density at harvest (3.5×10^7 cells) as those for histone analysis. Approximately 1.4×10^8 cells were removed from the bottles by scraping as described above for cell harvest. Following centrifugation, the cells were transferred using 10 mℓ Saline G (Puck et al., 1958) to a small tube where they were extracted three times using 0.33 mℓ 6% TCA (trichloroacetic acid) for 10 min, recovering the cells each time by centrifugation. The ATP content of the pooled TCA extracts was measured enzymatically using the reactions described by Bucher (1947) as modified by Adams (1963). The enzymes and reagents were supplied by Sigma Chemical Co., St. Louis, MO. The procedure employs the reaction between 3-phosphoglycerate (3-PGA) and ATP catalyzed by phosphoglycerate kinase to generate 1,3-diphosphoglycerate which is then dephosphorylated with concomitant oxidation of NADH to NAD^+. The amount of ATP present in the TCA extract was determined from the decrease in absorbance of NADH in the solution at 340 nm (Sigma, 1974) using a Gilford Model 240 spectrophotometer.

II.1.3 Results

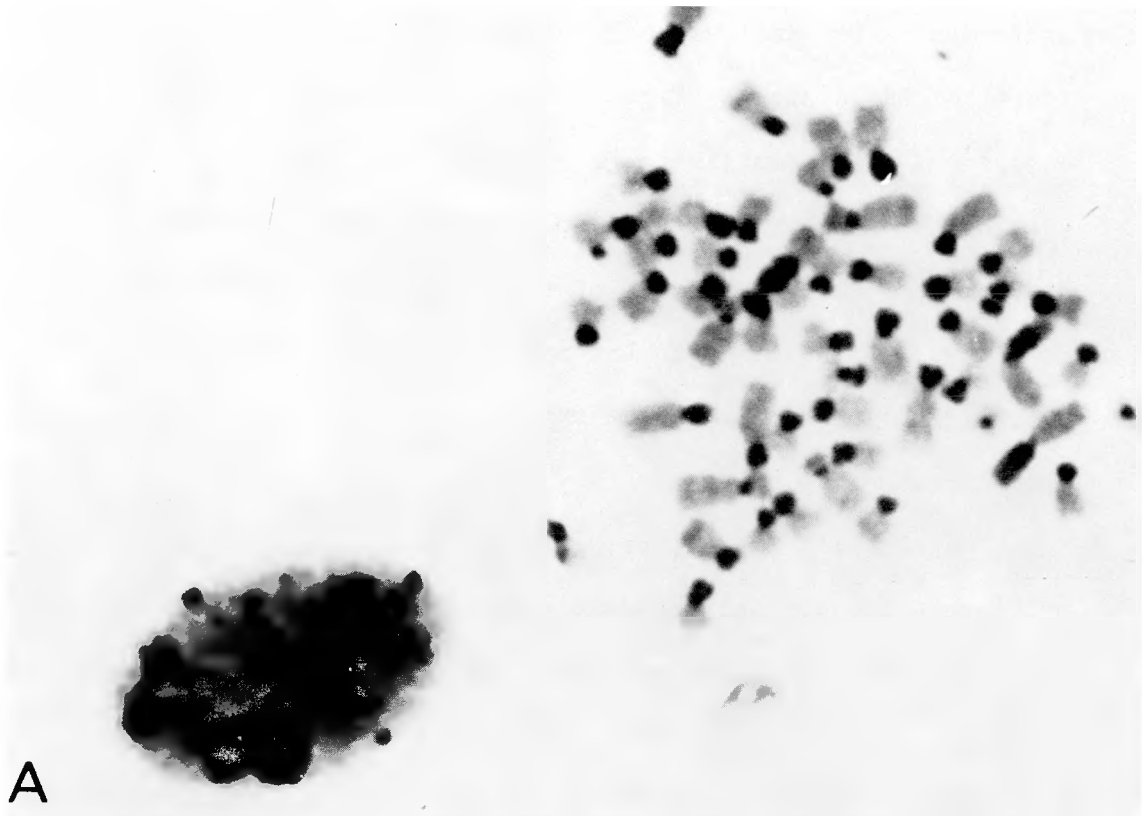
The genus of deer mice, Peromyscus, is composed of closely related animals whose cells contain essentially the same quantity and G-band pattern of genetically active euchromatin, but grossly different quantities of genetically inactive C-banded constitutive heterochromatin (Committee, 1977). Thus, cells of this genus can be used to advantage to investigate biochemical processes occurring in the heterochromatin of intact cells. By comparing measurements made on the chromatin of cultured fibroblasts of these different mice, it should be possible to

attribute specific elevated active biochemical processes to the excessive heterochromatin in those cells overly endowed with this highly condensed C-band positive chromatin. Indeed, comparative studies of histones in the high heterochromatin-containing cells of P. eremicus and the low heterochromatin-containing cells of P. crinitus have suggested that the amount of histone H2A phosphorylation is correlated with heterochromatin content (Gurley et al., 1978c). I have tested the generality of this observation by comparing a different low heterochromatin-containing Peromyscus species, P. boylii, with the highly heterochromatic P. eremicus. These two cell lines are then used to compare differences in histone H2A subfractions which might be attributed to heterochromatin.

C-banding analysis shows that each cell line has a diploid chromosome number of 48 (Fig. 1). However, P. eremicus chromosomes (Fig. 1A) contain a large number of extra darkly stained constitutive heterochromatic arms compared to the chromosomes of P. boylii (Fig. 1B). In interphase nuclei of both cell types, the constitutive heterochromatin remains highly condensed, existing as large clumps of darkly staining chromatin in P. eremicus (Fig. 1A) and smaller clumps in P. boylii (Fig. 1B). Thus, comparative heterochromatin measurements are possible in interphase nuclei as well as in mitotic chromosomes.

Comparison of P. boylii (Fig. 1B) to the P. crinitus cells previously studied (Gurley et al., 1978c) shows that this subspecies of P. boylii has 60 chromosome arms while P. crinitus has 56. The four extra arms of P. boylii do not C-band however, and likely represent pericentric inversions, rather than additions of genetic material (Hsu and Arrighi, 1971, Committee, 1977, Deaven et al., 1977). This was

Fig. 1. Constitutive heterochromatin in mitotic chromosomes and interphase nuclei of Peromyscus cells. Constitutive heterochromatin is stained darkly by the Giemsa C-band method. (A) Interphase nucleus and mitotic chromosomes of P. eremicus. Most chromosomes have short arms composed of darkly staining C-band positive heterochromatin. The nucleus contains large clumps of constitutive heterochromatin. (B) Interphase nucleus and mitotic chromosomes of P. boylii. Note the absence of C-banded short chromosome arms and localization of darkly staining areas to the centromeres. The nucleus shows small C-banded clumps of heterochromatin.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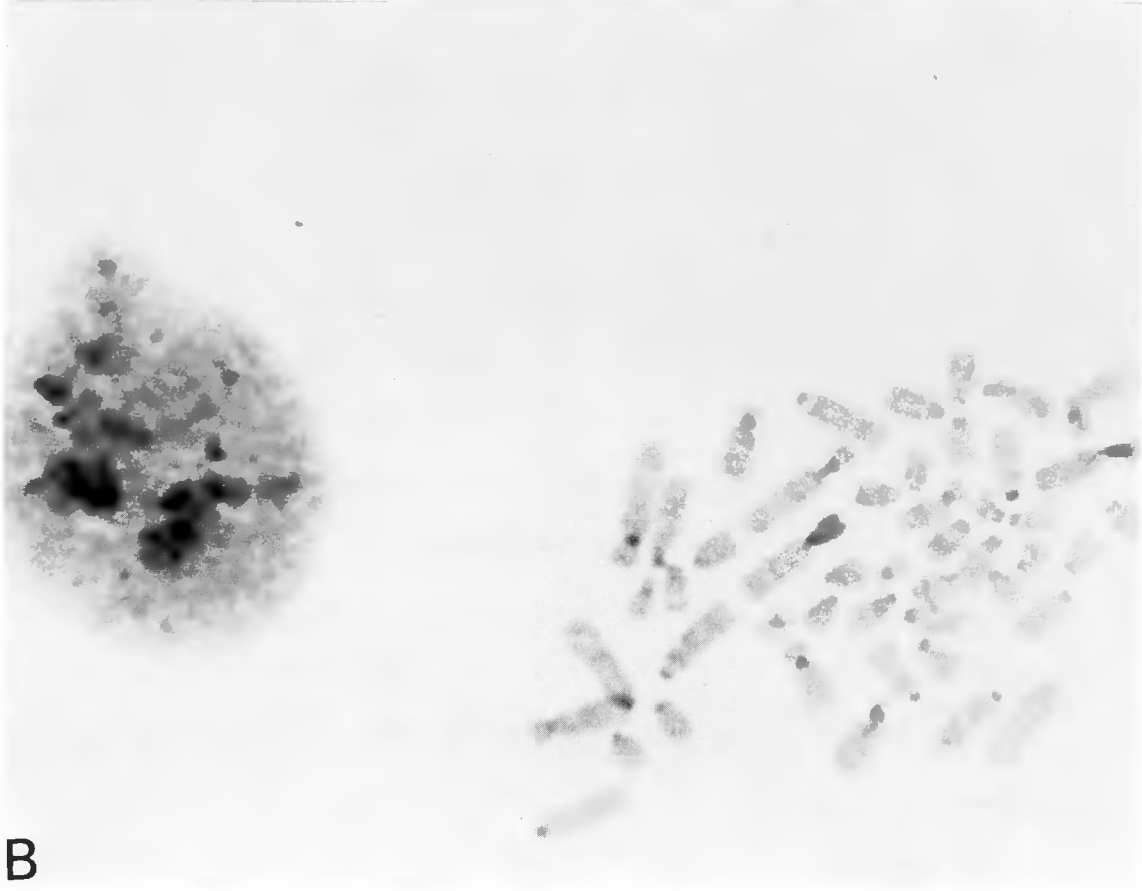
B

confirmed by FMF analysis. P. boylii and P. crinitus cells exhibited identical DNA contents (Fig. 2). For this reason, P boylii cells may be considered to be similar to P. crinitus, having an identical content of DNA and essentially the same G-banded euchromatin (Committee, 1977), but differing in the arrangement of some of the euchromatin in the chromosome arms. This rearrangement appears to be particularly common in P. boylii, for its subspecies exhibit a variety of karyotypes (Schmidly and Schroeter, 1974).

FMF analysis of a G_1-rich mixture of P. eremicus and P. boylii cells show two distinct quantities of DNA in the respective G_1 peaks of these cells, P. eremicus having a greater amount of DNA than P. boylii (Fig. 3). Based on this difference, it can be determined that P. eremicus cells contain 34.2% more DNA than P. boylii cells. Since P. eremicus and P. boylii contain essentially the same quantity of G-banded euchromatin (Committee, 1977) the extra DNA in P. eremicus must be due to a greater quantity of constitutive heterochromatin in P. eremicus than in P. boylii.

With these differences established, I followed the incorporation of [^{3}H]-lysine and $^{32}PO_4$ into the two cell lines (P. eremicus and P. boylii) and resolved the H2A histones on polyacrylamide gels in the presence of the nonionic detergent Triton DF-16 in order to compare the phosphorylation of the H2A subfractions. The two cell lines grew with identical doubling times and labeling was carried out such that each grew the same number of generations in [^{3}H]-lysine. Following a 2-hr exposure to $^{32}PO_4$, the histones were extracted, fractionated into the histone mixture containing H2A, H3 and H4 and the lysine-rich histone mixture containing H2B and H1, and purified by preparative gel electro-

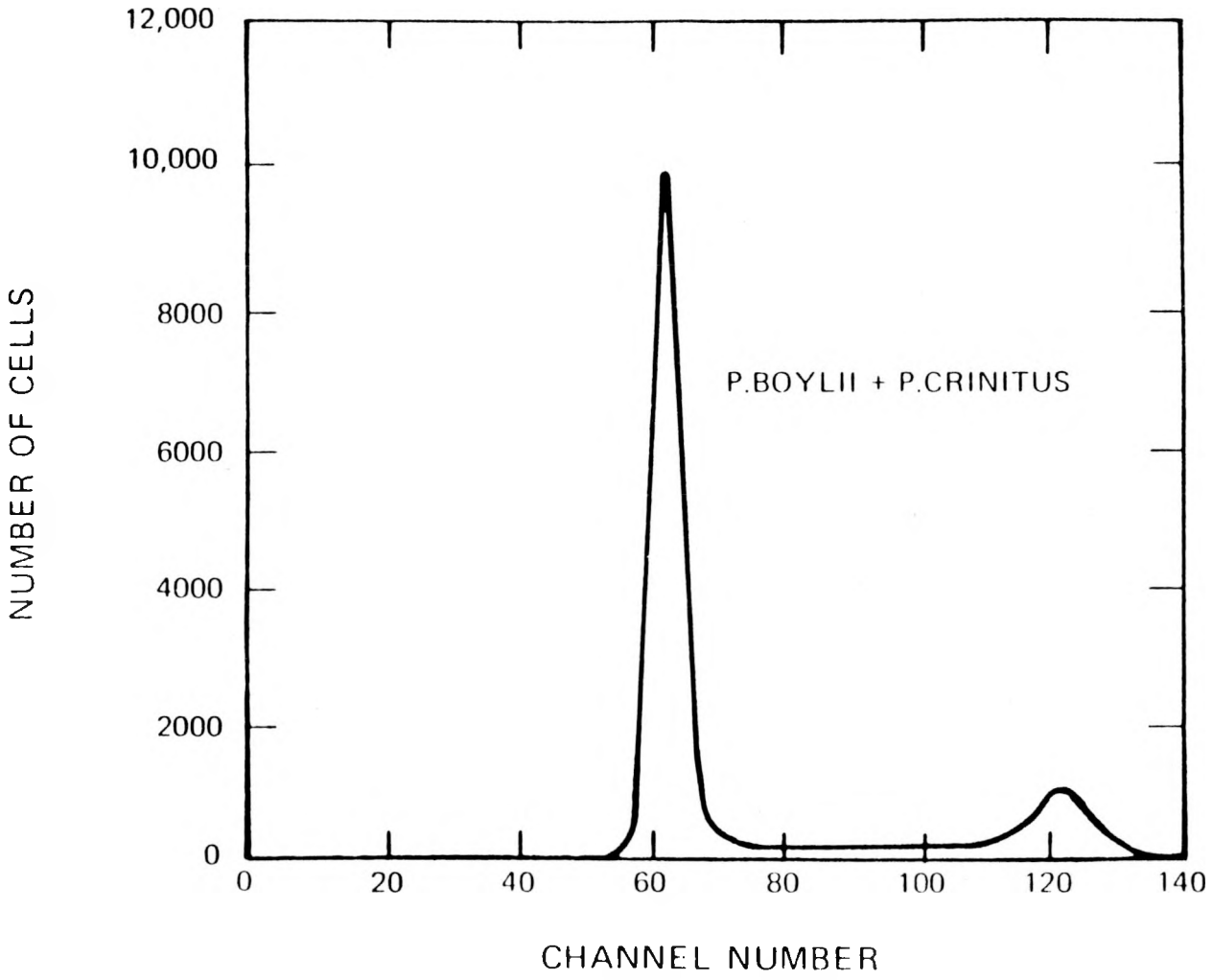

Fig. 2. Relative DNA content of P. boylii and P. crinitus cells measured by flow microfluorometry. FMF analysis of a mixture of these two cell lines show only one G_1 peak at channel 60 and only one G_2 peak at channel 120, indicating these two cell lines have identical DNA contents which are superimposed on one another.

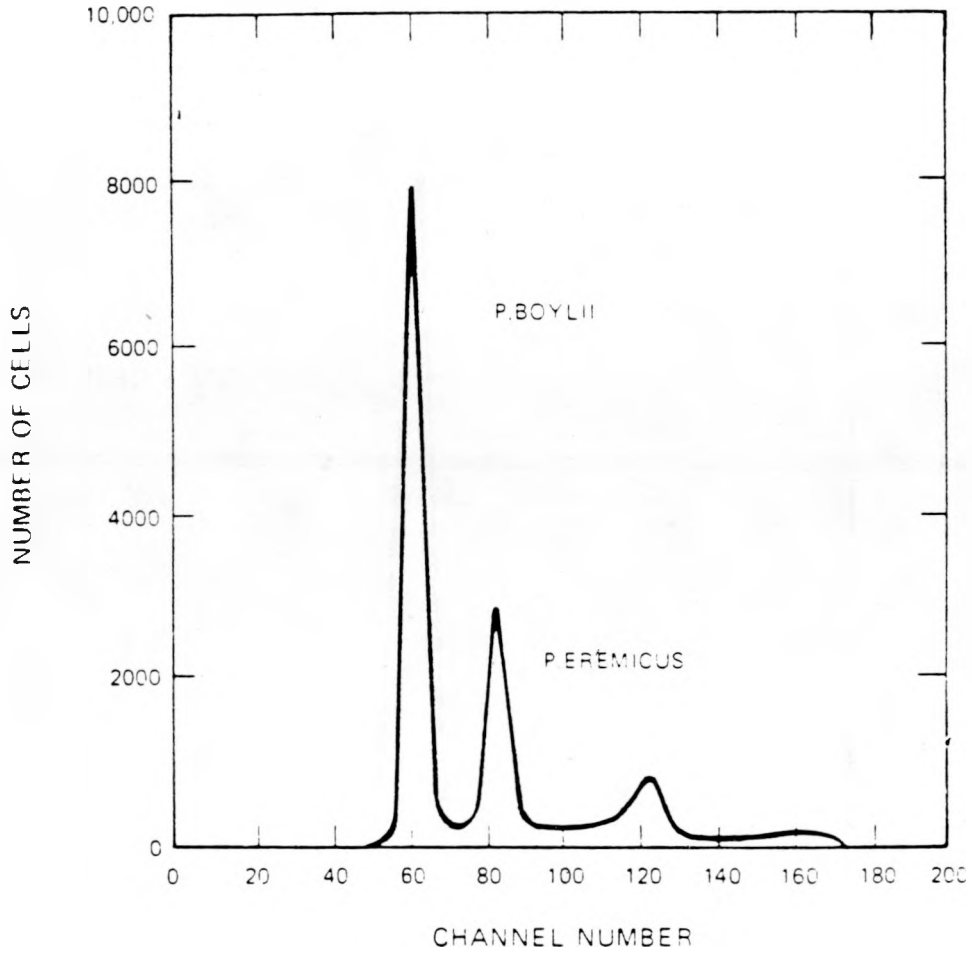

Fig. 3. Relative DNA content of *P. boylii* and *P. eremicus* cells measured by flow microfluorometry. Analysis of the difference between the *P. boylii* mean G_1 peak fluorescence (channel 61.75) and the *P. eremicus* mean G_1 peak fluorescence (channel 82.84) indicates that *P. eremicus* cells contain 34.2% more DNA than *P. boylii* cells. Peaks at about 120 and 160 represent the G_2 cells of *P. boylii* and *P. eremicus*, respectively.

phoresis. The distribution of the label in the various histones was determined as shown in Fig. 4 and 5. It was found that the H2A histone protein from both cell lines was resolved into two subfractions (Fig. 4), differing in their hydrophobic properties (Zweidler and Cohen, 1972; Gurley and Walters, 1973). In order to avoid the confusion generated by assigning the numbers 1 and 2 to these subfractions, which then becomes dependent upon which way the direction of migration is presented in the electrophoretograms, I have adopted the use of the terms "more hydrophobic" (MHP) and "less hydrophobic" (LHP) for the two H2A subfractions reported here. Zweidler and his colleagues (Franklin and Zweidler, 1977; Zweidler and Cohen, 1972) have shown that the electrophoretic mobility of the slower H2A subfraction is the most retarded due to its more hydrophobic (MHP) nature, while that of the faster H2A subfraction is less retarded due to its less hydrophobic (LHP) nature (Fig. 4). A comparison of the incorporated ^{32}P label to the ^{3}H label in each histone fraction allows one to measure the phosphorylation of these fractions. It was found that the $^{32}P/^{3}H$ ratio of the MHP-H2A subfraction was more than two times greater than that of the LHP-H2A subfraction (Fig. 4 and Table 1). This was true in both cell lines, although the total incorporated phosphate into all *P. eremicus* histones was much greater than that into *P. boylii* histones (Fig. 4 and 5 and Table 1). This is unlike the situation found by Gurley *et al.* (1978c), wherein the cell line with more heterochromatin (*P. eremicus*) had more H2A phosphorylation but the same H1 phosphorylation when compared to the cell line with less heterochromatin (*P. crinitus*). In the experiments reported here it was found that all of the histones (Fig. 4 and 5) were greater than two times more

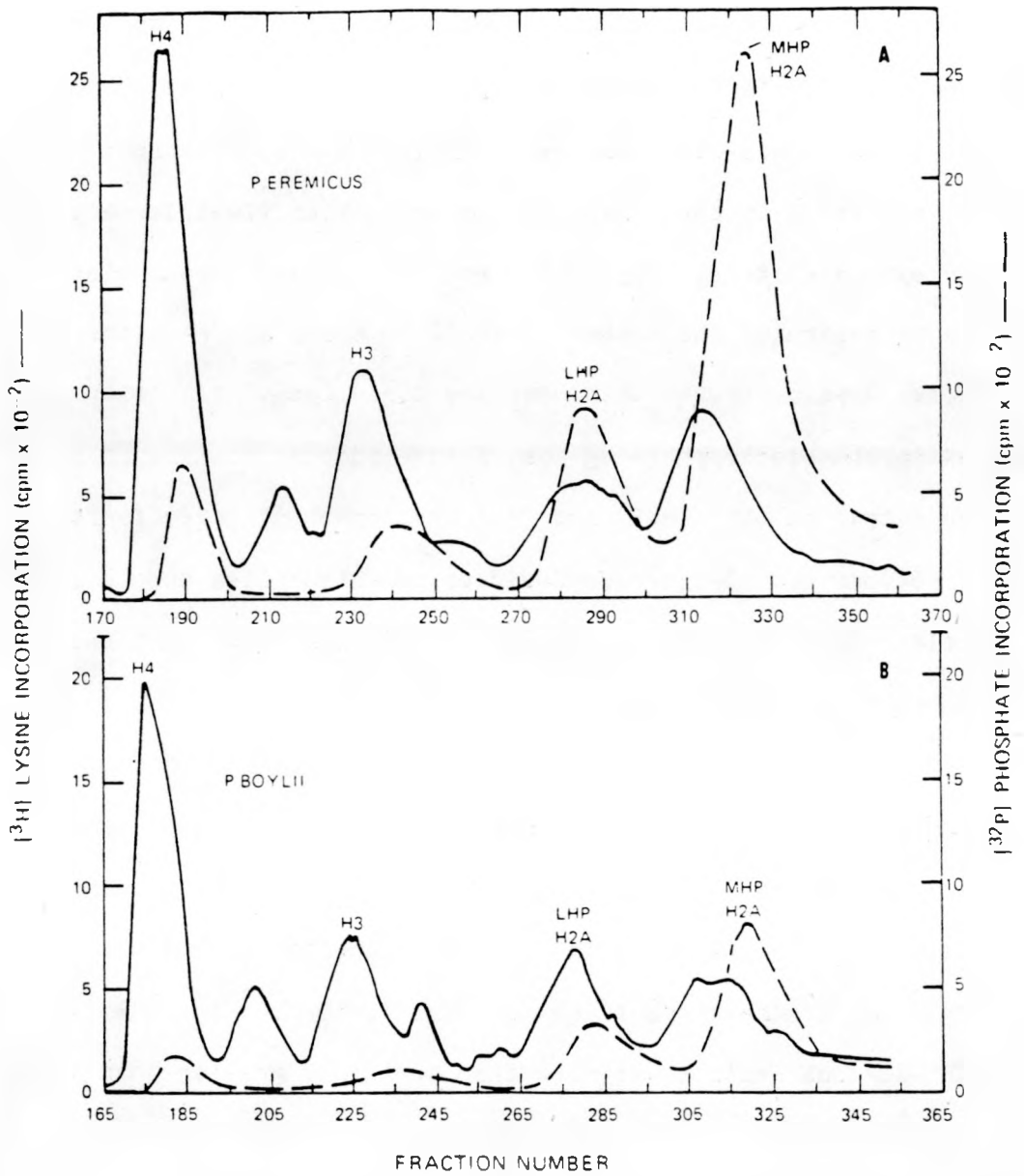

Fig. 4. Acid-urea-detergent preparative electrophoresis profile of histones H2A, H3 and H4 showing histone phosphorylation in exponentially growing cells. (A) Peromyscus eremicus cells. (B) Peromyscus boylii cells. Individual histone fractions are indicated by the 1.42-generation incorporation of [^{3}H]-lysine (——). Phosphorylation of each histone fraction is indicated by the 2-hr incorporation of $^{32}PO_4$ (---). Electrophoretic migration proceeds from right to left.

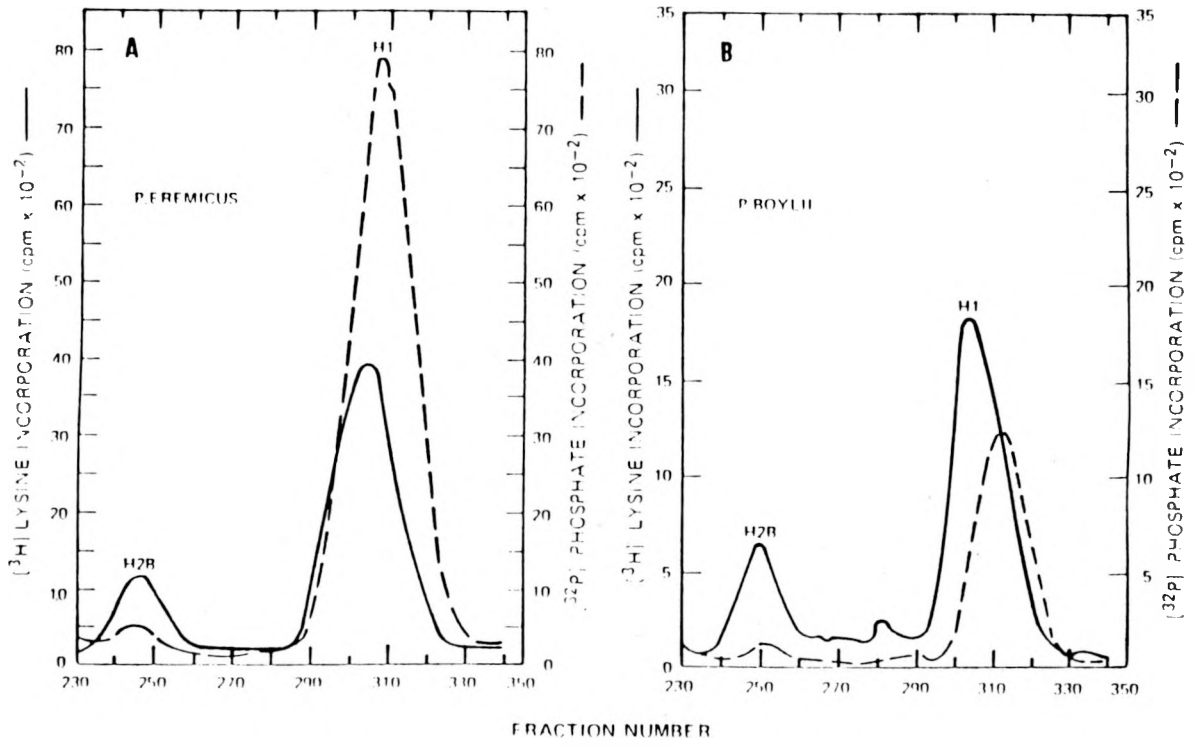

Fig. 5. Acid-urea preparative electrophoresis profile of histones H1 and H2B showing histone phosphorylation in exponentially growing cells. (A) Peromyscus eremicus cells. (B) Peromyscus boylii cells. Individual histones are indicated by the 1.42 generation incorporation of [^{3}H]lysine (——). Phosphorylation of each histone fraction as indicated by the 2-hr incorporation of $^{32}PO_4$ (---). Electrophoretic migration proceeds from right to left.

TABLE 1

HISTONE PHOSPHORYLATION IN EXPONENTIALLY GROWING CULTURES

	Phosphorylation of Histones ($^{32}PO_4$/[^{3}H]lysine)					
	H1	H2A			H3	H4
Cell Line		LHP	MHP	MHP/LHP		
P. boylii	0.67	0.49	1.08	2.20	0.13	0.08
P. eremicus	1.84	1.05	2.43	2.31	0.33	0.22

phosphorylated in the cell line with more heterochromatin (Table 1). The reasons for these differences are attributed to cell cycle distributions, which will be examined below. The apparent small phosphorylation of histone H2B seen in Fig. 5 is not considered here, since it has been shown to be due to contamination by phosphorylated H2A which has the same electrophoretic mobility as H2B in this gel system (Gurley and Walters, 1973). Also, the low-level phosphorylations of histones H3 and H4 are not considered here since the phosphorylation of H3 has been shown to be strictly a mitotic event (Gurley et al., 1978a) and the phosphorylation of H4 represents residual phosphate retained from the cytoplasm (Ruiz-Carrillo et al., 1975).

ATP analysis was carried out on the two cell lines in order to determine if they contained similar ATP pools at harvest density. The results of this analysis indicated that P. boylii cultures contained 4.1×10^{-10} µmol ATP per cell and P. eremicus cultures contained 4.3×10^{-10} µmol ATP per cell. Thus, the difference in phosphorylation of histones in these two cell lines does not appear to be a reflection of differences in cellular ATP pools.

Cell cycle analysis of both cell lines was carried out at the same harvest density used in the histone analysis in order to determine if exponential cultures of both cell lines had similar cell cycle distributions. It was found that the distributions were indeed different, (Table in Fig. 6) P. boylii (Fig. 6C) having many more cells in G_1 and much fewer cells in S than P. eremicus (Fig. 6D). Growth curves (Fig. 6A and B) show that the cells in both cell lines were in exponential growth. Since these two cultures had the same doubling

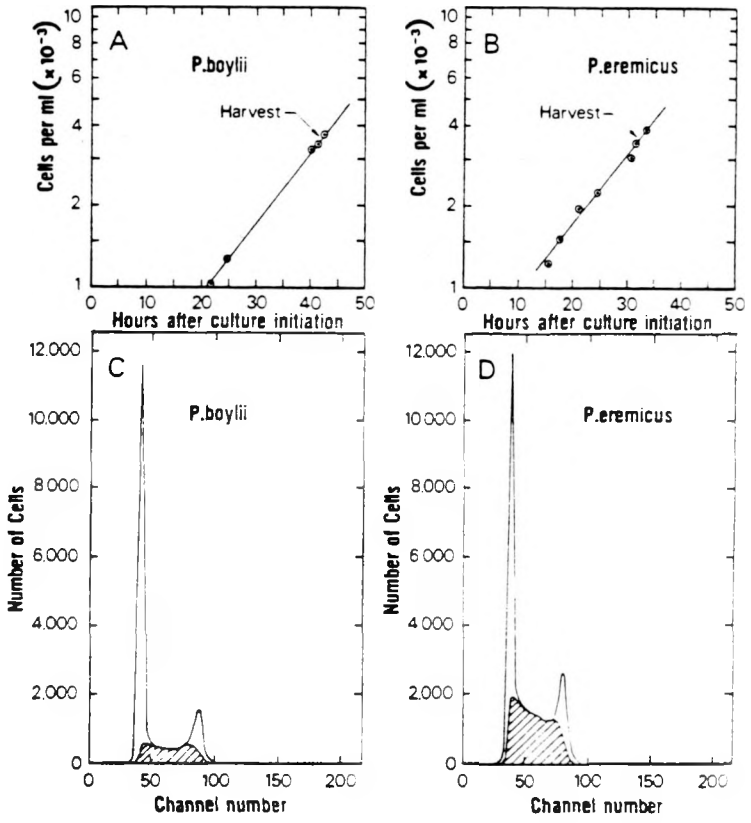

Fig. 6. Growth curves and cell cycle distribution of Peromyscus cells at harvest density. (A) Growth of P. boylii culture having an 11.5 hr generation time. (B) Growth of P. eremicus culture having an 11.5 hr generation time. (C) FMF profiles of the cell cycle distribution of P. boylii cultures. (D) FMF profiles of the cell cycle distribution of P. eremicus cultures. The G_1 peaks of both cell lines were aligned electronically on the same channel to assist comparison of the quantities of cells in each cell cycle phase. Cell cycle distributions were quantified as described by Dean and Jett (1974):

G1 determined from the peak at channel 40, S determined from the shaded area between channels 40 and 80, and G2 determined from the peak at channel 80.

	Percent G_1	Percent S	Percent G_2 + M
P. boylii	63.3	25.3	11.4
P. eremicus	36.7	52.0	11.3

times, the P. eremicus cells are judged to have a longer S phase and shorter G_1 phase than P. boylii cells.

Aside from the increased phosphorylation of P. eremicus histones as compared to P. boylii histones (Table 1), and their differences in cell cycle distribution (Fig. 6), another interesting difference observed in these two cell lines is that the mass ratio of the MHP to LHP-H2A subfractions is 29% greater in P. eremicus than in P. boylii although their relative amounts of total H2A to histones H3 and H4 were the same (Table 2). Thus it is found that the cell line with 34.2% more heterochromatin shows a 29% increase in the relative amount of the more phosphorylated MHP-H2A subfraction to the LHP subfraction when compared to the low heterochromatin-containing cell line (Table 2).

II.2.4. Discussion

The most striking difference observed in the phosphorylation of H2A histones in these two Peromyscus cell lines is that the two H2A subfractions are not phosphorylated equally. The more hydrophobic subfraction is phosphorylated more than twice as much as the less hydrophobic subfraction. This is true in both the high heterochromatic and low heterochromatic cell lines. Thus, this difference between the phosphorylation of the two H2A subfractions does not appear to be due to the total amount of condensed heterochromatin present.

A positive correlation between the amount of constitutive heterochromatin and H2A was made, however, when the masses of the two different H2A histones of these two cell lines were compared. The cell line with 34% more constitutive heterochromatin contained a similar excess (29%) in its ratio of the more hydrophobic H2A subfraction to the less hydrophobic subfraction. This correlation suggests that the

TABLE 2

HISTONE CONTENT OF EXPONENTIALLY GROWING CULTURES

	Mass ratio of histones		([^{3}H]lysine)/[^{3}H]lysine	
Cell Line	Total H2A/H3	Total H2A/H4	MHP H2A/LHP H2A	Relative Amount of Heterochromatin
P. boylii	1.93	1.20	1.04	1.00
P. eremicus	1.85	1.24	1.34	1.34
% Difference	4	3	29	34

MHP-H2A subfraction is directly associated with the higher heterochromatin content found in P. eremicus cells. This comparison may also be made in another way, by assuming that an addition of heterochromatin to the genome is in fact accompanied by a similar addition of the MHP-H2A subfraction:

$$\frac{CHC_E}{CHC_B} = \frac{MHP_E}{MHP_B}, \quad (1)$$

where CHC = constitutive heterochromatin
E = P. eremicus
B = P. boylii
and MHP = more hydrophobic H2A.

Secondly, we assume that both cell lines, (which contain the same amount of euchromatin) have the same content of the LHP-H2A subfraction:

$$[LHP_E] = [LHP_B] \quad (2)$$

where LHP = less hydrophobic H2A.

From FMF and C-band analysis we know that:

$$CHC_E = 1.34\ CHC_B. \quad (3)$$

From the results of [^{3}H]-lysine incorporation (Table 2) we also know that

$$\frac{\text{MHP-H2A}}{\text{LHP-H2A}} \text{ for } \underline{\text{P. boylii}} = 1.04 = \frac{MHP_B}{LHP_B}. \quad (4)$$

If the above assumptions (1,2) hold, we can predict the ratio MHP-H2A/LHP-H2A for P. eremicus, based on the data obtained for P. boylii, as follows:

Substituting (3) into (1)

$$\frac{CHC_E}{CHC_B} = 1.34 = \frac{MHP_E}{MHP_B}$$

so that

$$MHP_E = 1.34\ MHP_B.$$

Thus, the MHP-H2A/LHP-H2A ratio for P. eremicus is

$$\frac{MHP_E}{LHP_E} = \frac{1.34\ MHP_B}{LHP_E}$$

and, using (2)

$$\frac{MHP_E}{LHP_E} = \frac{1.34\ MHP_B}{LHP_B}$$

$$= (1.34)\ \frac{MHP_B}{LHP_B}\ .$$

Using (4)

$$\frac{MHP_E}{LHP_E} = (1.34)(1.04)$$

$$= 1.39 = \text{MHP-H2A/LHP-H2A predicted for } \underline{P.\ eremicus}$$

The actual measured value for the ratio of the more hydrophobic H2A subfraction to the less hydrophobic H2A subfraction in P. eremicus is 1.34 (Table 2), which is very close to the predicted value of 1.39. Similar analysis, using data from a second experiment where the MHP/LHP ratio for P. boylii was measured at 1.07, predicts that the MHP/LHP for P. eremicus should be 1.43. The actual measured value for P. eremicus in this second experiment is 1.37, which is, again, very close.

The close agreement of the experimental results with those predicted suggests that the initial assumptions (1,2) may be very good

ones. Therefore, the MHP-H2A subfraction may be associated in particular with chromatin condensation involved in constitutive heterochromatin structure. Thus, the high heterochromatin-containing cells contain more of the highly phosphorylated MHP-H2A subfraction than the low-heterochromatin-containing cells. This accounts for the original observation by Gurley et al. (1978c) that high heterochromatin-containing cells have more highly phosphorylated H2A histones than low heterochromatin-containing cells. These data suggest that constitutive heterochromatin may be enriched in the more phosphorylated, more hydrophobic H2A.

The above observations were based on comparisons of isotope incorporation into two subfractions of H2A within the same culture. Thus, the conclusions drawn from these observations are based on relative comparisons which are independent of intercultural variables such as precursor pools, cell cycle distribution of the population, etc. However, cell cycle analysis in these experiments illustrated the problem of comparing isotope incorporation between two different cultures. Although the doubling time of the two Peromyscus cultures was the same, the high heterochromatic cell culture contained twice as many cells in S and half as many cells in G_1 as did the low heterochromatic cell culture. The two cell lines also differed in that the $^{32}PO_4$ incorporation was higher into all the histones of P. eremicus than into those of P. boylii.

The different rates of phosphate incorporation into the histones of the two different cell lines cannot be attributed to differences in ATP pools, for analysis demonstrated that both cell lines had equivalent intracellular ATP levels. The different rates are more likely the

result of the differences in cell cycle distribution. For example, it has been shown that the phosphorylation of H1 is very sensitive to cell cycle position, being very low in early G_1 and greatly stimulated in S and G_2 (Gurley et al., 1973a, b; Gurley et al., 1974). Phosphorylation of the other histones is also increased as cells progress through the cell cycle. As a result of the high proportion of G_1 cells in exponential P. boylii cultures compared to P. eremicus cultures, the lower amounts of $^{32}PO_4$ incorporated into histones of P. boylii compared to P. eremicus appears to reflect more the cell cycle distribution differences between these two cell lines than the differences in their amount of heterochromatin.

In previous comparisons between the low heterochromatin-containing cells of P. crinitus and high heterochromatin-containing cells of P. eremicus, H2A phosphorylation in P. eremicus was observed to be elevated over that of P. crinitus, while H1 phosphorylation was found to be the same in both cell lines (Gurley et al., 1978c). In those experiments cell cycle analysis indicated the two cultures had more similar cell cycle distributions than did P. eremicus and P. boylii in the experiments reported here. Thus, those experiments (Gurley et al., 1978c) probably present a more accurate comparison for histone phosphorylation between cell lines than does that of P. eremicus and P. boylii. To reiterate, however, the comparison of isotope incorporation into the two subfractions of H2A in the same cultures presented in this report is independent of the above complications.

Comparisons of the [^{3}H]-lysine incorporation into the two H2A subfractions of a given cell line should be especially accurate since the labeling period is long and by comparison with other mammals, their

primary structures contain the same lysine content (Franklin and Zweidler, 1977; Blankstein et al., 1977). It was observed that the cell line with the greater heterochromatin content had a 29% greater MHP to LHP-H2A ratio than did the low heterochromatin-containing cell line, which exhibited essentially equimolar amounts of each. Redistributions of the ratios of H2A subfractions is a phenomenon that has been observed in other systems, also, and appears to be associated with differentiation, development, growth and aging. For example, (1) there are two subfractions of H2A in Friend erythroleukemia cells (Neumann et al., 1978; Blankstein and Levy 1976). When leukemic spleen tissue is placed in culture, the resulting Friend tumor cell line has a lower MHP to LHP-H2A ratio than its precursor tissue. With continued growth in culture the MHP to LHP-H2A ratio decreases further and the cells then become responsive to DMSO. Thus, there is an increase in the LHP subfraction during growth in culture which ultimately produces cells which are responsive to DMSO (Blankstein and Levy, 1976). It has been suggested that these H2A subfraction changes are prerequisite to chromatin structural changes which are then observed during DMSO induction of hemoglobin (Sato et al., 1971; Blankstein and Levy, 1976). (2) In other studies, it has been found in mammals that H2A ratios vary from tissue to tissue (Franklin and Zweidler, 1977), suggesting that the ratios of these various H2A's may be related to the differentiated state of the cells. (3) Comparative studies with mice (from embryos to 200 days old) have shown that the MHP to LHP-H2A ratio decreases with the changeover from a rapidly proliferating embryonic growth pattern to an adult limited growth pattern (Zweidler et al., 1978). An increase in the LHP-H2A subfraction was therefore associated with aging and/or

decreased cell proliferation. (4) Studies of the early development of the sea urchin have shown that as development and differentiation proceeds from fertilization to gastrula, different H2A subfractions are produced at various stages of embryogenesis (Cohen et al., 1975). This results in the gradual replacement of LHP-H2A's with MHP-H2A's.

I offer in this report another example of a difference in H2A subfraction ratios, but further, one which can be correlated with a visible difference in chromatin structure. This correlation suggests that such changes in H2A subfraction ratios may be associated with chromatin organization. Since it is thought that the processes of differentiation, development and growth may also be associated with chromatin organizational changes, the observations of this report, taken with those mentioned above, suggest a need to make direct measurements on chromatin structure in cell systems where changes in H2A subfraction ratios are occurring. In this way, changes in the nucleosomal components could be related to specific changes in higher orders of chromatin organization.

The observations presented here suggest that the increased amount of the more hydrophobic, more phosphorylated H2A subfraction can be correlated with an increased amount of constitutive heterochromatin. This observation opens several interesting questions. Are there specific domains of chromatin structure, such as euchromatin and heterochromatin, containing nucleosomes with different H2A molecules? Does the more hydrophobic nature of one H2A subfraction give it properties which assist heterochromatin aggregation? Does the phosphorylation of the MHP-H2A reenforce heterochromatin condensation? Are different H2A properties associated with only constitutive hetero-

chromatin, or with all genetically inactive chromatin, including facultative heterochromatin as well as inactive euchromatin?

In conclusion, the results of this work demonstrate that the increased phosphorylation of histone H2A found in Peromyscus cells containing excessive quantities of constitutive heterochromatin can be correlated with an enrichment of the chromatin with the more hydrophobic variant of H2A which is preferentially phosphorylated over the less hydrophobic variant. It is demonstrated that cell cycle analysis is required for the proper evaluation of histone phosphorylation data in order to include complications of cell cycle variations of histone phosphorylation in the interpretation of comparative experiments.

II.2. HISTONE ACETYLATION AND HETEROCHROMATIN CONTENT OF CULTURED *PEROMYSCUS* CELLS

II.2.1 Introduction

Histone acetylation, in particular the internal lysine acetylations of the arginine-rich histones (H3 and H4) and histone H2B, has often been correlated with the relative diffuseness and transcriptional activity of chromatin (Allfrey, 1971, 1973; Dixon *et al.*, 1975; Ruiz-Carrillo *et al.*, 1974), but the details of this association are still unknown. Recent work in a number of different cell systems has suggested that there may be an inverse relationship between amount of histone acetylation and extent of chromatin condensation. For example, during the maturation of avian erythrocytes, genetic inactivation of the nucleus and increased chromatin condensation is accompanied by a decrease in the rate and degree of H2B, H3 and H4 histone acetylation (Ruiz-Carrillo *et al.*, 1974, 1976; Sanders *et al.*, 1973). A comparison of the histones of sea urchin embryos and sea urchin sperm shows that the H3 and H4 histones associated with the highly condensed chromatin of sperm are unacetylated, while their homologous counterparts in embryonic chromatin are acetylated to a considerable extent (Wangh *et al.*, 1972; Ruiz-Carrillo and Palau, 1973). Similarly, the highly condensed nucleoprotamine of trout testis cells contains very little acetylated histone as compared to the multi-acetylated histones of the less condensed, putative transcriptionally active fraction of trout testis (Levy-Wilson *et al.*, 1979). A reduction in the degree of arginine-rich histone acetylation is also found in prophase and metaphase cells of Chinese hamster line CHO cells, when compared to interphase cells, indicating that highly condensed mitotic chromosomes contain more unacetylated histones (D'Anna *et al.*, 1977). Similar results have been

found in hepatoma tissue culture cells (Moore, et al., 1979). Another correlation between condensed chromatin and unacetylated histones is suggested by the finding that the condensed, facultatively heterochromatic paternal chromosome set of male mealy bugs is labeled considerably less by [^{3}H]acetyl Co-A than is the genetically active maternal chromosome set (Berlowitz and Pallotta, 1972).

Whether this correlation between unacetylated histones and condensed chromatin is primarily due to the genetically inactive state of genes in heterochromatin, or whether the tightly packed heterochromatin environment is simply inaccessible to histone acetylating enzymes is still unknown (Moore et al., 1979; Cousens et al., 1979). In order to further explore this relationship between unacetylated arginine-rich histones and condensed chromatin, I have compared histones from three species of Peromyscus. Further, I have hyperacetylated the histones of two of these species, through the use of sodium butyrate (Cousens et al., 1979; Riggs et al., 1977; Sealy and Chalkley, 1978) and found that in treated and untreated cells the amount of unacetylated H3 and H4 histone observed was proportional to the amount of constitutive heterochromatin present in the genome. The same relationship was found, to a lesser extent, for the acetylation of H2B. During the course of these studies I also found that the acetylated state of isolated histones was quite sensitive to the method of extraction. This sensitivity has not been previously noted in the literature and may be responsible for misleading conclusions if not taken into account during experimentation.

II.2.2. Materials and Methods

1. Cell Cultures. Cell cultures used in these experiments were from two sources. Ear fibroblast cultures of Peromyscus eremicus (strain 2352 ♀) and P. crinitus (strain 2350 ♀) were a generous gift of Dr. T. C. Hsu, Department of Biology, The University of Texas, M. D. Anderson Hospital and Tumor Institute, Houston, Texas. Ear fibroblast cultures of P. boylii were initiated in our laboratory from a female specimen which was trapped in Los Alamos County. Culture conditions were the same as described previously (II.1.2.1).

2. Growth Kinetics. Monitoring of the growth kinetics of the cultures was carried out using a series of small replica cultures as described previously (II.1.2.2).

3. Cell Harvest. Harvest of all three cell lines was carried out using saline G containing 0.05 M sodium bisulfite as previously described (II.1.2.5).

4. Histone Isolation. Histone fractions were extracted from approximately 1.5×10^8 cells by the first method of Johns (1964) as previously described for cultured cells by Gurley and Hardin (1968) with modifications described by Gurley et al. (1978c). In some experiments, nucleosomal core histones (H2A, H2B, H3 and H4) were extracted together after H1 was extracted in the above procedure. This was accomplished in the following manner. After treatment with 1.6 mℓ 5% $HClO_4$ containing 0.05 M sodium bisulfite to extract histone H1, the remaining cell pellet was treated with a total of 1.5 mℓ 0.4 N H_2SO_4 in three 0.5 mℓ extractions for 30 minutes each. The pooled supernatants (1.5 mℓ) were clarified by centrifugation and the core histones (H2A, H2B, H3 and H4) were precipitated overnight from this clear solution by adding 10

volumes of acetone. The resulting precipitate was recovered by centrifugation, washed two times with acetone, dissolved in water, and lyophilized to dryness.

5. Electrophoresis. The ethanol-extracted histones (H2A, H3 and H4) from the histone extraction method of Johns (1964), modified by Gurley and Hardin (1968), and the H_2SO_4-extracted core histones (H2A, H2B, H3 and H4) were separated analytically by electrophoresis on long (0.6 x 25 cm), 0.38% Triton DF-16, 6 M urea, 12% polyacrylamide gels. The gel formulation was the same as that developed by Alfageme et al. (1974). Gels were pre-electrophoresed for 20 hours at a constant current of 12 milliamps per 12 gels. Free radicals were scavenged from the gels by applying 0.1 mℓ per gel of 1 M β-Mercaptoethylamine-HCl to each gel and continuing a constant voltage of 210V per 12 gels for 40 minutes. Lyophilized histones were dissolved in sample buffer containing 4 M urea, 5% acetic acid and 4% β-Mercaptoethanol. Then 25-38 μg ethanol-extracted arginine-rich or H_2SO_4-extracted histone protein was applied to the gels. Electrophoresis was performed for 24 hours at a constant voltage of 210V per 12 gels. The gels were removed from the tubes, stained overnight with 0.2% Amido Black 10B in 30% methanol-9% Acetic acid, and then destained by diffusion for 30 hours. Profiles of the stained protein bands in the gels were obtained by measuring the absorbance at 630nm with a Gilford Model 240 spectrophotometer equipped with a linear transport attachment. The overlapping histone bands were resolved and quantified using a Dupont Model 320 Curve resolver (Ruiz-Carrillo, 1974; D'Anna et al., 1977; Panyim and Chalkley, 1969).

6. Hyperacetylation of Histones. Monolayer cultures of P. eremicus and P. boylii cells, seeded at an initial concentration of 6.5-9.5 x 10^6 cells per Blake bottle in 100 mℓ of growth medium were allowed to grow to 8 x 10^7 cells per Blake bottle. At this time the growth medium was made 15 mM with respect to sodium butyrate using a 1.0 M solution of sodium butyrate prepared by adjusting n-butyric acid (Sigma Chemical Co.) in physiological saline to pH 7.3 with NaOH (D'Anna et al., 1980). The cells were then allowed to grow for 24 hours, usually reaching a density of approximately 1.2 x 10^8 cells, at which time they were harvested as described above.

II.2.3. Results

I have compared acetylation of histones H2B, H3 and H4 in three cell lines, which have previously been shown to differ in their amount of constitutive heterochromatin (II.1.3). Results of acid-urea Triton DF-16 polyacrylamide gel electrophoresis of H_2SO_4-extracted core histones following removal of histone H1 are shown in Fig. 7. Quantitative analyses of the gel scans shown in Fig. 7 are shown in Tables 3, 4 and 5. Comparisons are made between the high heterochromatin-containing cell line Peromyscus eremicus and the low heterochromatin-containing line P. crinitus in Fig. 7A and Tables 3A, 4A, and 5A, and similar comparisons are made between P. eremicus and another low heterochromatin-containing cell line, P. boylii, in Fig. 7B and Tables 3B, 4B and 5B. The results (Tables 3, 4 and 5) are shown as percentages of the various acetylated species of histones H2B, H3 and H4, relative to the total amount of each of these histones.

It was found that the cell line with more heterochromatin (P. eremicus) always contained more unacetylated H3 and H4 histone than

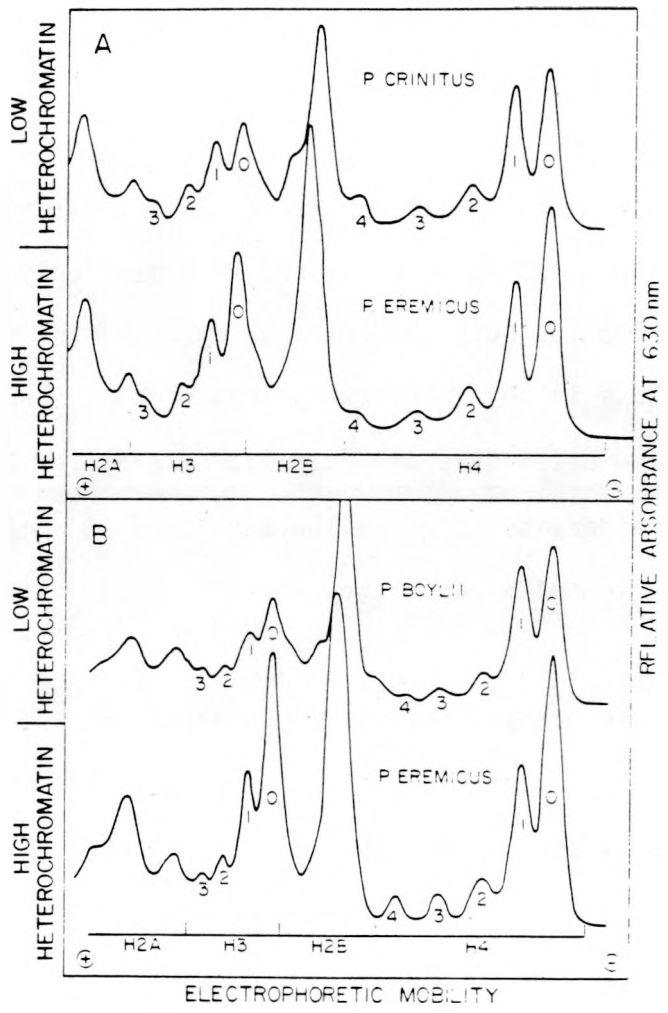

Fig. 7. Comparison of histone acetylation in low and high heterochromatin-containing cell lines. Spectrophotometer scans of 0.4N H_2SO_4-extracted histone distributions from (A) P. crinitus and P. eremicus cells and (B) P. boylii and P. eremicus cells following electrophoresis on acid-urea, DF-16, polyacrylamide gels. A and B represent two different gel runs, each made with histones from the high heterochromatin-containing cell line (P. eremicus) as a common sample for comparison.

TABLE 3

HISTONE H3 ACETYLATION RELATIVE TO HETEROCHROMATIN CONTENT

Electrophoresis Run	Cell Line	Number of Acetylations	Percent of Total H3	Relative Amount of Unacetylated H3	Relative Amount of Heterochromatin
A		0	39.2		
		1	36.7	1.00	1.00
	P. crinitus	2	16.9		
		3	7.2		
		0	47.7		
		1	27.7		
	P. eremicus	2	16.8	1.22	1.36
		3	7.1		
B		0	41.3		
		1	37.7	1.00	1.00
	P. boylii	2	13.8		
		3	7.2		
		0	53.3		
		1	25.0	1.29	1.34
	P. eremicus	2	17.8		
		3	3.7		

TABLE 4

HISTONE H4 ACETYLATION RELATIVE TO HETEROCHROMATIN CONTENT

Electrophoresis Run	Cell Line	Number of Acetylations	Percent of Total H4	Relative Amount of Unacetylated H4	Relative Amount of Heterochromatin
A		0	36.6		
		1	34.7		
	P. crinitus	2	17.9	1.00	1.00
		3	5.4		
		4	5.4		
		0	46.7		
		1	28.6		
	P. eremicus	2	14.8	1.28	1.36
		3	6.0		
		4	4.0		
B		0	37.1		
		1	34.2	1.00	1.00
	P. boylii	2	15.2		
		3	8.9		
		4	5.1		
		0	50.0		
		1	25.0	1.35	1.34
	P. eremicus	2	11.6		
		3	7.6		
		4	5.8		

TABLE 5

HISTONE H2B ACEYTLATION RELATIVE TO HETEROCHROMATIN CONTENT

Electrophoresis Run	Cell Line	Number of Acetylations	Percent of Total H2B	Relative Amount of Unacetylated H2B	Relative Amount of Heterochromatin
A	P. crinitus	0	68.8	1.00	1.00
		1	20.5		
		2	10.7		
	P. eremicus	0	81.2	1.18	1.36
		1	10.3		
		2	8.5		
B	P. boylii	0	64.8	1.00	1.00
		1	20.0		
		2	15.2		
	P. eremicus	0	79.0	1.22	1.34
		1	11.4		
		2	9.5		

either of the other two low heterochromatin cell lines. Peromyscus eremicus cells, which contain 36% more DNA as heterochromatin than P. crinitus cells (Gurley et al., 1978c), were found to contain an 18% greater proportion of unacetylated H2B, 22% more unacetylated H3, and 28% more unacetylated H4 than P. crinitus cells (Fig. 7A, Tables 3A, 4A and 5A). In a further comparison, P. eremicus cells, containing 34.2% more DNA as heterochromatin than P. boylii cells (see II.1.3), were found to contain 22% more unacetylated H2B, 29% more unacetylated H3, and 35% more unacetylated H4 than P. boylii cells (Fig. 7B, Tables 3B, 4B and 5B). Conversely, P. crinitus cells and P. boylii cells exhibit higher percentages of acetylated species of H2B, H3 and H4, having 75-99% more once acetylated H2B, 33-50% more once acetylated H3, 21 to 37% more once acetylated H4, and 21-31% more twice acetylated H4 than P. eremicus cells (Tables 3, 4 and 5).

This correlation between the proportion of unacetylated arginine-rich histones and relative constitutive heterochromatin content in these cell lines can be demonstrated in another way. If P. crinitus and P. boylii are theoretically increased in their heterochromatin content by amounts similar to that of the extra DNA in P. eremicus, and if all of the histone H4 in the added heterochromatin is assumed to be unacetylated, recalculation of the relative amounts of the various acetylated species for the two "theoretically converted" low heterochromatin cell lines results in percentages similar to those measured for P. eremicus (Table 6). Thus, an increase in constitutive heterochromatin content can be shown to be correlated with an increase in the proportion of unacetylated arginine-rich histone in Peromyscus cell lines.

TABLE 6

COMPARISON OF HISTONE H4 ACETYLATION FOLLOWING THEORETICAL CONVERSION OF LOW HETEROCHROMATIN CELLS TO HIGH HETEROCHROMATIN CELLS

Number of Acetylations	Percent of Total H4	
	P. eremicus	*P. crinitus* + 36% more DNA*
0	46.7	53.4
1	28.6	25.5
2	14.8	13.1
3	6.0	3.9
4	4.0	3.9

	P. eremicus	*P. boylii* + 34% more DNA
0	50.0	53.2
1	25.0	25.5
2	11.6	11.3
3	7.6	6.6
4	5.8	3.8

*This theoretical conversion was performed as follows: 36% more unacetylated H4 ($H4_0$) was added to the experimentally obtained proportion of $H4_0$ for *P. crinitus*. The relative percentages of all acetylated species of H4 ($H4_0$-$H4_4$) were then recalculated on the basis of a 136% total. The theoretical conversion of *P. boylii* was carried out in the same manner.

In order to further analyze the relationship between heterochromatin and unacetylated arginine-rich histones, the high heterochromatin cell line (P. eremicus) and a low heterochromatin cell line (P. boylii) were treated with 15 mM sodium butyrate for 24 hours. The selection of this concentration of sodium butyrate was based on results with other cell lines (Cousens et al., 1979; D'Anna et al., 1980) which indicated that acetylation of H4 histone reaches a constant maximum value at concentrations of sodium butyrate above 15 mM. It was found, as expected, that treatment of both cell lines, P. eremicus and P. boylii, resulted in a large increase in acetylated species of histones, in particular, those of histones H3 and H4 and, to a lesser extent, histone H2B (Figs. 8 and 9). Electrophoretic data is presented for histones extracted by two methods, ethanol extraction (Johns 1964; Gurley and Hardin, 1968) in Figs. 8A and 9A, and 0.4 N H_2SO_4 extraction in Figs. 8B and 9B. Since histone H2B also becomes hyperacetylated during sodium butyrate treatment (D'Anna et al., 1980) (see also Figs. 8B and 9B), removal of this histone through the use of ethanol proved to be a useful method to clear the H3 region of acetylated forms of H2B when examining arginine-rich histones from butyrate-treated cells (Figs. 8A and 9A). However, as will be discussed below, since ethanol extraction appears to cause deacetylation of arginine-rich histones during preparation, the data analysis discussed here is for the 0.4 N H_2SO_4 extracted histones shown in Figs. 8B and 9B. Quantitation of the gel scans shown in Figs. 8B and 9B appears in Table 7. It was found that in each cell line following treatment there was always a small amount of both histone H3 and H4 that was not acetylated, even at this high concentration of sodium butyrate. Considerably larger percentages

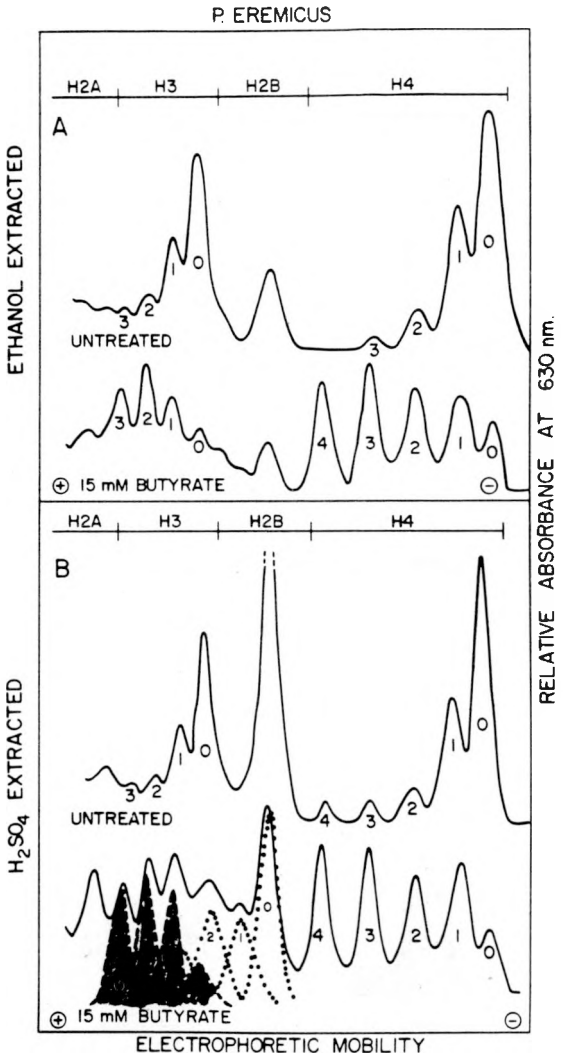

Fig. 8. Histone acetylation in the high heterochromatin-containing cell line, P. eremicus. Spectrophotometer scans of electrophoretograms of (A) ethanol-extracted and (B) 0.4 N H_2SO_4-extracted histones from untreated and 15 mM sodium butyrate treated P. eremicus cells. Dotted lines represent curves generated by a curve resolver. Shaded areas emphasize the curve resolved peaks for histone H3. Four modified species of histone H2B were resolved in butyrate treated cells based on the results of D'Anna et al. (1980). Three modified species of histone H3 were resolved. Note that only trace amounts of histone H2B are extracted with ethanol (A), but all the histone H2B is extracted using H_2SO_4 (B).

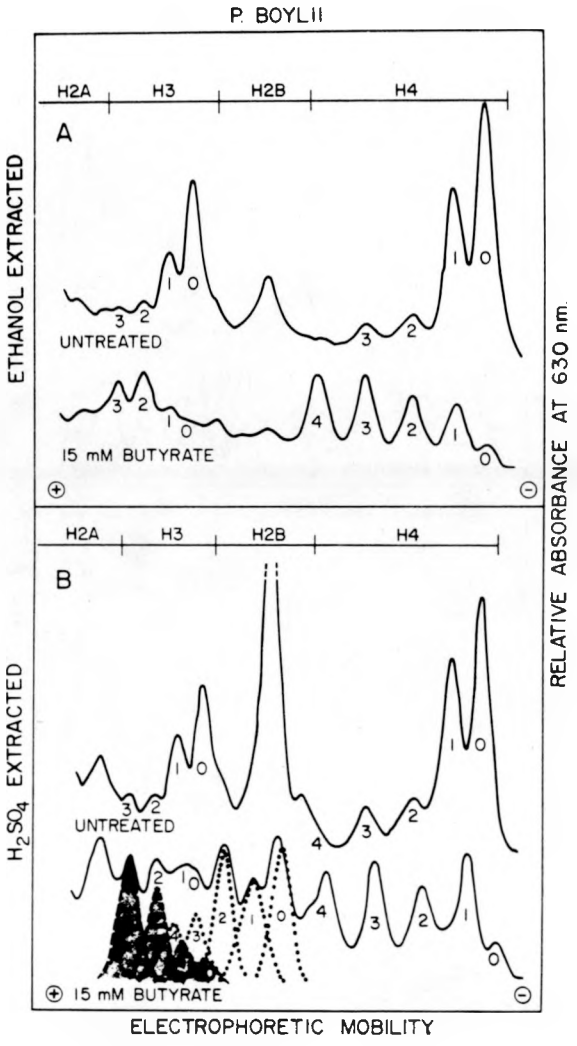

Fig. 9. Histone acetylation in the low heterochromatin-containing cell line P. boylii. Spectrophotometer scans of electrophoretograms of (A) ethanol-extracted and (B) 0.4 N H_2SO_4-extracted histones from untreated and 15 mM sodium butyrate treated P. boylii cells. Dotted lines represent curves generated by a curve resolver. Shaded areas emphasize the curves resolved for histone H3. Four modified species of histone H2B were resolved in butyrate treated cells based on the results of D'Anna et al. (1980). Three modified species of histone H3 were resolved. Note that only trace amounts of histone H2B are extracted with ethanol (A), but all the histone H2B is extracted using H_2SO_4 (B).

TABLE 7

BUTYRATE INDUCED HYPERACETYLATION OF H_2SO_4-EXTRACTED HISTONES

Cell Line	Number of Acetylations	Percent of Total H4 Untreated	Percent of Total H4 15 mM Butyrate
P. boylii	0	37.1	3.7
	1	34.2	25.7
	2	15.2	21.8
	3	8.9	23.1
	4	5.1	25.7
P. eremicus	0	50.0	9.8
	1	25.0	22.4
	2	11.6	20.9
	3	7.6	24.3
	4	5.8	22.6
		Percent of Total H3	
P. boylii	0	41.7	7.3
	1	28.1	11.7
	2	13.5	33.5
	3	16.7	47.5
P. eremicus	0	50.0	12.2
	1	26.1	26.2
	2	14.1	32.7
	3	9.9	28.8
		Percent of Total H2B	
P. boylii	0	64.8	24.0
	1	20.0	25.8
	2	15.2	23.1
	3	--	13.5
	4	--	13.5
P. eremicus	0	79.0	36.7
	1	11.4	21.1
	2	9.5	27.5
	3	--	10.1
	4	--	4.9

of unacetylated H2B remained after butyrate treatment, however. Further, in direct correlation with the results from untreated cells, following butyrate treatment there was more unacetylated H2B, H3 and H4 remaining in the high-heterochromatin cell line (P. eremicus) than in the low-heterochromatin cell line (P. boylii) (Figs. 8B and 9B). Quantitation of these results showed that there was 9.8% unacetylated H4, 12.2% unacetylated H3, and 36.7% unacetylated H2B remaining in the high-heterochromatin line, as opposed to only 3.7% unacetylated H4, 7.3% unacetylated H3, and 24.0% unacetylated H2B left in the low-heterochromatin line (Table 7).

Since the percentage of unacetylated arginine-rich histone in cells not treated with butyrate was found to be correlated with the relative constitutive heterochromatin content of these cells, is there a similar relationship demonstrated in butyrate-treated cells? Yes, in fact the percentages of unacetylated arginine-rich histones remaining following butyrate treatment can be correlated with the amount of constitutive heterochromatin present in the genomes of these mice. Arm-length measurements on C-banded chromosome preparations of untreated cells of the high-heterochromatin species (P. eremicus)(similar to those of Fig. 1A) indicate that constitutive heterochromatin represents about 35% of its genome. If we now set up a proportion between the amount of unacetylated H4 remaining after butyrate treatment and the percentage of constitutive heterochromatin in the genome of untreated cells, we can predict the percentage contribution that constitutive heterochromatin makes to the genome of P. boylii.

$$\frac{(H4_o)_E}{CHC_E} = \frac{(H4_o)_B}{CHC_B}$$

where: $H4_o$ = % unacetylated H4
CHC = % constitutive heterochromatin
E = P. eremicus
B = P. boylii

therefore: $\frac{9.8}{35} = \frac{3.7}{CHC_B}$

and: $CHC_B = 13\%$

Thus, the results of hyperacetylation of histone H4 in these cell lines predict that the low heterochromatin cell line should contain about 13% constitutive heterochromatin. A similar calculation can be made using data for the percentages of unacetylated H3 remaining following butyrate treatment.

$$\frac{(H3_o)_E}{CHC_E} = \frac{(H3_o)_B}{CHC_B}$$

$$\frac{12.2}{35} = \frac{7.3}{CHC_B}$$

and: $CHC_B = 18\%$

Thus, the results of hyperacetylation of histone H3 in these cell lines predict that P. boylii cells should contain about 18% constitutive heterochromatin. Arm length measurements on C-banded preparations of untreated P. boylii cells (similar to Fig. 1B) indicate that constitutive heterochromatin represents about 15% of its genome. The similarity between the results of arm length measurements (15%) and those predicted from hyperacetylation of the histones (13% and 18%) suggests that the proportion of unacetylated arginine-rich histone remaining following butyrate treatment can be correlated with the amount of constitutive heterochromatin in the genome.

A similar calculation, using the proportion of unacetylated H2B remaining following butyrate treatment, predicts a 23% constitutive heterochromatin content for P. boylii. This is a rather high estimate (compared to 15% from arm-length measurements) suggesting that the acetylation of histone H2B may not be related in the same way as arginine-rich histone acetylation is to constitutive heterochromatin content.

As mentioned above, these results also indicate that measurements of arginine-rich histone acetylation are sensitive to the method of extraction. Histones H2A, H3 and H4 extracted using ethanol (Johns, 1964; Gurley and Hardin, 1968) exhibited proportionately more of the unacetylated H3 and H4 species in all cell lines, when compared to those extracted by 0.4 N H_2SO_4 (Fig. 10, Table 8). Also, H4 in the ethanol-extracted histones contained fewer acetates per molecule than H4 in the H_2SO_4-extracted histones (Fig. 10, Table 8). In fact, no tetra-acetylated H4 histones are found in the ethanol extracts (Fig 10, solid lines), whereas they are present in the histones extracted using 0.4 N H_2SO_4 (Fig. 10, dashed lines). This comparison is especially striking with one of the low heterochromatin cell lines, P. crinitus (Fig. 10A). It can be seen that ethanol-extraction results in a relatively greater amount of unacetylated H3 and H4 than does H_2SO_4 extraction (Fig. 10A). This increase in the amount of unacetylated H4 in ethanol extraction is to such an extent that if ethanol-extracted histones from high and low heterochromatin cell lines are compared, P. eremicus (Fig. 10B, solid line) contains only 6% more unacetylated H4 than P. crinitus (Fig. 10A, solid line). These results are substantially different from the previously calculated 28% more unacetylated H4 in P. eremicus found when

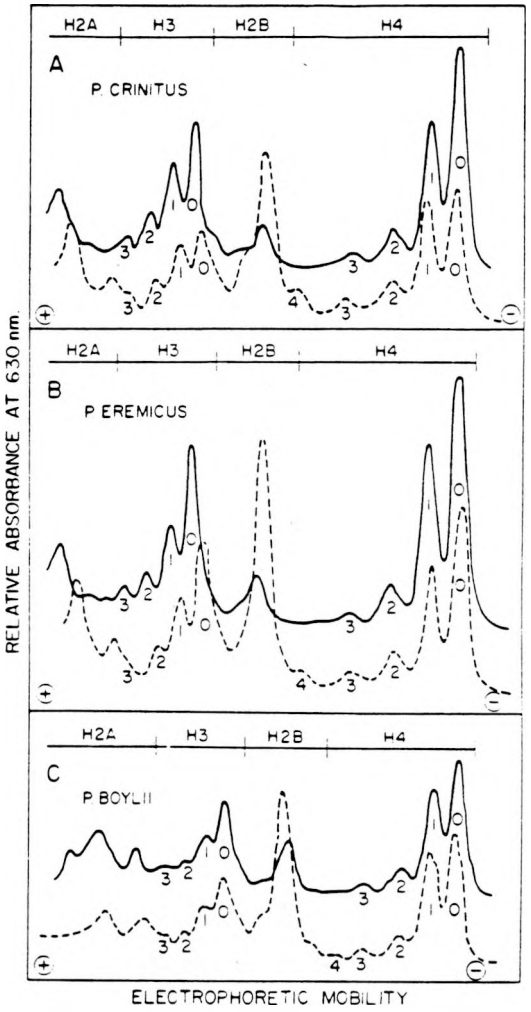

Fig. 10. Comparison of histone extraction methods. Comparison of histone extraction using ethanol (Johns, 1964, Gurley & Hardin 1968) [———] and by the use of 0.4 N H_2SO_4 [----]. Spectrophotometer scans of acid urea DF-16 polyacrylamide gels resulting from electrophoresis of from untreated (A) P. crinitus (B) P. eremicus and (c) P. boylii cells.

TABLE 8

COMPARISON OF HISTONE EXTRACTION METHODS

Number of Acetylations	Percent of Total H4 Histone		Acetates per 100 H4 Molecules		Percent Deacetylation
	Ethanol	H_2SO_4	Ethanol	H_2SO_4	
			A. P. crinitus		
0	48.9	36.6	0	0	
1	31.9	34.7	31.9	34.7	
2	12.2	18.9	24.2	35.8	
3	6.9	5.4	20.7	16.2	
4	0	5.4	0	21.6	
		Total =	76.8	108.3	41
			B. P. eremicus		
0	51.9	46.7	0	0	
1	34.8	28.6	34.8	28.6	
2	10.7	14.8	21.4	29.6	
3	2.7	6.0	8.1	18.0	
4	0	4.0	0	16.0	
		Total =	64.3	92.2	43
			C. P. boylii		
0	43.0	37.1	0	0	
1	31.5	34.2	31.5	34.2	
2	14.4	15.2	28.8	30.4	
3	11.0	8.9	33.0	26.7	
4	0	5.1	0	20.4	
		Total =	93.3	111.7	20

H_2SO_4-extracted histones from these cells are compared (Fig. 10A and B, dashed lines, Table 4A). Thus, the correlation between unacetylated arginine-rich histones and heterochromatin content would not have been observed if only ethanol-extracted histones were used in these experiments. This comparison between ethanol and H_2SO_4 extraction can also be expressed on the basis of the number of acetate groups obtained per 100 H4 molecules extracted. These results are shown in Table 8, where it can be seen that based upon the total number of acetate groups, deacetylation varies from 20-43% during ethanol extraction when compared to extraction in H_2SO_4. The range of this deacetylation over a number of experiments (not all shown in Table 8) was from 20%-64%, which would indicate that deacetylation tends to be variable, depending upon extraction conditions.

II.2.4. Discussion

Previous work (D'Anna et al., 1977; Moore, et al., 1979) has shown that histone acetylation is independent of interphase cell cycle position. Thus, any cell cycle distribution differences between P. eremicus, P. crinitus and P boylii cultures will have little effect on the histone acetylation measurements reported here. This independence of histone acetylation from cell cycle position in interphase also indicates that the hyperacetylation which occurs following butyrate treatment is not the result of cells arresting in G_1.

Evidence is presented in this work that the acetylated state of isolated histones is sensitive to the method of extraction. Clearly, the finding that ethanol extraction of histones H2A, H3 and H4 (Johns, 1964; Gurley and Hardin, 1968) results in about a 40% deacetylation during preparation as compared to extraction of core histones (H2A, H2B,

H3 and H4) with 0.4 N H_2SO_4 indicates that the advantages gained by the separation of histones must be carefully weighed against the possibility of acetate losses during preparation. For this reason, measurements correlating histone acetylation with heterochromatin content were made exclusively on H_2SO_4-extracted histones from the Peromyscus cell lines.

The main objective of this work was to determine if a relationship exists between arginine rich histone acetylation and condensed chromatin. The cell lines from Peromyscus offer a unique system in which to test this hypothesis because they contain essentially the same quantity and G-band pattern of genetically active euchromatin, but contain grossly different quantities of genetically inactive, C-bandable, constitutive heterochromatin (see II.1.3, Gurley et al., 1978c; Committee, 1977). The work presented here demonstrates a clear correlation between amount of unacetylated arginine-rich histone and constitutive heterochromatin content in cell lines from different Peromyscus species. This difference in unacetylated arginine-rich histone content was demonstrable when the high heterochromatin cell line was compared to cell lines from two different low heterochromatin species. Similar differences in some unacetylated arginine-rich histones and heterochromatin content were found by Holmgren et al. (1976) in a comparison of histones from Drosophila species. Although they found no correlation with histone H4 acetylation, they reported that the proportion of acetylated H3 molecules was significantly lower in D. virilis, which contains more heterochromatin and rapidly reassociating DNA than either D. melanogaster or D. hydei. The differences in unacetylated arginine-rich histones were clearly shown in both H3 and H4 histone in the Peromyscus system, however. This failure

to show any correlation between histone H4 acetylation and heterochromatin content in the Drosophila species may have been due to the method of histone extraction used by these workers. Histones used in their experiments were extracted using HCl, which may cause either differential extraction of these histones, or allow deacetylase activity during preparation. These results emphasize the need to clearly establish the effect the various methods of histone extraction have on the acetylated state of isolated histones, such as was presented here.

The concepts that lack of acetylation of some arginine-rich histones may reflect their occurrence in heterochromatin, or that the condensed heterochromatin environment may not be favorable for the acetylation of histones are not new ones. It has been proposed (Ruiz-Carrillo et al., 1974, 1976; Ruiz-Carrillo and Palau, 1973; Sanders et al., 1973; Levy-Wilson et al., 1979), that histone acetylation is somehow related to RNA synthetic activity in cells. If we assume that only 5-20% of the genome in mammalian cells is transcribed (Pederson, 1978), the results presented here, that 50-60% of arginine-rich histones are normally acetylated in these cells, would support the idea that a much larger proportion of histones is involved in acetylation and deacetylation than would be necessary if this process were involved only in the activation of genes (Moore, et al., 1979). Thus, it seems more likely that histone acetylation may play a structural role in chromatin. I found with the Peromyscus lines that no matter how far acetylation is pushed in these cells through hyperacetylation with sodium butyrate, there is always a small amount of arginine-rich histone and a larger amount of histone H2B that cannot be

acetylated. During the course of these experiments, Cousens *et al.* (1979) reported similar results with cultured rat hepatoma cells. Thus, there must be a portion of histones in chromatin that is inaccessible to acetylation, perhaps because it is located in condensed heterochromatin. It has been demonstrated that the cell line with 34-36% more constitutive heterochromatin contains a similar excess of unacetylated arginine-rich histones (28-35% for H4, 22-29% for H3), when compared to low heterochromatin cell lines. It has also been demonstrated that if one hyperacetylates the histones of both high and low heterochromatin cell lines through treatment of the cells with sodium butyrate, there is always a small portion of arginine-rich histone that remains unacetylated. Further, this portion is present in an amount proportional to the amount of condensed constitutive heterochromatin in the genome. It should be noted, however, that following sodium butyrate treatment the percentage of unacetylated arginine-rich histone remaining is really quite small, compared to untreated cells. Further, concerning these percentages remaining after butyrate-treatment; although they are proportional to, they are much less than the percentage of constitutive heterochromatin in these cell lines. This suggests that a significant portion of arginine-rich histone in constitutive heterochromatin (70-90%) is, apparently, *available* to be acetylated under hyper-acetylating conditions, to at least the monoacetylated state. These results raise the question whether an increase in acetylated arginine-rich histones during sodium butyrate hyperacetylation also decreases the amount of C-bandable material in these cells. Work is in progress to further elucidate this relationship.

Observations on the acetylation of histone H2B deserve further comment. The work presented here on histone H2B acetylation represents one of the few investigations involving modification of this histone and condensed chromatin. A decrease in H2B acetylation was noted in the development of avian erythrocytes leading to mature cells with condensed, inactive chromatin (Ruiz-Carrillo *et al.*, 1976; Sanders *et al.*, 1973). In the *Peromyscus* system, although a greater percentage of unacetylated histone H2B can be demonstrated in the high heterochromatin cell line when compared to two low heterochromatin cell lines, these differences are *not* strictly proportional to the differences in heterochromatin content observed in these cell lines. Further, the extent of histone H2B acetylation is not as great as that of the arginine-rich histones (H3 and H4) in *Peromyscus* cells. Even when hyperacetylated using butyrate, a significantly large proportion (24-26.7%) of histone H2B remains unacetylated. These results are consistent with the idea that histone H2B may be even more inaccessible to histone acetylating enzymes than H3 and H4, perhaps by being buried deeper within the nucleosome core.

Part III. FRACTIONATION OF *P. EREMICUS* CHROMATIN

III.1. Introduction

The demonstration that there are morphologically, biochemically, and genetically distinct regions of chromatin in the interphase nucleus has led recently to the development of procedures for fractionating interphase chromatin. Since cytological and biochemical evidence indicates that genetic activity is associated with extended, euchromatic regions of chromatin and not with condensed, heterochromatic chromatin masses, various attempts have been made to physically separate these chromatin types and to biochemically characterize them. The idea of chromatin fractionation was first described by Frenster *et al*. (1963), who fragmented lymphocyte chromatin and separated two fractions which, by ultrastructural and biochemical criteria appeared to be hetero-chromatin and euchromatin. Since that time a plethora of such fractionation schemes has appeared in the literature. The basic approach offered by all of these schemes is to first physically break chromatin, based on the differential resistance of hetero- and euchromatin, using shear or nuclease attack. The fractions are then separated, using some physical or biochemical property (density, size, ionic properties of associated proteins, DNA base content) associated with these fractions. Once separation is achieved, proof of the fractionation is generally demonstrated by ultrastructural and biochemical criteria. Methods vary with the source of the chromatin and whether the focus of interest is on structual, genetic, or biochemical aspects of the fractionated chromatin.

Current methods for the physical breaking of chromatin vary from mechanical disruption of nuclei or chromatin to enzymatic digestion of

isolated chromatin. Chromatin has been broken by sonication (Frenster et al., 1963; Tata and Baker, 1974), motor-driven homogenization (Rodriguez and Becker, 1976; Duerkson and McCarthy, 1971) pressure (Yasmineh and Yunis, 1974; Murphy et al., 1973), gentle, hand-driven glass-on-glass homogenization (Chance et al., 1974), and limited enzymatic digestion (Paul and Duerksen, 1976; Marushige and Bonner, 1971). Separation of the fractions has been achieved by methods as simple as differential centrifugation (Frenster et al., 1963; Comings and Harris, 1975) or by sedimentation through sucrose (Chalkley and Jensen, 1968) or glycerol gradients (Murphy et al., 1973). Ion exchange chromatography (Reeck et al., 1972), hydroxylapatite thermal chromatography (McConaughy and McCarthy, 1972) and differential solubility in either saline (Marushige and Bonner, 1971) or divalent cations (Gottesfeld et al., 1974) have also been used to separate fractions. Identification of the obtained fractions as heterochromatin or euchromatin, or as active or inactive chromatin, is usually based on such criteria as DNA base composition and repetitiveness, nascent RNA content, template activity, and endogenous RNA polymerase association.

Before choosing a chromatin fractionation scheme for these experiments many possible choices were considered and a variety of methods were examined before using the method presented here. As mentioned above, methods for fractionating chromatin have been developed basically with the idea of separating transcriptionally active and inactive chromatin components. These approaches have had two rationales; separation of chromatin into two distinct morphological types--condensed heterochromatin and extended euchromatin, or separation based on the unique physical and biochemical properties of transcrip-

tionally active chromatin (Pederson, 1978). These distinctions are important, since, as was shown previously (I.2 and I.3), just as there has been confusion surrounding the definition of heterochromatin, the definition of euchromatin has also been a problem. The concept that there is a strict correlation between morphological chromatin structure (heterochromatin and euchromatin) and gene transcription is now generally considered to be faulty. The observation that the proportion of transcribed DNA in chromatin is far less than the amount of euchromatin in most mammalian cells (Pederson, 1978) suggests that the majority of euchromatin DNA, like heterochromatin, is genetically inert. Thus fractionation schemes need to be judged on the basis of the purity of the desired end product. The main objective in fractionating Peromyscus eremicus chromatin, which has been shown to contain large amounts of constitutive heterochromatin, was to obtain a relatively pure fraction of this constitutive heterochromatin in order to then study its histone modifications versus those of total (bulk) chromatin. For this reason, an attempt was made to judge fractionation schemes on the relative merits of separating highly condensed chromatin from bulk chromatin, without much concern for the genetic activity of the euchromatin fraction. Many of the fractionation schemes mentioned above were developed with interest in the active fraction of chromatin, thus the procedures developed here were somewhat different.

Because fractionation of unperturbed native chromatin was a prime consideration, nuclei were used as a starting material, rather than isolated chromatin. It was considered important that relatively mild conditions be used for the preparation of these nuclei and that protection from phosphatase and deacetylase activity be provided by

inhibitors as well. Some of my early fractionation attempts employed the use of nuclease digestion by DNAase II to fragment chromatin, and differential solubility in magnesium ion to fractionate chromatin (Gottesfeld et al., 1974). This method was abandoned, however, when excessive degradation of the histones was encountered. I also employed sonication to fragment chromatin. This method was eventually abandoned due to lack of reproducibility caused by excessive wear of the sonicator microtip. Attempts were also made to fragment chromatin using a glass-to-glass tissue homogenizer to avoid excessive shear (Anderson et al., 1975). This method was also abandoned when it, too, proved to be unreliable due to a lack of reproducibility. Mechanical disruption of nuclei using a speed controlled Virtis homogenizer eventually became the method of choice to fragment chromatin.

In early experiments separation of the fractions was accomplished by differential centrifugation as a result of the success of Frenster et al. (1963) and Comings and Harris (1975). Although successful in some ways, this method was abandoned, due to suspected cross-contamination of the fractions. It was finally established that sedimentation through steep sucrose gradients provided the most reproducible method to separate chromatin fractions.

Following establishment of a relatively reproducible method of chromatin fractionation (homogenization with a Virtis homogenizer and sedimentation through sucrose gradients), the fractions were examined for their content of heterochromatin and euchromatin, based on various biochemical criteria. The nature of the histone modifications in these chromatin fractions was then examined to determine if histone phosphorylation, acetylation, and H2A variation did indeed correlate with

the hetero and euchromatin fractions, as suggested by the experiments presented in II.1 and II.2 of this work.

III.2. Materials and Methods

1. Cell Culture. Cell cultures used in these experiments were exclusively ear fibroblast cultures of Peromyscus eremicus (strain 2352 ♀), described previously (II.1.2.1, II.2.2.1).

2. Growth Kinetics. Monitoring of the growth kinetics of the cultures was carried out using small replica cultures as described previously (II.1.2.3).

3. Isotope Incorporation for Labeling of DNA in Chromatin Fractions. Cells to be isolated for chromatin fractionation were seeded in 4-8 Blake bottles, each at an initial density of 5-7 x 10^6 cells in 100 mℓ of growth medium containing 0.002 μCi/mℓ methyl [^{14}C]-thymidine. After incorporation of [^{14}C]-thymidine during growth for approximately 48 hours, the cells were harvested at approximately 3.5 x 10^7 cells per bottle.

4. Isotope Incorporation for Labeling of Histones. P. eremicus cells to be used for the isolation of histones from chromatin fractions were labeled with [^{3}H]-lysine and $H_3{}^{32}PO_4$ in the same manner previously described for the study of histones from whole cells of Peromyscus (II.1.2.4).

5. Isotope Incorporation for Labeling of Nascent RNA. Cells to be used for the labeling of nascent RNA in chromatin fractions were treated for 30 min with 0.04 μg/mℓ Actinomycin D to inhibit ribosomal RNA synthesis (Perry and Kelley, 1970). The cells were then pulsed for 30 sec with 10 μCi/mℓ [5-^{3}H]-Uridine (28.5 Ci/mMole) prior to harvest

and chromatin fractionation. All solutions and glassware used for subsequent manipulations were heat treated to destroy RNase activity.

6. Isotope Incorporation for Labeling of Late Replicating DNA. P. eremicus cells to be used for the isolation of late-replicating DNA were obtained by a method which selects for mitotic cells containing portions of chromosomes radioactively labeled during the very last portion of the previous S phase. This is accomplished by a short pulse-label of [^{3}H]-thymidine which labels cells in S, followed by incubation in fresh medium for the length of G_2 phase (2 hr). Since this incubation includes one hour in Colcemid, the cells become blocked in mitosis. These loosely attached mitotics are shaken off, released from Colcemid and allowed to pass into G_1 phase before harvesting. Labeled mitotics so obtained could only have come from very late S phase.

The cells to be used for the labeling of late replicating DNA in chromatin fractions were grown in 15 Blake bottles, each containing 100 mℓ growth medium. Cells were seeded at an initial density of 6.2 x 10^6 cells per bottle and 12 of these cultures were labeled from the time of initiation with 0.002 μCi/mℓ methyl [^{14}C]-thymidine. The cells were allowed to grow until they reached a density of 3.7 x 10^7 cells per bottle. At this time the volume of the growth medium in the 12 bottles was reduced to 17 mℓ and the cells were pulse-labeled with 10 μCi/mℓ [^{3}H]-thymidine (60 Ci/mMole) for 15 minutes. The cells were rinsed twice with fresh medium and incubated in 17 mℓ regular growth medium for 1 hour. The 12 cultures were then vigorously shaken for 10 seconds by mechanical agitation and loosely attached cells were then discarded by decantation (Tobey et al., 1967). Then 25 mℓ fresh medium containing

0.04 μg/mℓ Colcemid were added and the cells were incubated for another hour. Mitotic cells were then gently shaken off by mechanical agitation for 3 sec, reseeded in 3 Blake bottles and incubated for 2 hours. These cells (approximately 9.1×10^6 in number) were then harvested as previously described and mixed with a total of 1.4×10^8 unlabeled carrier cells harvested from the remaining 3 Blake bottles from the original 15. A total of 1.5×10^8 cells resulted from this procedure which were then subjected to nuclear preparation and chromatin fractionation, to be described.

In order to be certain that only mitotic cells, and no labeled interphase cells were harvested by this method using mechanical agitation, P. eremicus cells obtained from the shake-off, before release, were monitored by autoradiography. Cells obtained from the mitotic shake were mixed with unlabeled carrier Chinese hamster line CHO cells in order to provide enough cellular material during the procedure, fixed in 3:1 methanol-acetic acid and mounted on slides as described by Deaven and Petersen (1974). Slides were dipped in Kodak NTB emulsion, exposed for 4 days at room temperature, developed using Kodak D19 developer and stained with Giemsa stain. These autoradiographs were useful only to determine whether any contaminating interphase cells of P. eremicus were shaken off during the procedure which uses mechanical agitation. Due to the presence of the 48 hour [^{14}C]-thymidine bulk label, as well as the [^{3}H]-thymidine pulse label, we would expect all P. eremicus cells to be labeled in these autoradiographs. Since only mitotic P. eremicus cells should have been harvested by this procedure, and since the carrier CHO cells were unlabeled, any labeled interphase cell in this autoradiographs represents a possible contamination of

non-late S phase labeled chromatin. Autoradiographs obtained in this way were used only to monitor this contamination. However, due to the presence of both [^{14}C]-thymidine and [^{3}H]-thymidine in these preparations, late labeling patterns could not be analyzed from these autoradiographs. Thus, a separate experiment was carried out where four unlabeled P. eremicus cultures in exponential growth were labeled with 10 μCi/mℓ [^{3}H]-thymidine for 1, 2, 3, and 4 hours, respectively. Each culture was treated for one hour with 0.06 μg/mℓ Colcemid, harvested by trypsinization and prepared for autoradiography as described above. Late-labeling patterns of P. eremicus were analyzed from these autoradiographs.

7. Cell harvest. Because chromatin fractions prepared from these cells were to be analyzed for their extent of histone phosphorylation it was necessary to harvest these cells in the presence of phosphatase inhibitors. Due to the difficulty in later preparing nuclei from cells harvested using the phosphatase inhibitor sodium bisulfite, an excess of phosphate ion was used to inhibit the phosphatase reaction by overloading it with its product. Thus, harvest of P. eremicus cells to be used to prepare nuclei was modified from the method described earlier (II.1.2.5) in the following manner. The growth medium on each Blake bottle to be harvested for chromatin fraction was poured off and replaced with 25 mℓ ice cold Saline G (Puck et al., 1958) containing 100 mM potassium phosphate at pH 7.0. The cells were harvested by scraping with a rubber policeman. The cells were then decanted and each bottle was rinsed with 50 mℓ saline G containing 100 mM potassium phosphate pH 7.0. The resulting cell suspension was made 20% with

respect to calf serum (Gurley et al., 1978) and centrifuged to recover the cells.

8. Preparation of nuclei. Nuclei were prepared by a method modified from that of Penman (1966). Between 1.25 x 10^8 and 1.5 x 10^8 cells to be used for the preparation of nuclei were transferred in 10 mℓ Saline G + 100 mM potassium phosphate to 40 mℓ glass centrifuge tubes. Following centrifugation, the cells were resuspended in 8 mℓ of the hypotonic buffer "RSB-K" (0.01 M KCl, 0.01 M Tris, 0.0015 M $MgCl_2$ pH 7.4; a modification of the well-known Reticulocyte Standard Buffer, RSB, prepared using KCl instead of the NaCl in RSB) containing 100 mM potassium phosphate to inhibit histone dephosphorylation during preparation. After allowing the cell suspension to stand 10 minutes to produce cell swelling, 1 mℓ of a mixture made from one part of a 10% w/v solution of the ionic detergent sodium deoxycholate and two parts of a 10% v/v solution of the non-ionic detergent Tween 40 (polyoxyethylene sorbital monopalmitate, Sigma Chemical Co.) was added to lyse the cells. The cellular suspension was then vortexed for one minute at top speed using an eccentric Vortex mixer to remove attached cytoplasm from the nuclei by gentle shear. After allowing the cellular suspension to stand for 5 minutes, the suspension was vortexed again for one minute to remove any remaining cytoplasmic tags. The nuclear suspension underlaid with 5 mℓ of a 0.25 M sucrose, 100 mM potassium phosphate (pH 7.0) solution. The nuclei were pelleted by centrifugation at 1000 g for 5 minutes and then resuspended in 8 mℓ RSBK containing 100 mM potassium phosphate and treated with the sodium deoxycholate-Tween 40 detergent mixture a second time, as described above. Nuclei were pelleted through a 0.25 M sucrose, 100 mM potassium phosphate (pH 7.0) pad, as before.

Nuclei prepared for experiments to study acetylated histone species in chromatin fractions were protected from deacylation of the histones by the addition of 6 mM sodium butyrate during the above nuclear preparation (Perry et al., 1979). Extent of cytoplasmic contamination of nuclear preparations was judged by phase contrast microscopy and fluorescence microscopy using Acridine Orange as described by Gurley et al. (1973a). Cytoplasmic contamination was observed as red-fluorescing material attached to green-fluorescing nuclei.

9. Nuclear fractionation. Nuclei from each nuclear preparation were suspended in 3 mℓ 0.001 M Tris (pH 8.0) and blended at 4°C for 1 min at 15,000 RPM in a 5 mℓ capacity, fluted, glass microflask, using a Virtis 60 homogenizer equipped with a tachometer. Following homogenization, the nuclear chromatin suspension was transferred to another tube, the microflask rinsed with 1 mℓ 0.001 M Tris pH 8.0, and this wash was also added to the homogenate. Varying speeds of homogenation were studied to determine the proper amount of shear needed to fractionate the chromatin.

10. Separation of Nuclear-Chromatin Fractions. Following a method modified from that of Anderson et al. (1975) the nuclear-chromatin homogenates (4 mℓ each) were layered over 34 mℓ 0.4 M to 2.8 M sucrose (in 0.004 M Tris) gradients prepared using a model 570 gradient former (Instrumentation Specialties Co.). The gradients were centrifuged for 30 minutes at 24,000 RPM in a Beckman Spinco SW27 rotor at 4°C. Fractionation of the gradients was accomplished at room temperature by pumping the sucrose from the bottom of the tube through a 2 mm bore stainless steel tube using a Peristaltic pump, Model P-3, (Pharmacia) at a flow rate of 1 mℓ/min. One mℓ fractions were collected every minute

and stored at 4°C. Aliquots of chromatin fractions labeled with ^{3}H and ^{14}C double label were treated overnight with 0.075 mℓ, 100 mM EDTA, 0.075 mℓ 1% SDS 0.075 mℓ 5 mg/mℓ Pronase, and 1.175 mℓ H_2O, before addition of scintillation fluor and counting. Aliquots of 100 μℓ were removed for scintillation counting, a total of 2 mℓ water and 13 mℓ of either Aquasol (New England Nuclear Corp.) or Hydrofluor (National Diagnostics Corp.) scintillation fluor were added and the ^{14}C and ^{3}H present in these fractions were counted simultaneously in a Packard Tri-Carb Spectrometer (Gurley and Walters, 1971). Chromatin peaks generated in the gradient, were identified by the presence of ^{14}C. These peaks were then pooled from the remaining portion of the sucrose fractions, diluted 1:1 with 0.004 M Tris (pH 7.8) to reduce sucrose viscosity and centrifuged at 50,000 RPM in a Beckman Spinco 60 Ti rotor to pellet the chromatin fractions. RS (rapidly sedimenting) fractions were thus derived from the lower portion of the gradient and SS (slowly sedimenting) fractions from the upper portion of the gradient.

Chromatin fractionated for the purpose of histone phosphorylation analysis was fractionated from the gradient into tubes containing 2 mℓ of H_2O containing 0.05 M sodium bisulfite, which inhibits phosphatase activity.

11. Isolation of the Histones. Histones were extracted from the pelleted chromatin fractions by the first method of Johns (1964), with modifications described by Gurley et al. (1978), except that the saline washes were omitted because cytoplasm had been removed during the nuclear isolation procedure. In certain experiments following extraction of H1 by the method of Johns (1964), core histones (H2A, H2B,

H3 and H4) were extracted using 0.4 N H_2SO_4, as previously described (II.2.2.4).

12. Fractionation and Purification of Histones by Gel Electrophoresis.

(a) Preparative gel electrophoresis--Lysine-rich histones H1 and H2B from chromatin fractions were mixed together and subjected to preparative urea acetic-acid polyacrylamide gel electrophoresis without detergent (Panyim and Chalkley, 1969). The histone mixture containing H2A, H3 and H4 was subjected to urea-acetic acid polyacrylamide gel electrophoresis which was carried out in the presence of 0.38% of the non-ionic detergent Triton DF-16 (Alfageme et al., 1974). Both procedures were as previously described for histones from whole cells (II.1.2.8).

(b) Analytical polyacrylamide gel electrophoresis--Histone H1 samples from chromatin fractions were subjected to high resolution electrophoresis on long (0.6 x 25 cm) urea, acetic acid polyacrylamide gels according to the method of Panyim and Chalkley (1969), as described by Gurley et al. (1978a). Lyophilized samples were dissolved in 6 M urea, 0.9 M acetic acid, 4% 2-mercaptoethanol at such a concentration that a 25 µl sample applied to each gel produced a 12.5 µg load. Electrophoresis was performed in 2.5 M urea, 0.9 M acetic acid, 15% polyacrylamide gels. Following pre-electrophoresis for 12 hours at a constant current of 20 milliamps per 12 gels, and prior to application of the experimental H1 sample, a mobility marker (8 µg of BioRex 70 purified calf thymus histone H1) was loaded on the gels and electrophoresis was carried out for 4 hours at a constant current of 10 milliamps per 12 gels, with a total voltage of 240. Histone H1 from

P. eremicus whole cells, nuclei and chromatin fractions was then applied to the gels and electrophoresis was continued for 28 hours. Gels were stained overnight in 0.2% Amido Black 10B dissolved in 30% methanol, 9% Acetic acid. Destaining was then accomplished by diffusion for 30 hours in a circulating bath of 30% methanol in 9% acetic acid. Absorbance profiles of the stained protein bands in the gels were measured and quantified as described previously for analytical gel electrophoresis of histones from whole cells of Peromyscus cell lines (II.2.2.5).

Core histones (H2A, H2B, H3 and H4) extracted from chromatin fractions, with 0.4 N H_2SO_4, were separated analytically by electrophoresis on long (0.6 x 25 cm) 0.38% Triton DF16, 6 M urea 12% polyacrylamide gels as described previously for histones from whole cells (II.2.2.5).

13. Determination of Protein, DNA and RNA Content. Analysis of the protein content of chromatin fractions was carried out by the method of Lowry (1951) on 1.5 mℓ aliquots from RS and SS pellets which had been sheared with 3 mℓ 1 mM Tris (pH 8.0) in a Sorvall Omni-Mixer with a microblender attachment for one minute at 50,000 RPM (top speed). The protein standard used was bovine serum albumin.

DNA and RNA content of the chromatin fractions was measured on 1.5 mℓ aliquots, from the same sheared pellets by a modified Schmidt-Tannhauser procedure described by Walters et al. (1974), in which RNA was hydrolyzed with 0.3 N KOH at 37°C for 18 hr and DNA was hydrolyzed with Deoxyribonuclease I for 4 hrs at 37°C. The RNA and DNA content of samples (which were in 0.2 M $HC1O_4$ and 0.5 M $HC1O_4$, respectively) was determined from the absorbance of these solutions at 260 nm, read on a

Gilford Spectrophotometer. Values for RNA content were calculated using extinction coefficients in acid for a equal mixture of 2' and 3'-ribonucleotides ($A_{260\ nm}$ = 31.9 OD for a 1 mg/mℓ sample). Values for DNA content were calculated using extinction coefficients in acid for an average mixture of deoxyribonucleotides for mammalian DNA ($A_{260\ nm}$ = 30.9 OD for a 1 mg/mℓ sample). DNA content was also measured on the same hydrolyzed samples using the colorimetric assay based on the diphenylamine reaction as described by Friesen (1968).

14. Isolation of DNA. DNA was isolated by two procedures, both based on the method of Marmur (1961). In experiments to isolate DNA for CsCl density gradient satellite analysis chromatin pellets and whole cells were treated overnight with 1.32 mℓ H_2O, 0.075 mℓ 100 mM EDTA, 0.075 mℓ 1% SDS, 0.075 mℓ nuclease-free Pronase (5 mg/mℓ) at 37°C, afterwhich at 4°C 1.5 mℓ chloroform: phenol (1:1) was added, with vortexing. Following centrifugation to separate the phases, the aqueous phase (top) was drawn off and added to 1.5 mℓ chloroform: isoamyl alcohol (24:1). The mixture was vortexed again, centrifuged and the aqueous phase drawn off and added to 6 mℓ 100% ethanol to precipitate nucleic acids. The organic phases were than re-extracted using 1.5 mℓ 1 M NaCl which was then added to the same 6 mℓ 100% ethanol to precipitate nucleic acids. Following precipitation in the freezer (-15°C to -20°C) overnight, nucleic acids were pelleted by centrifugation, washed in 95% ethanol-2%potassium acetate and air dried. The pellet was then dissolved in 1.35 mℓ 20 mM NaCl - 20 mM Tris, 0.15 mℓ of a 200 µg/mℓ Ribonuclease A solution were added, and the solution was incubated for 2 hours at 48°C. Following the incubation, DNA was extracted from the solution using chloroform: phenol (1:1) and

chloroform: isoamyl alcohol (24:1), in the same manner described above. Precipitation of DNA was accomplished as before in 6 mℓ 100% ethanol overnight in the freezer (-15°C to -20°C). Following centrifugation, the DNA pellet was washed with 95% ethanol-2% potassium acetate and dissolved in 50 mM NaCl-50 mM Tris for CsCl density gradient analysis.

DNA to be used for melting experiments, in which greater protection from DNA shearing during isolation was needed, was prepared by a procedure modified from that described above as follows (also increasing the volumes of solvents used). Whole cells or chromatin pellets were suspended in 10 mℓ 10 mM Tris, 150 mM NaCl, 10 mM EDTA, pH 8.0 and 2.5 mℓ of a 1 μg/mℓ Proteinase K solution in 10 mM Tris, 150 mM NaCl, 10 mM EDTA, pH 8.0, 2.5% SDS, were added dropwise, while mixing. This solution was incubated overnight at 37°C. Following incubation, the solution was cooled to 4°C and added to an equal volume (12 mℓ) of chloroform: phenol (1:1) and shaken to mix the phases. Following centrifugation the aqueous phase (top) was drawn off and added to an equal volume (12 mℓ) of chloroform: isoamyl alcohol (24:1) and shaken. Following centrifugation, the aqueous phase (top) was drawn off and added to 24 mℓ 100% ethanol. The nucleic acids were precipitated (at -15°C to -20°C) in the freezer overnight. Re-extraction of the organic phases with an equal volume of 10 mM Tris, 150 mM NaCl, 10 mM EDTA (pH 8.0) led to no further precipitation of nucleic acids in alcohol. Following precipitation, the nucleic acids were pelleted by centrifugation, washed with 95% ethanol-2% potassium acetate, and air dried. The pellet was dissolved in 4 mℓ of the Tris-NaCl-EDTA buffer, 0.1 mℓ of a 1.5 mg/mℓ Ribonuclease A solution was added, and the solution was incubated at 37°C for 4 hours or overnight. Following the incubation,

8 mℓ of the Tris-NaCl-EDTA buffer were added and the DNA was extracted from the solution at 4°C, using chloroform: phenol (1:1) and chloroform: isoamyl alcohol (24:1) in the same manner described above. DNA was precipitated from the aqueous phase in 24 mℓ 100% ethanol in the freezer (at -15°C to -20°C) overnight, pelleted by centrifugation, and dissolved in 0.015 M NaCl, 0.0015 M sodium citrate (pH 7.0) for satellite analysis and melting experiments. DNA isolated by this procedure precipitated as long stringers and was not as sheared as that isolated by the first method.

15. CsCl Equilibrium Sedimentation for Satellite Analysis. Identification of satellite and main band DNA and the determination of their buoyant densities were obtained by equilibrium centrifugation in CsCl. The sample in either 50 mM Tris, 50 mM NaCl or 0.015 M NaCl, 0.0015 M sodium citrate (pH 7.0) was adjusted to a density of 1.700 g/mℓ with CsCl. The DNA concentration of the sample was 4 μg/mℓ. DNA from Micrococcus lysodeikticus (ρ = 1.731 g/mℓ) at 1 μg/mℓ was used as a density marker. Centrifugation was performed in a Beckman Model E analytical ultracentrifuge, equipped with a photoscanner, using either an AN-G Ti or AN-F Ti rotor with double-sectored cells. Equilibrium was reached after 24 hours at 44,000 RPM at 25°C.

16. Thermal Denaturation of the DNA. The temperature of the DNA in a 1 cm light path curvette was increased at 0.5°C/min from 20°C to 95°C using a Perkin-Elmer model C-570-0701 Digital controller. Temperature and the absorbancy change at 260 nm were recorded using a Perkin-Elmer model 552 Spectrophotometer and model 561 recorder.

17. Dephosphorylation of Histone H1. In order to determine which stained protein bands following analytical acid-urea polyacrylamide gel

electrophoresis were due to phosphorylated H1 species and which were due to parental H1 species, H1 histone from whole P. eremicus cells was deliberately dephosphorylated by treatment with alkaline phosphatase in the following manner. H1 histone from 2.1 x 10^8 cells was dissolved in 0.5 mℓ 0.1 M Tris pH 8.0, and 5 μℓ E. coli alkaline phosphatase (Worthington Biochemical Co.) were added. The solution was incubated at 37°C for 8 hours at which time another 5 μℓ alkaline phosphatase was added. Incubation was continued for another 16 hours, afterwhich a 3rd addition of 5 μℓ alkaline phosphatase was made. Three hours later the sample was made 5% with respect to $HClO_4$ by adding 0.04 mℓ 70% $HClO_4$. The sample was made up to 3.2 mℓ by adding 2.66 mℓ 5% $HClO_4$ and placed on ice to precipitate the alkaline phosphatase, which was removed by centrifugation at 8,000 g for 15 minutes. H1 histone was precipitated overnight by adding 0.77 mℓ 100% TCA. The precipitate was washed one time with acidified acetone, taken up in H_2O and lyophilized to dryness.

III.3. Results

1. Method of Chromatin Fractionation

The primary considerations for the development of a chromatin fractionation scheme for P. eremicus were that the method be reproducible and that histone modifications be preserved as much as possible during isolation of chromatin fractions. For these reasons, fractionation was carried out using whole nuclei isolated under conditions designed expressly for inhibiting phosphatases and deacetylases. By blending the nuclei for 1 min at 15,000 RPM in a Virtis homogenizer and then centrifuging the homogenate through a sucrose gradient a reproducible fractionation was obtained, as depicted in Fig. 11. The shape of the sucrose gradient is shown superimposed upon the chromatin dis-

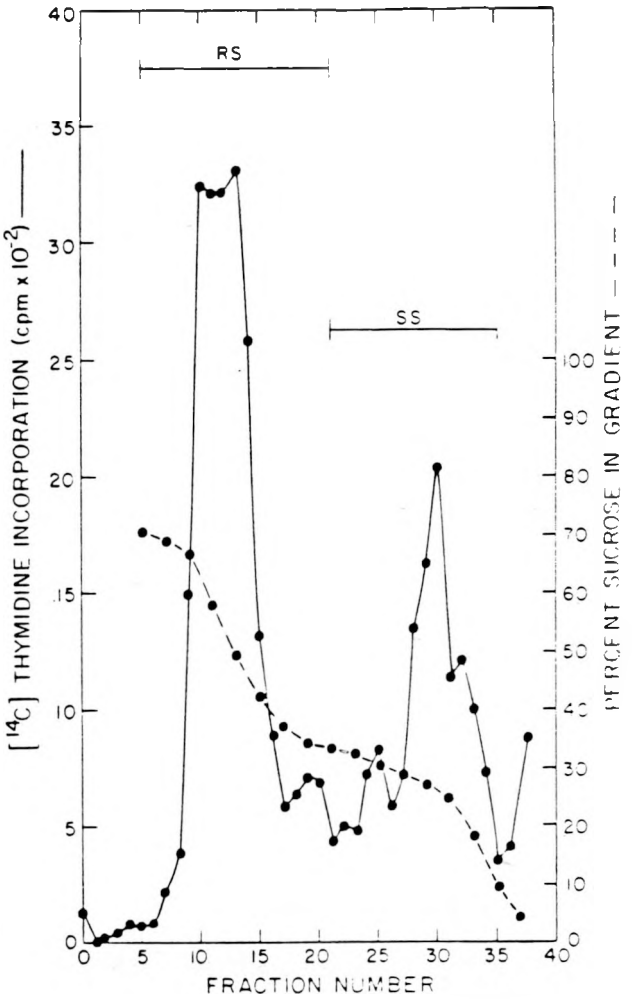

Fig. 11. Fractionation of *P. eremicus* chromatin. Distribution of [^{14}C] thymidine-labeled, sheared, chromatin in fractions from sucrose gradients, collected from the bottom of the tube following centrifugation. Rapidly sedimenting (RS) and slowly sedimenting (SS) chromatin peaks are shown superimposed upon the shape of the sucrose gradient (expressed as percent concentration of sucrose determined by refractive index).

tribution (Fig. 11). This homogenization condition (15,000 RPM for one minute) was chosen on the basis of fractionation distributions generated by increasing the speed of the blender from 5,000 to 30,000 RPM in 5,000 RPM increments. These results are shown in Fig. 12. A blender speed of 15,000 RPM appears to be the speed below which RS (rapidly sedimenting) and SS (slowly sedimenting) peaks are not completely separated, and above which no further separation is accomplished, but the SS peak is increased at the expense of the RS peak. In order to be sure that blending caused breakage of only the least condensed chromatin, and left the most condensed chromatin in the heavy, RS peak, the blender speed was chosen that broke up P. eremicus chromatin just enough to separate it into two recognizable peaks on the sucrose gradients. Blender speeds between 10,000 and 15,000 RPM did not always separate the peaks, thus 15,000 RPM was established as the homogenization speed for these experiments expected to give the most reproducable results. Even at 15,000 RPM there was some variability in blending between experiments. On the average, however, based on ^{14}C thymidine incorporation in 23 experiments, 65.5% of the chromatin was found in the RS fraction and 34.5% was found in the SS fraction, with a standard deviation of ± 4.5%.

Many attempts were made to harvest cells, prepare nuclei and obtain chromatin fractions under conditions normally used to inhibit phosphatases and proteases. These included the use of both 0.05 M sodium bisulfite (as discussed in II.1.2.5), and a mixture of 10^{-4} M cadmium sulfate, 10^{-3} M sodium bisulfite, and 1 μg/mℓ soybean trypsin inhibitor (Comings and Harris, 1975). It was found that the preparation of clean nuclei was impossible using detergent techniques involving these

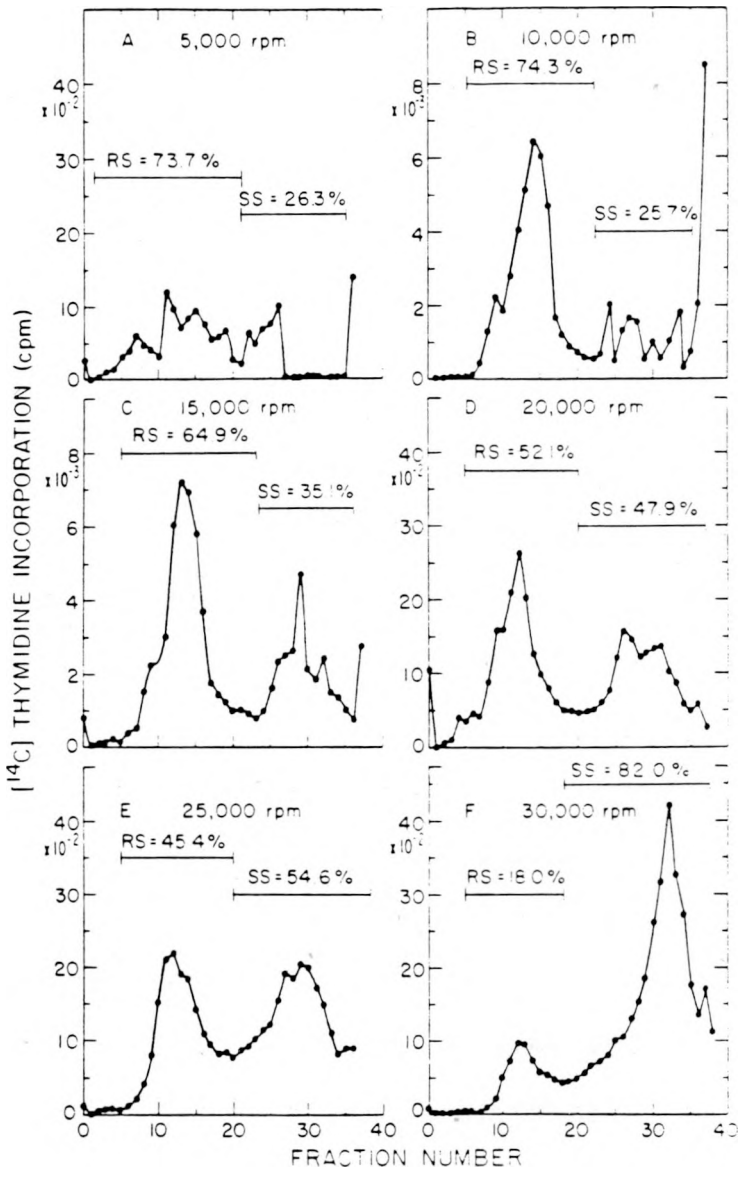

Fig. 12. Differential fractionation of P. eremicus nuclei by increased shearing speed. The distribution of rapidly sedimenting (RS) and slowly sedimenting (SS) chromatin fractions is indicated by [^{14}C] thymidine incorporation into chromatin DNA. Distributions are shown for each 5,000 RPM increase in homogenizer speed from 5,000 RPM to 30,000 RPM.

inhibitors. In order to overcome this problem, cells were harvested and nuclei were prepared in the presence of 100 mM potassium phosphate (pH 7.0). The rationale behind the use of excess phosphate ion was to inhibit phosphatases by overloading the dephosphorylation reaction with its product. In order to determine the effectiveness of phosphate ion as a phosphatase inhibitor, nuclei were prepared from P. eremicus cells using various concentrations of potassium phosphate. H1 histone was then isolated from these preparations and examined by analytical, acetic acid-urea, polyacrylamide gel electrophoresis. Previous work from this laboratory has shown that phosphorylated species of H1 histone are very susceptible to degradation by phosphatases (Gurley et al., 1975), thus preservation of H1 phosphorylated bands was used as a measure of phosphatase inhibition. The only adverse effects of the increased concentrations of phosphate ion on nuclear preparation were that nuclei appeared to be more delicate and more easily broken. Examination of phosphate prepared nuclei using phase contrast and acridine orange fluorescent microscopy revealed the presence of many clumps of nuclei, indicating some nuclear breakage. Phosphate ion did, however, appear to inhibit phosphatase activity, as shown in Fig. 13. In this Figure spectrophotometer scans of H1 histones extracted from nuclei in increasing concentrations of potassium phosphate from 0 to 100 mM are compared to those from whole cells harvested with or without 0.05 M sodium bisulfite. As expected, the best protection against dephosphorylation is afforded by sodium bisulfite during cell harvest (Fig. 13, top), as shown by the presence of 6 H1 bands. Quantitation of the gel scans presented in Fig. 13 is shown in Table 9. Six bands are still resolvable in H1 histone extracted from whole cells without sodium

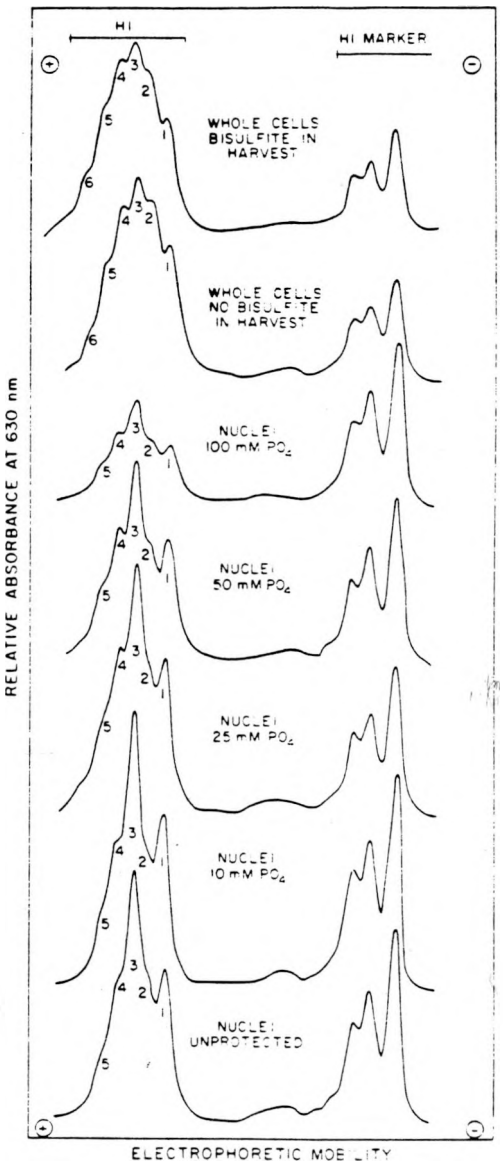

Fig. 13. Effectiveness of sodium bisulfite and increased phosphate ion concentration as inhibitors of H1 dephosphorylation in P. eremicus whole cells and nuclei. Spectrophotometer scans of $HClO_4$-extracted H1 histone from whole cells and nuclei with various degrees of phosphatase protection, following electrophoresis on 25 cm acetic acid, urea, polyacrylamide gels (peaks on left side of scan). Peaks on right side of scan are H1 histones added to the gels as a mobility reference before the experimental samples (left side) were added.

TABLE 9

COMPARISON OF THE DISTRIBUTION OF THE PHOSPHORYLATED SPECIES OF H1 RELATIVE TO PHOSPHATASE PROTECTION

	Percent of Maximum Percent in Each Band*					
Protection	1	2	3	4	5	6
Whole Cells Bisulfite	63.0	96.9	63.7	96.7	100	100
Whole Cells No Bisulfite	69.6	100	64.2	98.1	89.2	87.7
Nuclei 100 mM PO_4	76.1	91.2	85.0	100	80.3	0
50 mM PO_4	85.2	85.6	85.5	98.6	73.9	0
25 mM PO_4	88.3	90.8	89.3	98.6	54.8	0
10 mM PO_4	100	84.6	100	84.3	39.5	0
None	91.3	93.8	94.2	89.0	47.8	0

*Calculations were performed as follows: The percentage contribution of each band (1-6) to the total H1 distribution was determined for each protection method (whole cells-bisulfite, whole cells--no bisulfite, Nuclei-100 mM PO_4, etc.). Each of these percentages in every band set (for example, in band 1) was then divided by the largest percentage in the set, in order to determine which protection method maximizes the relative amount of protein in each band.

bisulfite during harvest (bisulfite was still present during H1 extraction). However, there was some dephosphorylation without bisulfite as indicated by the increase in the relative proportion of the unphosphorylated H1 (Band No. 1, Table 9). Comparison of H1 from whole cells with H1 extracted from nuclei (Fig. 13) shows a definite change in the phosphorylation pattern in H1 isolated from nuclei. Nuclei isolated in the presence of excess phosphate ion contain H1 with only 5 bands. Relative amount of protein present in each band is listed as the percent of the maximum percentage of protein found in the band, when H1 histones from phosphate prepared nuclei and whole cells are compared (see Table 9).

One of the difficulties in analyzing the phosphorylation of protein like H1, without label, is the identification of parental, unmodified species as opposed to the phosphorylated species. In order to determine which of the 6 bands seen in H1 from whole cells are due to phosphorylated species and which represent unphosphorylated species, H1 histone extracted from whole cells was dephosphorylated using alkaline phosphatase (Gurley et al., 1975). Results of this treatment are shown in Fig. 14. Bands 1 and 3 appear to be the parental, unphosphorylated bands, although there does seem to be some persistence of bands 2 and, possibly, 5, following phosphatase treatment. Either these bands (2 and 5) represent phosphorylated species not as suceptible to phosphatase action or, more probably, they are small parental species overwhich phosphorylated species are superimposed in untreated preparations. The results of this experiment suggest that dephosphorylation of H1 histone from P. eremicus is characterized by an increase in bands 1 and 3 with concomitant loss of phosphorylated protein in bands 2, 4, 5

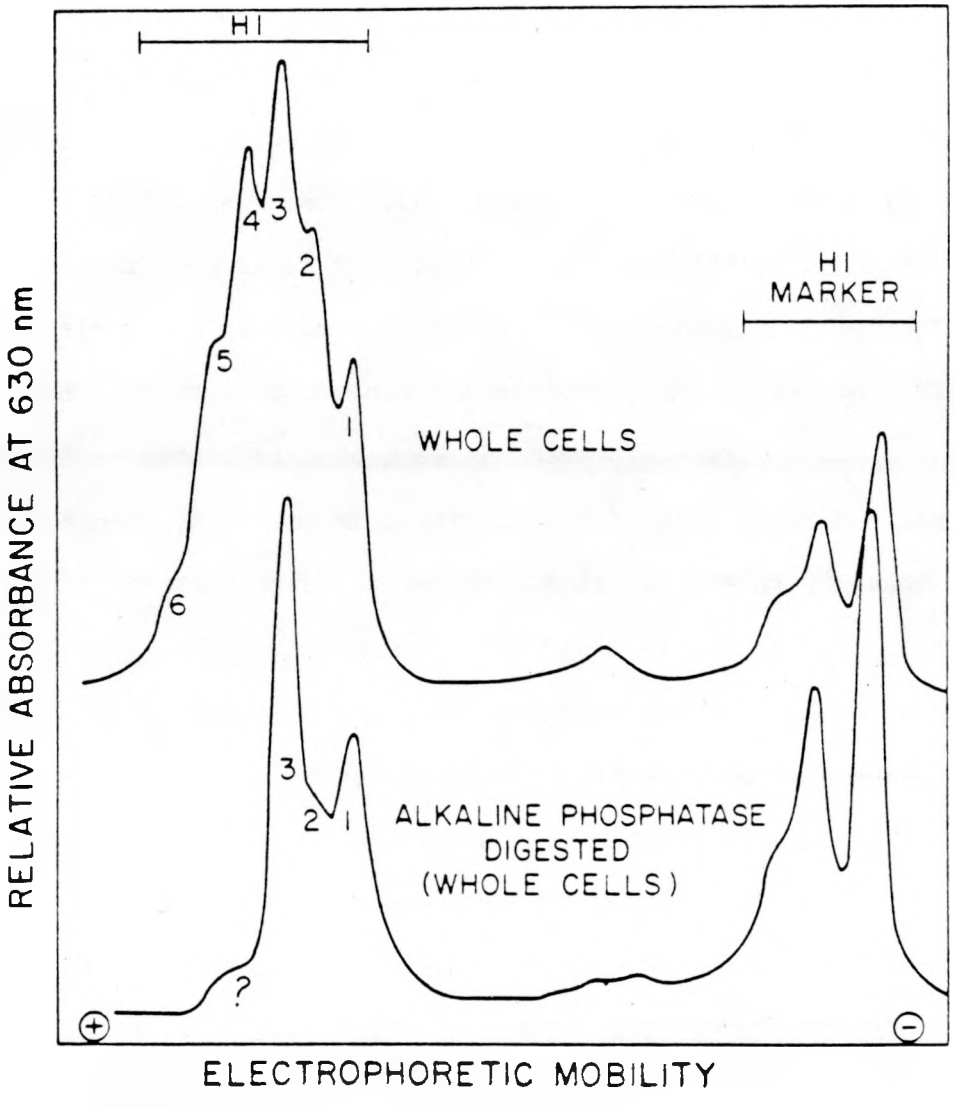

Fig. 14. Distribution of phosphorylated and parental species of histone H1 in P. eremicus cells. Spectrophotometer scans of $HClO_4$-extracted H1 histone from whole cells following electrophoresis on acetic acid, urea, polyacrylamide gels, comparing untreated H1 histone with H1 that was deliberately dephosphorylated by extensive treatment with alkaline phosphatase.

and 6. A comparison of the gel scans (Fig. 13), their quantitation (Table 9), and the graphical representation of that quantitation (Fig. 15), from nuclei prepared with varying concentrations of phosphate ion indicates that 100 mM phosphate affords the best protection against dephosphorylation although it is not as effective as sodium bisulfite. However, since nuclei cannot be isolated in the presence of sodium bisulfite, 100 mM potassium phosphate was used as a phosphatase inhibitor, although it was expected that there would still be some losses due to dephosphorylation in these experiments. Attempts to add sodium bisulfite to the sucrose gradient during chromatin fractionation resulted in shifts of the chromatin peaks to more dense positions, suggesting cross-linking and fixing of the chromatin which was undesirable (L. R. Gurley, unpublished results with CHO). However, immediately upon obtaining chromatin fractions from the sucrose gradients, 0.05 M sodium bisulfite was added to the preparation in order to further inhibit phosphatase activity.

2. Histone Modifications.

1. Phosphorylation

Once a reproducible chromatin fractionation scheme was obtained, experiments were run to determine the extent of histone modification in the two fractions. Results of [^{3}H]-lysine and $^{32}PO_4$ incorporation into P. eremicus cells, which were then fractionated into RS and SS chromatin fractions, are shown in Figs. 16 and 17 and Tables 10 and 11. In this experiment the cells were grown for 1.65 generations in [^{3}H]-lysine and exposed to $^{32}PO_4$ for two hours. Nuclei were prepared, in the presence of 100 mM phosphate, fractionated, and histones were extracted from the chromatin fractions by the method of Johns (1964) as modified by Gurley

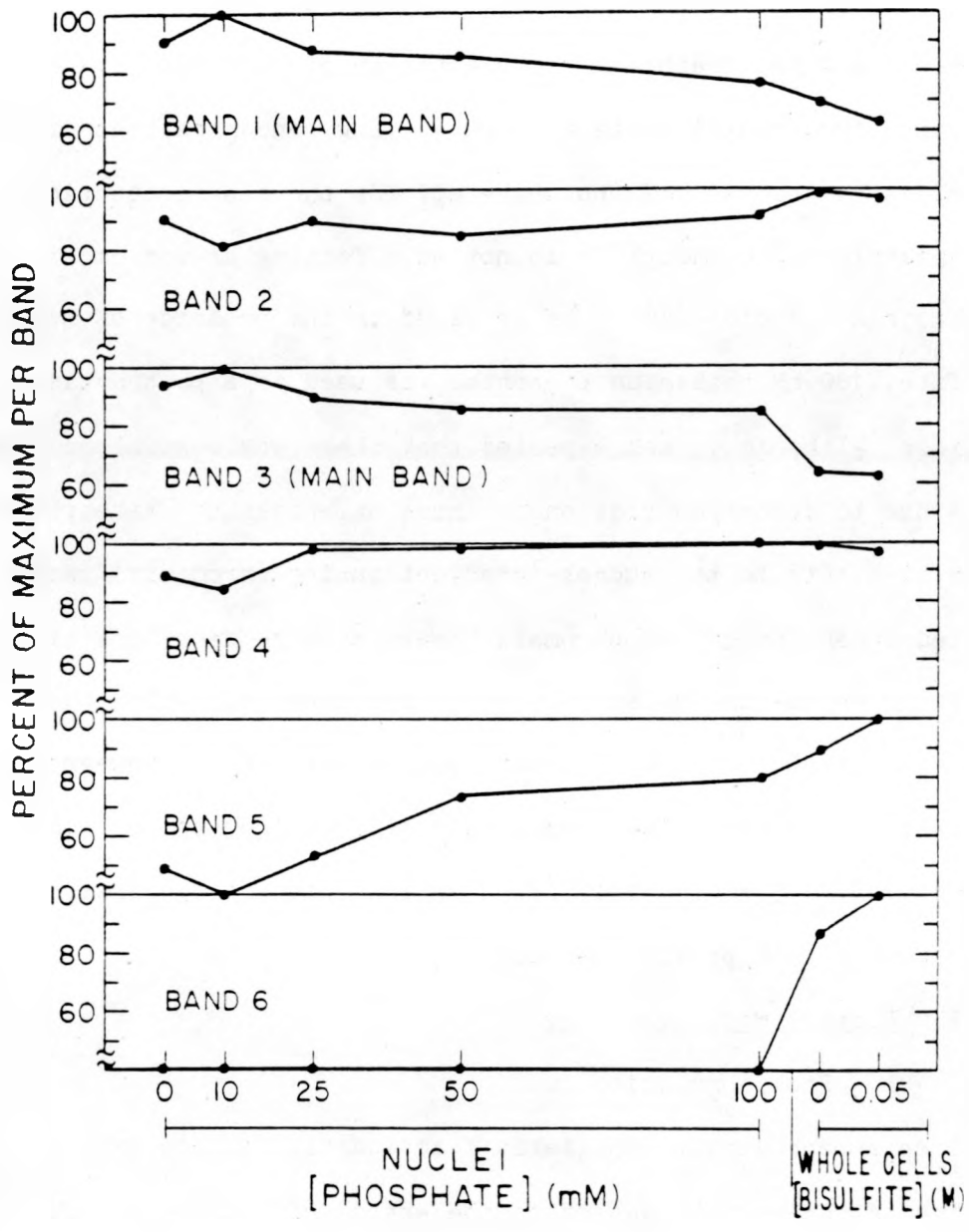

Fig. 15. Comparison of increased phosphate ion concentration and 0.05 M sodium bisulfite as phosphatase inhibitors; shown as a function of the percentage of the maximum relative absorbance of stained protein found in each band when all samples were compared. This is a graphical representation derived from the quantitation (Table 9) of spectrophotometer scans (Fig. 14).

and Hardin (1968) and Gurley et al. (1975). Histones were then purified by preparative acetic acid-urea polyacrylamide gel electrophoresis, either with (for histones H2A, H3, and H4) or without (for histones H1 and H2B) Triton DF-16 detergent. The distribution of label in the various histone fractions obtained from sheared (unfractionated) nuclei, and from RS and SS chromatin fractions are shown in Figs. 16 and 17. Sheared, unfractionated nuclei (Figs. 16C and 17C) were run as a control, and would be expected to show histone distributions similar to those from whole P. eremicus cells (Figs. 4 & 5). It was found that although the histone species distribution, both from the chromatin fractions and from the unfractionated sheared nuclei, looked the same as that from whole cells, differences were noted when these distributions were quantified (Tables 10 and 11). The most striking difference between labeled histones extracted from whole cells and those derived from sheared nuclei or fractions is seen in the phosphorylation of all histones (Table 10). When the ^{32}P to ^{3}H ratios for histones extracted from whole cells, labeled with [^{3}H]-lysine for the same number of generations as those used in fractionation experiments, are compared to those from sheared fractionated and unfractionated nuclei there appears to be a large amount of dephosphorylation during shearing. This was true even with 100 mM PO_4 present during nuclear preparation, and 0.05 M sodium bisulfite added following fractionation. This dephosphorylation ranges from two to three fold, the largest amount being associated with H3 and H2A (Table 10). The phosphorylation of the RS and SS fraction histones, although considerably lower than those from whole cells, are similar to each other, however. Histones from these chromatin fractions are also phosphorylated to about the same extent as those from whole

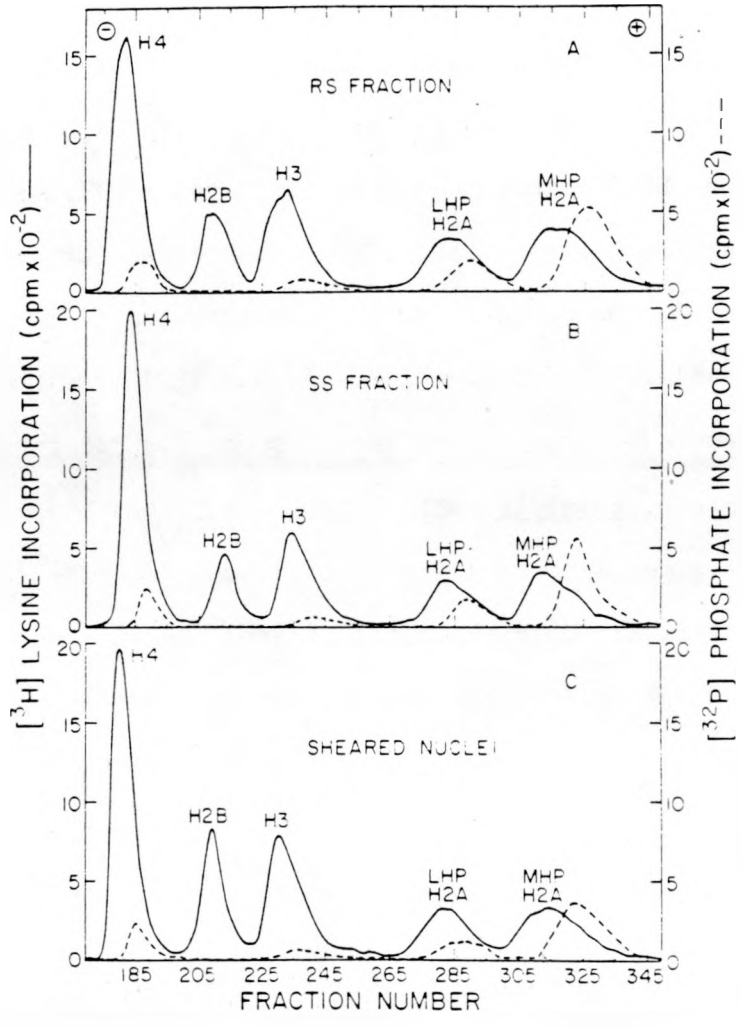

Fig. 16. Acetic Acid-urea-detergent preparative electrophoresis profiles of the histone H2A, H3 and H4 ethanol-extracted mixture, showing histone phosphorylation in sheared nuclei and chromatin fractions from exponentially growing P. eremicus cells. (A) Rapidly sedimenting (RS) chromatin fraction. (B) Slowly sedimenting (SS) chromatin fraction. (C) Sheared nuclei (whole chromatin). Individual histones are indicated by the incorporation of [^{3}H] lysine (———) into them over a time period of 1.65 cell generations. Phosphorylation of each histone fraction is indicated by the 2-hour incorporation of $^{32}PO_4$ (----). Electrophoretic migration proceeds from right to left. LHP and MHP represent the less hydrophobic and more hydrophobic subfractions of H2A.

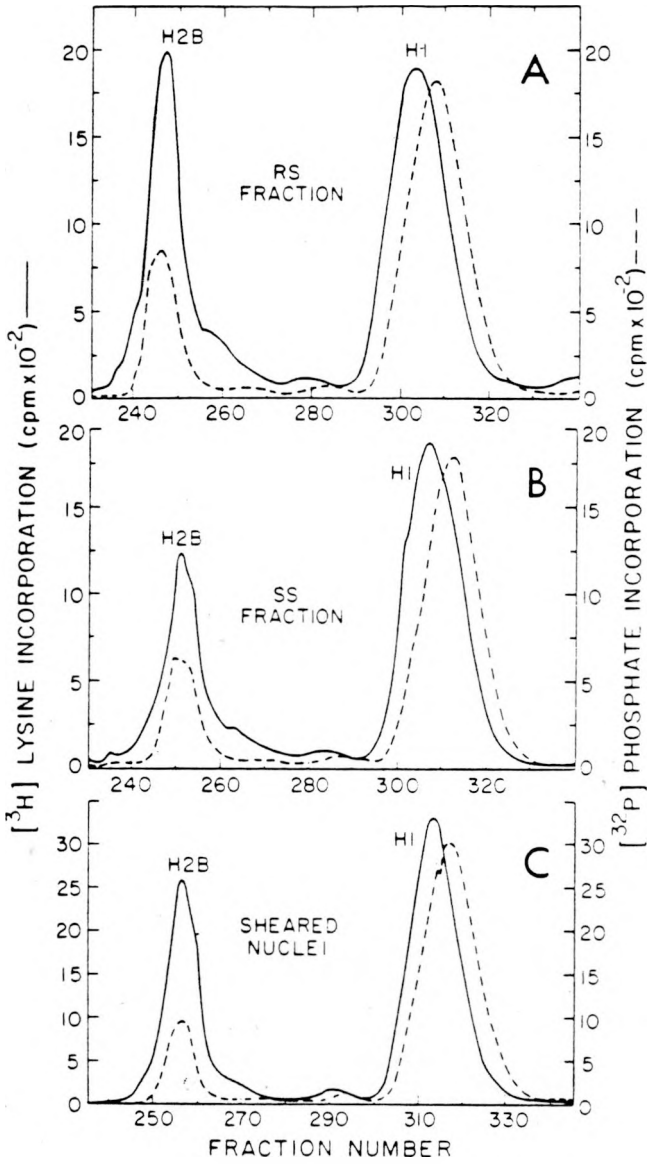

Fig. 17. Acid-urea-preparative electrophoresis profile of histones H1 and H2B, showing histone phosphorylation in sheared nuclei and chromatin fractions from exponentially growing P. eremicus cells. (A) Rapidly sedimenting (RS) chromatin. (B) Slowly sedimmenting (SS) chromatin. (C) Sheared nuclei (whole chromatin). Individual histones are indicated by the incorporation of [^{3}H] lysine (———) into them over a time period of 1.65 cell generations. Phosphorylation of each histone fraction is indicated by the 2-hour incorporation of $^{32}PO_4$ (----). Electrophoretic migration proceeds from right to left.

TABLE 10

HISTONE PHOSPHORYLATION IN P. EREMICUS

Sample	Generations in [^{3}H] lysine	[^{32}P]/[^{3}H]							
		H1	H3	H4	H2B	H2A			
						Total	MHP	LHP	MHP/LHP
Whole cells	1.62	2.16	0.43	0.27	0.56	2.29	3.20	1.20	2.67
Sheared Nuclei	1.65	0.92	0.10	0.10	0.34	0.63	0.85	0.35	2.43
RS Fraction	1.65	0.93	0.15	0.12	0.35	0.82	1.03	0.52	1.98
SS Fraction	1.65	0.94	0.14	0.11	0.45	0.83	1.14	0.46	2.48

TABLE 11

MASS ACCOUNTING OF HISTONES IN P. EREMICUS
WHOLE CELLS AND FRACTIONS

	Relative Percent of Histones ([^{3}H] cpm/Total [^{3}H] cpm) X 100					
	H1	H3	H4	H2B	H2A	$\frac{H2A}{[^3H]MHP/[^3H]LHP}$
Whole Cells						
Experiment #1	45.7	9.5	14.1	13.3	17.5	1.34
Experiment #2	40.3	11.8	16.0	13.7	18.1	1.37
Whole Sheared Nuclei	29.4	12.1	19.7	23.1	15.7	1.30
RS Fraction	31.7	9.2	16.0	26.8	16.3	1.44
SS Fraction	35.9	9.2	18.9	21.0	14.9	1.20

sheared nuclei, although the overall phosphorylation of histone H2A and H3 is less in the sheared nuclei. Thus, these results do not indicate any differences in histone phosphorylation between the RS and SS chromatin fractions. They do, however, indicate that the chromatin fractionation scheme does allow significant dephosphorylation of the histones.

Comparisons of the [^{3}H]-lysine incorporation into the histones of the chromatin fractions and sheared nuclei with those from whole P. eremicus cells (2 experiments) are shown in Table 11. In relative proportion, there appears to be less H1 and H2A but more H2B and H4 extracted from sheared and fractionated nuclei than from whole cells. In this representative experiment, (Figs. 16 & 17 and Table 11) the proportion of histones extracted from the two chromatin fractions is different, there being more histone H1 and less histone H2B and H2A extracted from the SS fraction than from either the RS fraction or sheared, unfractionated nuclei. Since these histone compositions varied between experiments, small differences in these proportions are probably due to differential extractability and recovery of the histones in the fractions, rather than due to actual differences in histone composition.

The subspecies or variants of histone H2A can also be compared between sheared nuclei, fractionated nuclei, and histones extracted from whole cells. When histones from whole Peromyscus cells are used, H2A is split into two subspecies, differing in their hydrophobicity which facilitates their separation by urea polyacrylamide gel electrophoresis in the presence of detergent (Fig. 4). This was also true with nuclei and chromatin fractions (Fig. 16). As expected, histone H2A from whole cells, sheared nuclei, and fractionated nuclei is composed of pro-

portionately more MHP-H2A than LHP-H2A (Figs. 4, 16 and Table 11). The ratio of MHP to LHP histone H2A subspecies for sheared nuclei (1.30) was similar to that found using whole P. eremicus cells (1.34, 1.37), but differed from the ratios derived from the chromatin fractions (Table 11). The RS and SS chromatin fractions also differed from each other, there being more of the MHP-H2A subspecies in the RS fraction, resulting in about a 20% difference in their MHP to LHP H2A ratios. Two separate experiments gave the same results. The RS chromatin fraction contained proportionately more of the MHP-H2A subspecies compared to whole, sheared nuclei while the SS fraction contained proportionately less MHP-H2A than these nuclei (Table 11). Evidence that the MHP to LHP H2A ratios of these chromatin fractions (RS and SS) represent fractionations of the MHP to LHP H2A ratios from whole, sheared nuclei is provided by the following prediction. The MHP to LHP ratio of histones from a mixture composed of 63.4% RS fraction (with MHP/LHP = 1.44) and 36.6% SS fraction (with MHP/LHP = 1.20) should be 1.35. (The percentages of the RS and SS chromatin fractions were derived from DNA distribution measured by [^{14}C]-thymidine incorporation for this experiment.) This predicted ratio compares favorably with the MHP/LHP = 1.30 measured for unfractionated nuclei used in this experiment (Table 11). Thus, the RS fraction differs from the SS fraction in its content of the MHP subspecies, and both of these fractions differ from whole unfractionated nuclei, suggesting an enrichment of the more phosphorylated, MHP-H2A subspecies in the RS fraction. These results are similar to the observed increase in the MHP/LHP-H2A ratio in whole P. eremicus cells over that in P. boylii cells, where the cell line with more constitutive heterochromatin had a greater MHP/LHP-H2A ratio.

2. Acetylation

P. eremicus chromatin fractions were also analyzed for arginine-rich acetylation, in a manner similar to that applied to whole cells from Peromyscus cell lines. Core histones (H2A, H2B, H3 and H4) extracted from nuclei and chromatin fractions with 0.4 N H_2SO_4, following perchloric acid extraction of histone H1, were subjected to analytical Triton DF-16, acetic acid-urea, polyacrylamide gel electrophoresis. The relative absorbance at 630 nm of stained protein bands in the resulting gels was then determined (Fig. 18 and 19). Before a comparative study of histone acetylation in the chromatin fractions could be made, however, the procedure for preparing nuclei was modified to prevent histone deacetylation. Based on the results of Perry et al. (1979), nuclei were prepared in the presence of 6 mM sodium butyrate (which inhibits deacetylation) and the 0.4 N H_2SO_4-extracted histones from these nuclei were then compared to histones from whole cells and nuclei not protected by butyrate. The results are shown as spectrophotometer scans of the polyacrylamide gels (Fig. 18). Quantitation of these scans is shown in Tables 12 and 13. An estimate of the extent of histone deacetylation occurring during nuclear preparation with and without 6 mM sodium butyrate protection is provided by a comparison of the total number of acetyl groups obtained per 100 H3 or H4 molecules (Tables 12 and 13). These data suggest that there is considerable deacetylation during nuclear preparation without sodium butyrate protection (54.8% for H3 and 38.3% for H4), whereas this deacetylation is diminished to 16.2% for H3 and 6% for H4 when 6 mM sodium butyrate is present (Tables 12, 13). For this reason, in these experiments, nuclear

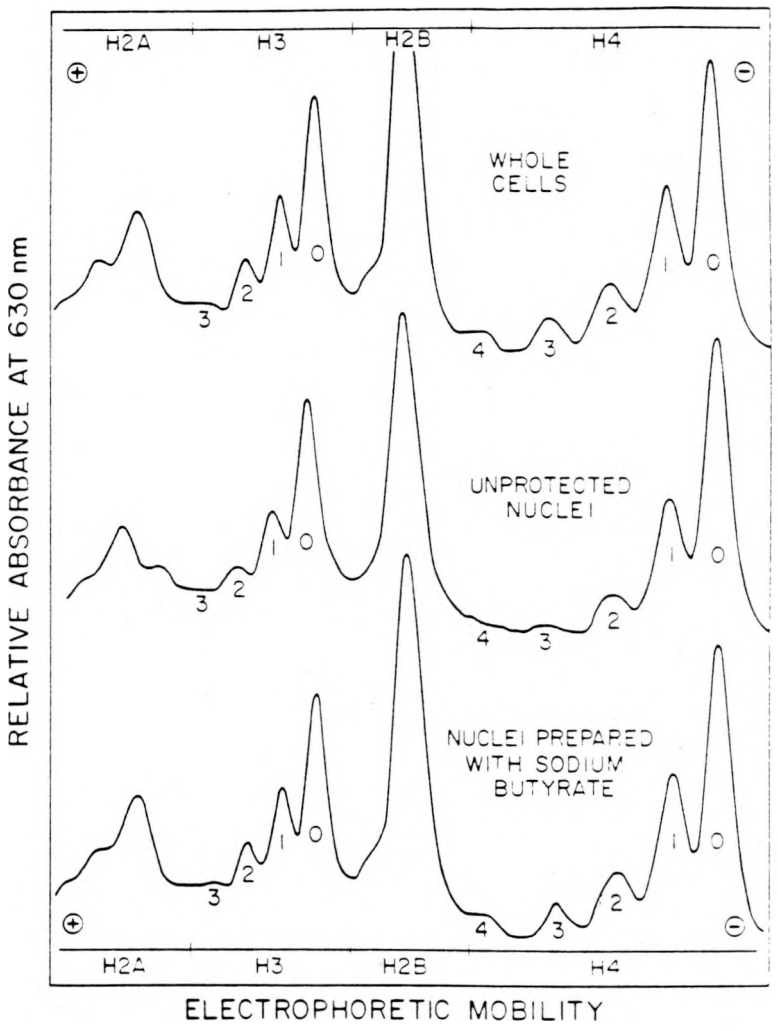

Fig. 18. Evaluation of histone deacetylation during nuclear preparation of P. eremicus cells. Spectrophotometer scans comparing 0.4 N H_2SO_4-extracted histone distributions from whole cells; unprotected nuclei; prepared without deacetylase inhibition; and nuclei prepared in the presence of 6 mM sodium butyrate (a deacetylase inhibitor). These distributions were generated by electrophoresis of acid-extracted histones on acetic acid, urea, DF-16 polyacrylamide gels.

TABLE 12

EFFECTIVENESS OF SODIUM BUTYRATE IN PREVENTING DEACETYLATION OF HISTONE H3 DURING NUCLEAR PREPARATION FROM *P. EREMICUS* CELLS

Sample	Number of Acetylations	Percent of Total H3	No. Acetates Per 100 H3 Molecules	Percent Deacetylation Compared to Whole Cells
Whole cells	0	52.5	0	
	1	28.5	28.5	
	2	15.2	30.4	
	3	3.8	11.4	
			Total = 70.3	0
Unprotected nuclei	0	66.2	0	
	1	23.7	23.7	
	2	8.6	17.2	
	3	1.5	4.5	
			Total = 45.4	54.8%
Nuclei prepared with 6 mM Na butyrate	0	57.1	0	
	1	27.3	27.3	
	2	13.6	27.2	
	3	2.0	6.0	
			Total = 60.5	16.2%

TABLE 13

EFFECTIVENESS OF SODIUM BUTYRATE IN PREVENTING DEACETYLATION OF HISTONE H4 DURING NUCLEAR PREPARATION FROM *P. EREMICUS* CELLS

Sample	Number of Acetylations	Percent of Total H4	No. Acetates Per 100 H4 Molecules	Percent Deacetylation Compared from Whole Cells
Whole cells	0	48.7	0	
	1	27.7	27.7	
	2	14.1	28.2	
	3	5.8	17.4	
	4	3.7	14.8	
			Total = 88.1	0
Unprotected nuclei	0	59.4	0	
	1	24.2	24.2	
	2	11.5	23.0	
	3	3.1	9.3	
	4	1.8	7.2	
			Total = 63.7	38.3%
Nuclei prepared with 6 mM Na Butyrate	0	51.9	0	
	1	25.9	25.9	
	2	13.5	27.0	
	3	4.3	12.9	
	4	4.3	17.2	
			Total = 83.0	6%

preparation and fractionation was carried out in the presence of 6 mM sodium butyrate.

The results of histone acetylation analysis from whole nuclei and the RS and SS fractions prepared with butyrate in this manner are shown in Fig. 19. Quantitation of the H3 region of these scans is shown in Table 14 and quantitation of the H4 region in Table 15. These results indicate that the RS chromatin fraction contains proportionately more unacetylated H3 and H4 histone than either whole nuclei or the SS fraction. The SS fraction, however, contains proportionately less unacetylated H3 and H4 than whole nuclei (Tables 14 and 15). The relative amount of unacetylated H3 and H4 obtained from whole nuclei, however, (57.1% $H3_o$ and 51.9% $H4_o$) are consistent with the percentages (61.1% $H3_o$ and 50.8% $H4_o$) predicted if a chromatin mixture composed of 67.9% RS ($H3_o$ = 64.4% $H4_o$ = 53.8%) and 32.1% SS ($H3_o$ = 54.1% $H4_o$ = 44.4%) were analyzed. (Chromatin fraction percentages for RS and SS fractions were derived from [^{14}C]-thymidine incorporation for this experiment.) Thus, whole nuclei have been fractionated into two fractions, RS and SS, in which the RS fraction displays about a 20% increase in unacetylated arginine-rich histones when compared to the SS fraction (Tables 15 and 16). This suggests that the chromatin fractionation procedure results in an enrichment of unacetylated arginine-rich histones in the RS fraction relative to the SS fraction and whole nuclei. These results are similar to the increase in unacetylated arginine-rich histones observed when whole cells from P. eremicus (the cell line with extra constitutive heterochromatin) are compared to cells from P. boylii (the cell line with less constitutive heterochromatin).

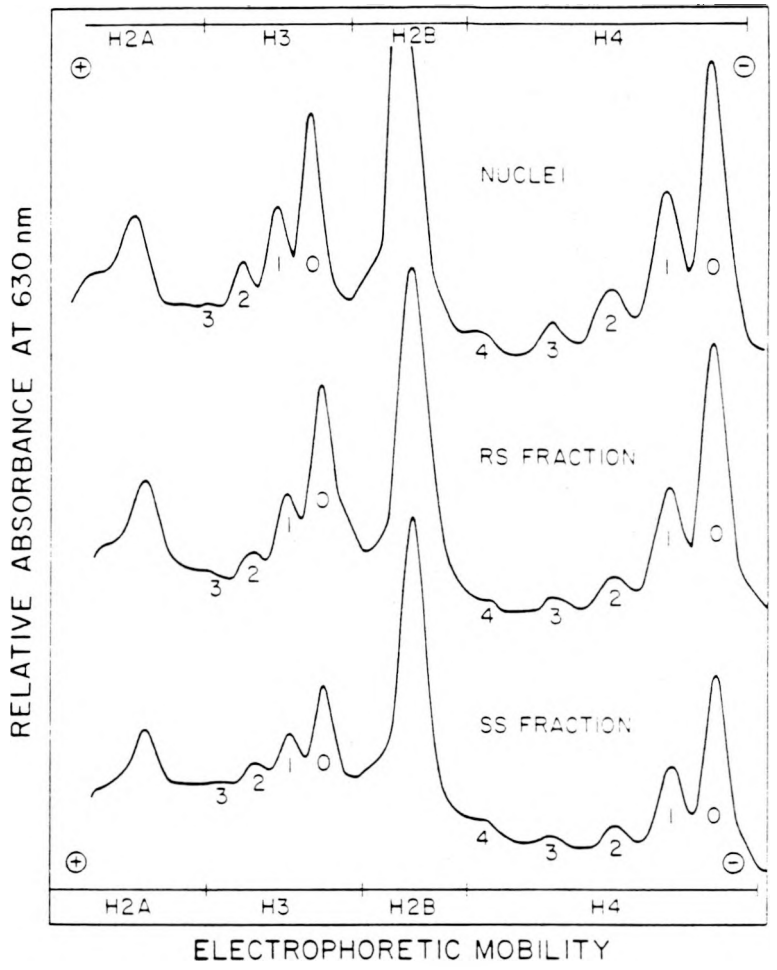

Fig. 19. Comparison of histone acetylation in nuclei and chromatin fractions from P. eremicus cells. Spectrophotometer scans comparing 0.4 N H_2SO_4-extracted histone distributions from nuclei prepared with 6 mM sodium butyrate, from rapidly sedimenting (RS) chromatin, and from slowly sedimenting (SS) chromatin. These distributions were generated by electrophoresis of acid-extracted histones on acetic acid, urea, DF-16 polyacrylamide gels.

TABLE 14

HISTONE H3 ACETYLATION IN CHROMATIN FRACTIONS FROM *P. EREMICUS* CELLS

Sample	No. of Acetylations	Percent Total H3	Relative Amount of Unacetylated H3
RS Fraction	0	64.4	1.19
	1	25.9	
	2	8.1	
	3	1.5	
Nuclei	0	57.1	1.06
	1	27.2	
	2	13.6	
	3	2.0	
SS Fraction	0	54.1	1.00
	1	30.4	
	2	13.5	
	3	2.0	

TABLE 15

HISTONE H4 ACETYLATION IN CHROMATIN FRACTIONS FROM *P. EREMICUS* CELLS

Sample	No. of Acetylations	Percent of Total H4	Relative Amount of Unacetylated H4
RS Fraction	0	53.8	1.21
	1	29.0	
	2	12.4	
	3	3.0	
	4	1.8	
Nuclei	0	51.9	1.17
	1	25.9	
	2	13.5	
	3	4.3	
	4	4.3	
SS Fraction	0	44.4	1.00
	1	29.1	
	2	15.3	
	3	6.6	
	4	4.6	

3. Identification of the Fractions

It was next necessary to determine if the differences in histone modifications in the chromatin fractions resulted from an enrichment of condensed, constitutive heterochromatin in the RS fraction, compared to the SS fraction. In order to determine this, the RS and SS chromatin fractions were tested in several ways in order to establish their relative content of heterochromatin and euchromatin. Three different approaches were used to establish the presence or absence of heterochromatin in these fractions. Two of these approaches were used to test for the presence of the GC-rich satellite known to be characteristic of P. eremicus cells (Mace et al., 1974). In a third approach, the fractions were tested for the presence of late-replicating DNA known to be characteristic of P. eremicus heterochromatin (Pathak et al., 1973; Kuo, 1979).

The first approach used neutral CsCl buoyant density analysis of DNA extracted from whole cells and both chromatin fractions as shown in Fig. 20. The values obtained for the bouyant density of the main band DNA (ρ = 1.699) and satellite DNA (ρ = 1.703) peaks from whole P. eremicus cells are similar to those values reported by others (Mace et al., 1974, main band ρ = 1.698, satellite ρ = 1.703; Hazen et al., 1977, main band ρ = 1.699, satellite ρ = 1.705). The neutral CsCl profiles for DNA from the RS and SS chromatin fractions both clearly show the presence of a GC-rich satellite (Fig. 20). However, quantitation of these profiles (Table 16) using the DuPont curve resolver indicated that the SS fraction contains proportionately less of the GC-rich satellite (26.9%) than the RS fraction (41.7%). Both fractions contained a substantial amount of the satellite, however.

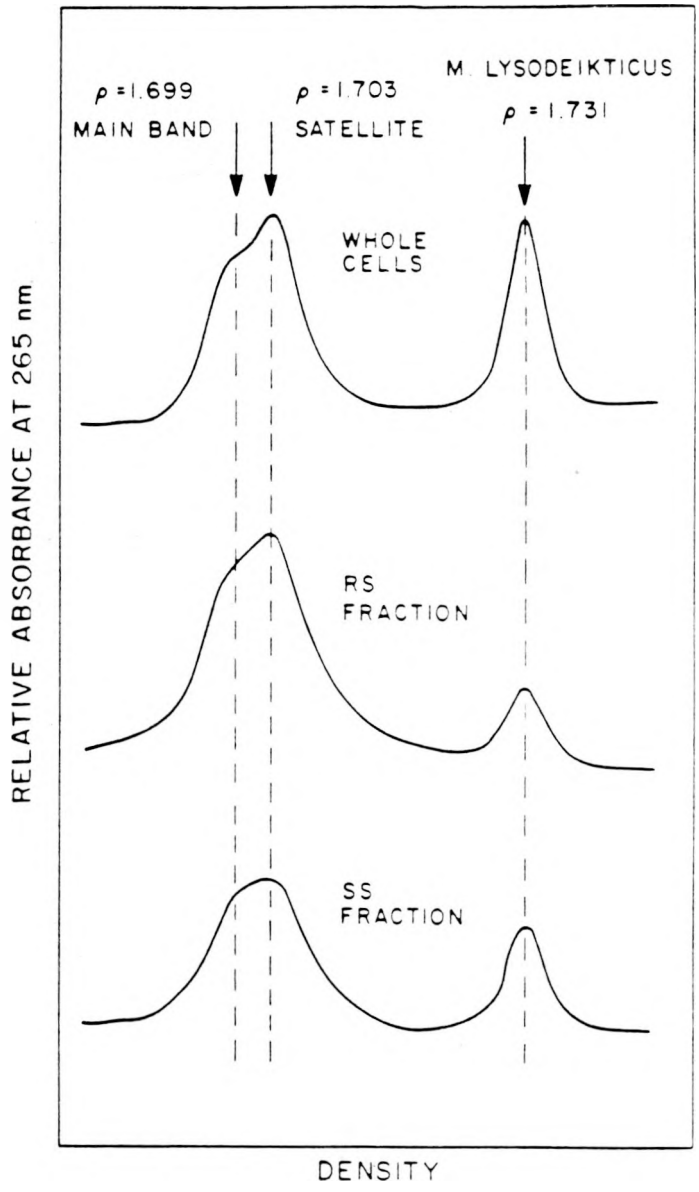

Fig. 20. Buoyant density ultracentrifugation analysis of P. eremicus DNA. CsCl density gradient analysis of DNA extracted from whole cells, the rapidly sedimenting chromatin fraction (RS) and slowly sedimenting chromatin fraction (SS). The main band DNA has a buoyant density = 1.699 g/cc and satellite DNA has a buoyant density = 1.703 g/cc. DNA from Micrococcus lysodeikticus (ρ = 1.731 g/cc) was used as a density marker.

TABLE 16

CsCl SATELLITE ANALYSIS - P. EREMICUS

	Percentage Contribution to Profile	
DNA Source	Main Band	Satellite
Whole Cells	65.0	35.0
RS Fraction	58.3	41.7
SS Fraction	73.1	26.9

The second approach to test for the presence of a GC-rich satellite in the DNA from the chromatin fractions was to analyze the DNA thermal denaturation as shown in Fig. 21. DNA isolated from whole cells, RS, and SS fractions, dissolved in dilute saline citrate buffer was melted in a temperature controlled recording spectrophotometer. The results revealed some very slight differences between DNA from the fractions and from whole cells. The T_m values for the three DNA samples varied by only a few degrees. The T_m for RS DNA was the highest at 72.5°C and that of SS DNA was the lowest at 70°C. The T_m for DNA isolated from whole cells (71.5°C) was between the T_m values observed for the fractions. Based on the equation given by Mandel and Marmur (1968) for the GC content of DNA, based on its thermal denaturation in the same dilute saline citrate buffer used in these experiments:

$$GC = (T_m - 53.9)\ 2.44$$

the RS fraction is calculated to have a GC content of 45.3% and the SS fraction 39.3%. Whole cell DNA is calculated to contain 42.9% GC. Again, although these results suggest an enrichment of GC-rich satellite DNA characteristic of heterochromatin in the RS fraction, satellite sequences are undoubtedly present in both fractions.

The third approach to test for the presence or absence of heterochromatin in the chromatin fractions was a method for measuring chromatin labeled with [^{3}H]-thymidine specifically in the heterochromatin region replicating late in S phase of the cell cycle (Kuo, 1979). P. eremicus cells bulk-labeled with [^{14}C]-thymidine, as before, were pulse-labeled with high specific activity [^{3}H]-thymidine for

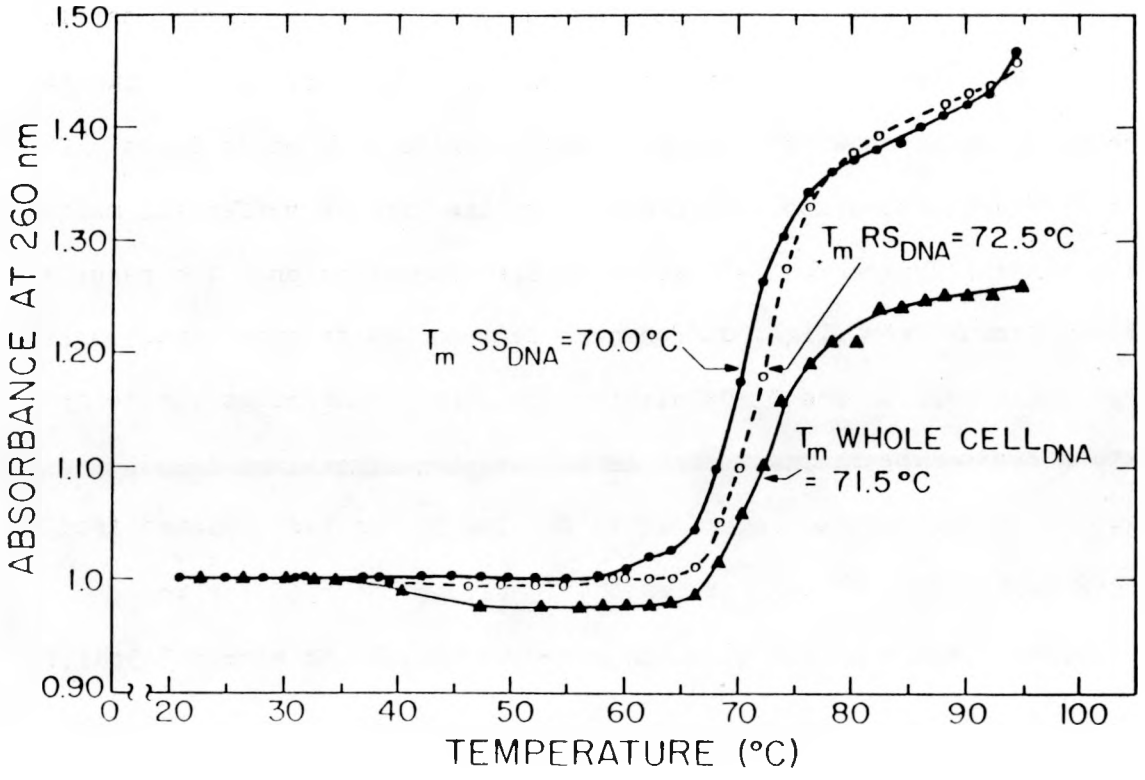

Fig. 21. Thermal denaturation profile of DNA extracted from P. eremicus whole cells (—▲—▲—▲—); from the (RS) rapidly sedimenting chromatin fraction (o--o--o); and from the (SS) slowly sedimenting chromatin fraction (●—●—●) in 0.015 M NaCl, 0.0015 M sodium citrate, pH 7.0.

15 minutes and then allowed to grow for 2 hours (1 hour in Colcemid) following the pulse. Mitotic cells were then removed by mitotic detachment using mechanical agitation. Since P. eremicus cells are assumed to have a 2 hour G_2 phase (Kuo, 1979 and FMF analysis) mitotic cells thus obtained must have come from either G2 or very late S. Mitotic cells from late S phase can be distinguished from those originating in G_2 by the presence of [^{3}H]-thymidine label in autoradiographs. The mitotic cells were then allowed to grow in fresh medium for another hour to allow them to pass into G_1. These cells were then harvested with unlabeled carrier cells. Nuclei were prepared and fractionated into the RS and SS chromatin, as before. Results of this experiment are shown in Fig. 22, where the distribution of the late replicating [^{3}H]-thymidine pulse-label is shown relative to the distribution of the [^{14}C]-thymidine long-labeled bulk chromatin. The distribution of the [^{3}H]-pulse-labeled DNA in the chromatin fractions follows exactly the distribution obtained for the [^{14}C] bulk chromatin label, indicating that the late-replicating DNA is found in both the RS and SS fractions. Autoradiographic analysis of P. eremicus cells labeled with high specific activity [^{3}H]-thymidine and harvested 2 hours following label addition (including a 1 hour Colcemid treatment) indicates that most of the label is incorporated into the constitutively heterochromatic, short arms of chromosomes (Fig. 23A, also see Kuo, 1979). However, the facultative heterochromatin of the X chromosome is found to replicate very late also (Fig. 23B, and also Kuo, 1979). Thus DNA from this type of heterochromatin would also be included in the [^{3}H] label. The [^{3}H] to [^{14}C] ratio, a measure of the amount of late replicating DNA compared to the bulk DNA in the chromatin fractionation

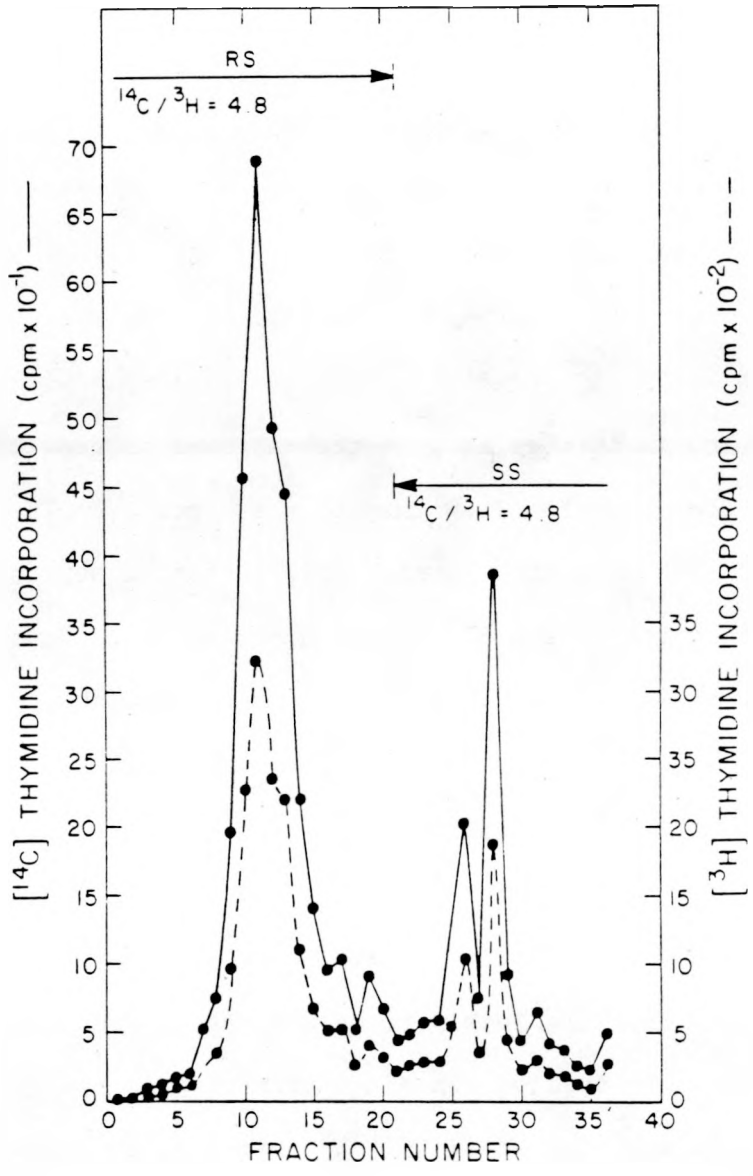

Fig. 22. Distribution of late-replicating DNA in P. eremicus chromatin fractions. Sucrose gradient fractionation of sheared nuclei with distribution of bulk chromatin DNA indicated by [^{14}C] thymidine incorporation (———) and distribution of late replicating DNA by [^{3}H] thymidine incorporation (------).

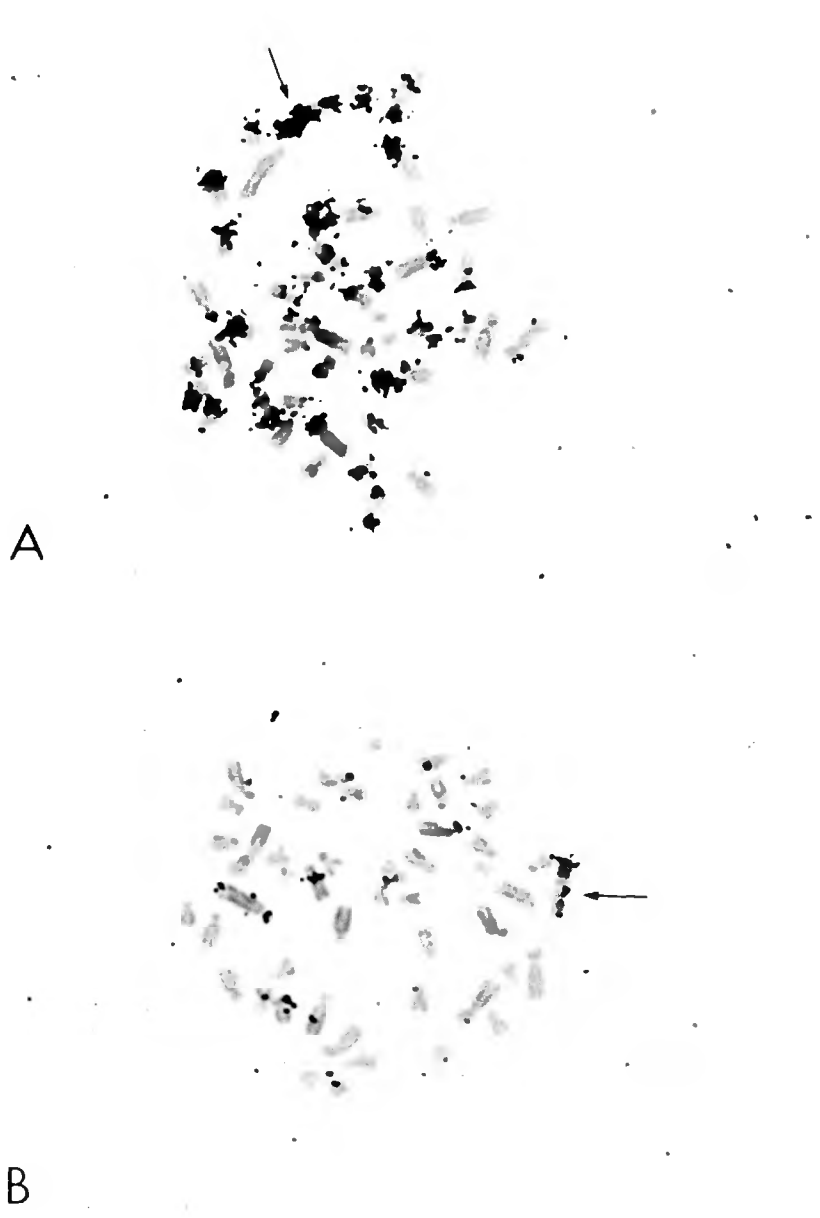

Fig. 23. Autoradiographs showing the late-S replicating DNA in P. eremicus chromosomes. (A) Late-S replicating DNA, showing label over the constitutive heterochromatin in the short arms and centromeres of many chromosomes. Note heavy label over late replicating faculative heterochromation of one X chromosome (arrow). (B) Very late S replicating DNA, showing facultative heterochromatin of X chromosome (arrow) replicating last.

experiment, was identical for both the RS and SS chromatin fractions (Fig. 22). If the mitotic detachment by shaking harvest procedure used to select for cells specifically pulse labeled in late-S was effective, these results indicate that there is no enrichment of late-replicating DNA in either the RS or SS fraction, but that it occurs in both. Autoradiographic analysis of cells obtained during mitotic detachment in this experiment, however, revealed the presence of about 12% labeled interphase cells. Since these cells could have incorporated the [^{3}H] label anytime during S phase, they represent a possible contamination of [^{3}H] label from earlier replicating DNA. The extent of this effect is unknown.

In order to further characterize the RS and SS chromatin fractions by criteria considered to be associated with heterochromatin or euchromatin the fractions were assayed for their relative content of nascent RNA. Nascent RNA should be intimately associated with active genes in euchromatin. P. eremicus cells, with [^{14}C]-thymidine incorporated into bulk DNA, were pulse labeled for 30 sec with [^{3}H]-uridine before harvest and chromatin fractionation. The distribution of the resulting [^{3}H]-uridine label versus the [^{14}C]-thymidine bulk label of chromatin DNA is shown in Fig. 24. It was found that, although ^{3}H label was incorporated into the chromatin in both fractions, the ^{3}H to ^{14}C ratio of the fractions was not the same (Fig. 24). These ratios indicate almost a twofold enrichment of nascent RNA in the SS fraction compared to the RS fraction. These data suggest that the chromatin of the SS fraction may contain a greater proportion of active genes than

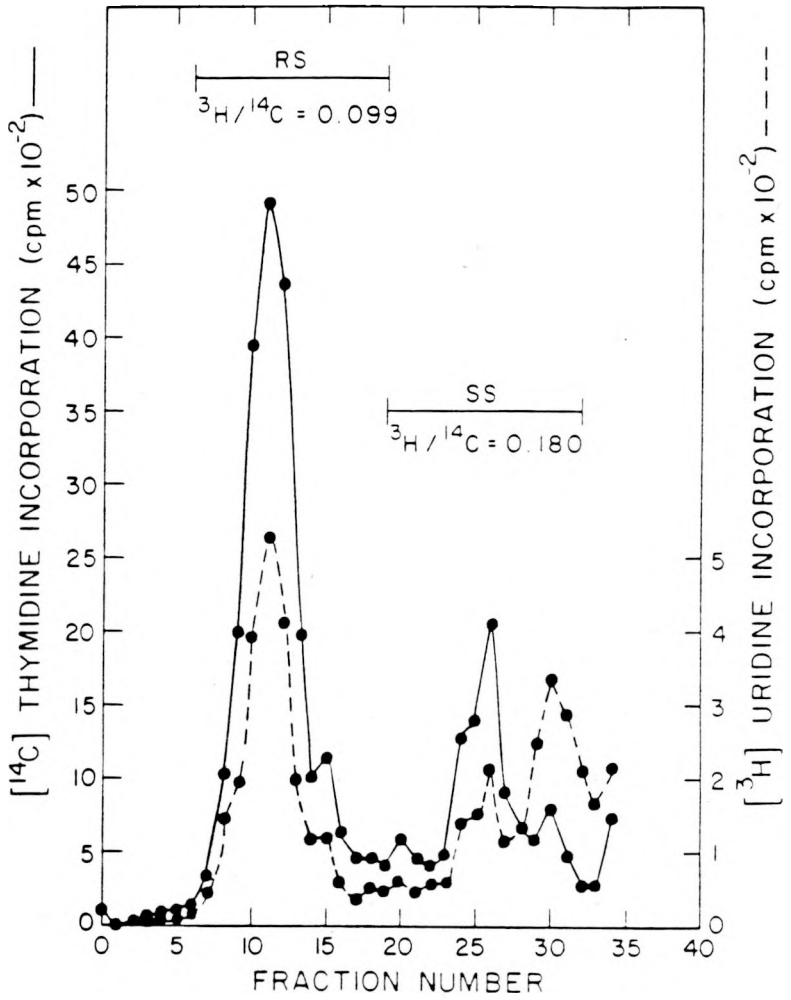

Fig. 24. Rapidly labeled nascent RNA in P. eremicus chromatin fractions. Sucrose gradient fractionation of chromatin from sheared nuclei with the distribution of chromatin DNA indicated by [^{14}C] thymidine incorporation (———) and the distribution of nascent RNA indicated by [^{3}H] Uridine incorporation during a 30 second pulse (------).

the chromatin of the RS fraction, but, again, both fractions show the presence of this probe.

A final characterization of the chromatin fractions was made by determining their relative content of DNA, RNA and protein. Results of these analyses are shown in Table 17. These results indicate that the SS chromatin fraction contains approximately 25% more protein to DNA and almost 65% more RNA to DNA than the RS fraction. Results for the RNA to DNA content are consistent with the nascent RNA labeling data presented previously (Fig. 24).

4. Discussion

The main objective of the chromatin fractionation studies was to provide another approach to the analysis of histone modifications in heterochromatin. This approach would be independent of intra cell line metabolic differences, yet would provide another set of chromatin samples, one of which was enriched in heterochromatin relative to the other, in which histone modifications could be compared. It was hoped that the trends observed in whole cells from the different Peromyscus cell lines could then be tested in this system. It was also hoped that if a reproducible, clean separation of heterochromatin and euchromatin could be developed it would prove very useful for other types of research. In the final analysis, however, the fractionation scheme appears to have been only partially successful. A clean separation of heterochromatin and euchromatin was not obtained, but the fractionation apparently does result in an enrichment of more condensed heterochromatin in the RS fraction versus the SS fraction. This was first suggested by the results of the histone analysis.

TABLE 17

PROTEIN, DNA AND RNA CONTENT OF CHROMATIN FRACTIONS

Analysis	RS Fraction	SS Fraction
Protein by Lowry (μg)	881.3	985.7
DNA by Schmidt-Tannhauser (μg)	229.3	209.1
DNA by Diphenylamine (μg)	209.3	178.3
RNA by Schmidt-Tannhauser (μg)	55.8	81.2
$Protein_L$: DNA_{S-T}	3.8	4.7
RNA_{S-T}: DNA_{S-T}	0.24	0.39

One of the constraints placed upon the analysis of modified histones in chromatin fractions derived from whole nuclei or isolated chromatin is the need to provide proper protection from phosphatase, protease, and deacetylase activity. These precautions are, of course, necessary with whole cells, but the use of many of these inhibitors [sodium bisulfite or phenylmethyl sulfonyl fluoride (PMSF), for example] interferes with the preparation of clean nuclei and chromatin. Use of 100 mM potassium phosphate, although shown to provide ample protection from histone dephosphorylation during the preparation of nuclei in preliminary experiments, was not effective when these nuclei were fractionated. This was evidenced by a 2 to 3 fold dephosphorylation seen in chromatin, relative to whole cells. This dephosphorylation probably occurred when nuclei were sheared using the Virtis, since 100 mM PO_4 was not present during that step, since it interferes with the fractionation in the sucrose gradient. Since some phosphatases are thought to be chromatin bound, these may have been released when the nuclei were broken, thus having plenty of opportunity to dephosphorylate the histones. The results of the histone phosphorylation analysis demonstrated that, although as was shown in histones from whole Peromyscus cells, the MHP-H2A subspecies is always more phosphorylated than the LHP-H2A subspecies, results from the fractionation experiments show that, if the RS fraction is enriched in heterochromatin, the H2A associated with it is not more phosphorylated than that in the SS fraction. However, because of the dephosphorylation discussed before, comparisons of the extent of histone phosphorylation in the fractions are probably minimized. Thus, the results of Gurley et al. (1978), which show a correlation between extent of histone H2A phosphorylation

and constitutive heterochromatin content could not be verified with this system.

The distribution of histones in fractionated heterochromatin and euchromatin has been examined by several investigators. A wide range of results have been reported concerning the overall histone content of these fractions and the distribution of the five major histone classes. Some have reported no differences between the histones of active and inactive, or diffuse and condensed chromatin (Comings, 1967, Pallota et al., 1970, Paul and Duerksen, 1977). However, Gottesfeld et al. (1975) and Montagna et al. (1977) found a depletion of total histone protein in euchromatin fractions when compared to heterochromatin fractions. There have also been several reports that histone H1 is either very much reduced relative to the other histones (Shirley and Anderson, 1977; Simpson and Reeck, 1973; Pederson and Bharjee, 1975; Lau and Ruddon, 1977) or absent (Berkowitz and Doty, 1975) in transcriptionally active euchromatin fractions. In direct contrast, H1 has also been reported to be reduced or absent in a nuclease digested fraction consisting of constitutive heterochromatin, relative to bulk chromatin (Musich et al., 1977). Others have reported an enrichment of H1 in euchromatin fractions, apparently generated by a selective depletion of core histones in these regions (Ryffel et al., 1975). The results of the experiments reported here show that the RS fraction, which is believed to be enriched in heterochromatin, is depleted of H1 histone, relative to the other histones when compared to the SS fraction. Both chromatin fractions and sheared nuclei were depleted of H1 histone relative to whole cells, however, suggesting a selective loss during the fractionation procedure. Concerning the individual core histones,

condensed heterochromatin fractions have been reported to be enriched in H4 and depleted in H2A (Johnson et al., 1973), while some transcrip tionally active euchromatin fractions have been found to have increased amount of H2A, H3 and H2B (Bonner et al., 1973; Shirley and Anderson, 1977). In contrast to these results, the RS fraction of this report appears to be depleted in histone H4 and enriched in histones H2A and H2B relative to the SS fraction.

The discussion above suggests that there have been as many variations in histone distribution between chromatin fractions as there have been fractionation methods. These varying results probably reflect the use of different tissues, varying details of chromatin preparation, the extent to which cross-contamination of fractions occurred, and the methods in which the histones were extracted and analyzed. Also, although there have been numerous reports concerning the overall histone content of heterochromatin and euchromatin, very little information has been available concerning the modification of histones in chromatin fractions. The advent of more sensitive gel electrophoresis methods (Alfageme et al., 1974) has provided greater resolution of histone species necessary for this type of analysis. Thus, the study of the subspecies of H2A in chromatin fractions presented here represents the first report of histone H2A variants in fractionated chromatin. In addition, the fractionation of P. eremicus chromatin, already shown to contain a large amount of heterochromatin displaying a particular MHP to LHP H2A ratio, provides a baseline for comparison. The same trend towards an increase in the MHP-H2A subspecies with increasing hetero-chromatin content found in whole Peromyscus cells was found in the

chromatin fractions, suggesting that an enrichment of heterochromatin in the RS fraction occurs in the fractionation scheme.

Histone modification, via acetylation, in chromatin fractions has been investigated before, however. Johmann et al (1973) reported that the histones from condensed and extended chromatin fractions prepared by sonication and differential centrifugation of mouse liver were quantitatively similar when compared on long, polyacrylamide gels. Those results do not support the evidence presented here that heterochromatin contains more unacetylated arginine-rich histone. The electrophoretic gel system used by Johmann et al. (1973) did not employ detergents and, thus, did not produce the high resolution produced by present gel systems. In the absence of detergent, H2A runs between H2B and H4, possibly interferring with the acetylated species of H4. In addition, no attempt was made by those investigators to prevent histone deacetylation during the preparation of nuclei, a procedure later shown to be necessary (this report and Perry et al., 1979). Possibly as a result of these problems, only two species of H4 (only one of them modified) were resolved from their chromatin fractions. Others have shown the presence of at least 3 or 4 modified species of histone H4 in eucaryotic cells (D'Anna et al., 1977; Davie and Candido, 1978; Simpson, 1978; Treigyte and Gineitis, 1979; Cousens et al., 1979; Levy-Wilson et al., 1979). Finally, greater resolution of overlapping peaks can be obtained by the use of a curve resolver (Panyim and Chalkley, 1969; Ruiz-Carrillo et al., 1974; D'Anna et al., 1977), a procedure not available to those investigators. Acetylation of histones in fractionated chromatin was also investigated in a very general way by Levy-Wilson et al. (1977). Overall acetylation of histones was compared

in chromatin fractions obtained by either DNAase II or hydrodynamic shearing of Drosophila chromatin labeled with [^{3}H]-acetate. It was shown that labeled acetate was associated with Drosophila histones H2B, H3 and H4, the highest specific activity being associated with H3. Further, the specific activity of acetate was highest in histones recovered from the template-active, euchromatin fraction, suggesting a greater proportion of acetylated species in that fraction. By comparison, the bulk chromatin, presumably containing heterochromatin, was less acetylated. These results support in a general way the evidence presented here that heterochromatin displays a higher percentage of unacetylated arginine-rich histone than bulk chromatin. In a more recent report, Davie and Candido (1978) found that multi acetylated species of histone H4 were enriched in a template-active fraction prepared by DNAase II digestion of trout testis chromatin. In fact, most of the tri- and tetra-acetylated species of H4 appeared to be in this fraction, which represented 7-10% of the total chromatin. Results similar to these were obtained by Levy-Wilson et al (1979), using a micrococcal nuclease digested fraction of trout testis shown to be enriched in template-active chromatin. Thus, although there have been some investigations concerning arginine-rich histone acetylation in putatively active chromatin fractions, until this work on Peromyscus chromatin, no attempt has been made to characterize arginine-rich histone acetylation in a system enriched in heterochromatin. The results of the present work suggest that condensed heterochromatin is depleted in acetylated species of arginine-rich histones and that these unacetylated histones are enriched in the rapidly sedimenting chromatin fraction of P. eremicus cells.

Identification of the RS and SS chromatin fractions by satellite DNA analysis, DNA base content, content of late replicating DNA, and presence of nascent RNA proved to be difficult. All of these tests indicated there was a cross-contamination between fractions. Thermal denaturation analysis of the DNA from the fractions indicated the presence of a GC-rich satellite in both fractions with a possible enrichment in the RS fraction although this could not be quantified. In experiments to show the presence of late replicating DNA the failure to show any enrichment of this late replicating DNA in either of the chromatin fractions is inexplicable if, indeed, there is an enrichment of heterochromatin in the RS fraction and late replicating DNA is present in this type of chromatin. Experimental design problems, as discussed in section III.3.3, may have been the cause. Tests for the presence of nascent RNA also indicated a cross-contamination of the fractions, since it was found in both. Nascent RNA did appear to be enriched in the SS fraction, although not to the extent described by others. For example, Berkowitz and Doty (1975) found two thirds of the total labeled nascent RNA was associated with an active chromatin fraction representing only 10% of sonicated chick reticulocyte chromatin. About one half of the labeled nascent RNA was found associated with the SS fraction representing about 30% of fractioned P. eremicus chromatin in the experiments reported here. Experiments of this nature to establish the presence of nascent RNA may not be valid, however, if, as suggested by Savage and Bonner (1978) and Seidman and Cole (1977), nascent RNA transcripts are dislocated from the chromatin during preparation and preferentially co-sediment with slowly sedimenting chromatin during fractionation. Since a large part of the

rapidly labeled RNA in the experiments reported here is located in the upper part of the gradient this may be occurring here, also. The presence of some rapidly labeled RNA in both fractions would suggest that not all of the RNA has become dislocated however.

One of the approaches used to test for presence of the GC-rich satellite in the chromatin fractions was relatively successful, however. Although density gradient analysis of P. eremicus DNA using CsCl did not completely separate the satellite DNA from the main band DNA, quantitation of the relative amount of this satellite DNA was possible using the curve resolver. Attempts to separate the GC-rich satellite from main band DNA, using the Ag^{+}-$CsSO_4$ gradient method described by Jensen and Davidson (1966), as applied to P. eremicus DNA by Hazen (1977), were unsuccessful, however. Using CsCl gradient analysis, the satellite DNA was clearly present in both fractions, although enriched in the RS fraction. These results were similar to those obtained by others, using other fractionation schemes, where satellite DNA is found to be enriched in the rapidly sedimenting or nuclease-resistant fraction (Johmann et al., 1973; Paul and Duerksen, 1976; Godsen and Mitchell, 1975; Mattoccia and Comings, 1971). Quantitation of the relative amount the satellite DNA to total DNA in the experiments reported here demonstrated that P. eremicus DNA contains about 35% satellite. This satellite has been shown, by in situ hybridization to be located in the constitutively heterochromatic short arms of P. eremicus chromosomes (Hazen et al., 1977), thus measurement of the relative proportion of satellite DNA to total DNA is probably a good estimate of heterochromatin content. Indeed, the percentage of satellite DNA in whole P. eremicus cells is identical to the percentage of constitutive

heterochromatin in P. eremicus cells (determined by arm length measurements on C-banded chromsome preparations). Thus, measurement of the relative amount of satellite DNA extracted from the chromatin fractions provides a method for estimating the constitutive heterochromatin content of these chromatin fractions. These data suggest then that the RS chromatin fraction is enriched in constitutive heterochromatin, containing about 42% heterochromatin relative to whole P. eremicus cells containing 35% heterochromatin. The SS fraction however is depleted in constitutive heterochromatin, containing about 27%.

It is clear that the chromatin fractions obtained by the method reported here are not cleanly separated inactive, GC-rich satellite-containing heterochromatin and template-active, satellite-free euchromatin. As a result, there is a substantial amount of heterochromatin in both fractions. Another, independent, assessment of the heterochromatin composition of these fractions can be made on the basis of their extent of histone modification. This is accomplished using results from whole Peromyscus cells in which P. boylii and P. crinitus cells were compared to P. eremicus cells. The H2A histone data is expressed in terms of the percentage contribution of the MHP-H2A to total H2A histone versus the percentage of heterochromatin present in the genomes of these mice. When this is done, a relationship such as that shown by the solid line in Fig. 25 is developed. All data from P. boylii and P. eremicus whole cells and nuclei, representing four experiments, have been included. If now the ratios of MHP H2A to total H2A obtained for the chromatin fractions are located on this line, the percentage contribution that heterochromatin makes to these chromatin

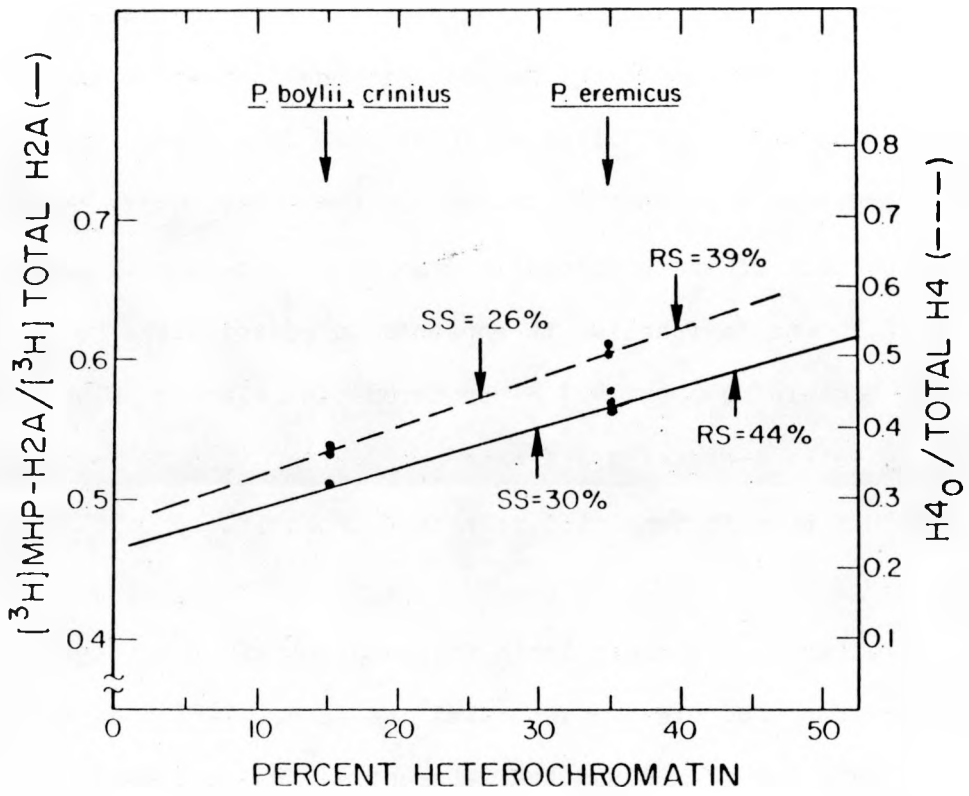

Fig. 25. Heterochromatin content of rapidly sedimenting (RS) and slowly sedimenting (SS) P. eremicus chromatin fractions based on histone modifications. The contribution of [^{3}H] lysine incorporation into the MHP-H2A variant to total H2A[^{3}H]lysine incorporation versus constitutive heterochromatin content, based on C-banding, (———) is shown for P. boylii whole cells and P. eremicus whole cells and nuclei. The contribution of unacetylated H4 ($H4_o$) to total H4 histone versus constitutive heterochromatin content, based on C-banding, (------) is shown for P. boylii, P. crinitus whole cells, and P. eremicus whole cells and nuclei. Location of the contribution of the MHP-H2A to total H2A for each chromatin fraction on the solid line predicts that the RS fraction is composed of 44% heterochromatin while the SS fraction contains 30% heterochromatin. Similarly, location of the percentage contribution of unacetylated H4 obtained for each chromatin fraction on the dashed line predicts that the RS fraction is composed of 39% heterochromatin while the SS fraction contains 26% heterochromatin.

fractions can be determined (Fig. 25). Histone H2A subspecies results thus indicate that the RS fraction contains 44% heterochromatin, while the SS fraction contains 30%. A similar, yet independent, analysis can be made with histone H4 acetylation data derived from Peromyscus cell lines. The dashed line in Fig. 25 represents the relationship between the percentage contribution of unacetylated species of H4 ($H4_o$) to total H4 histone and the percentage of heterochromatin in the genome of P. crinitus, P. boylii and P. eremicus. Location of the percentage contribution of unacetylated H4 obtained for each chromatin fraction on this line predicts that the RS fraction is composed of 39% heterochromatin while the SS fraction contains 26% heterochromatin (Fig. 25). Thus, by two independent measurements of unrelated histone modifications, the RS chromatin fraction of P. eremicus is found to contain 39%-44% heterochromatin. This does, indeed, represent an enrichment over the 35% heterochromatin content of whole P. eremicus cells. The SS fraction, however, by these measurements contains 26-30% heterochromatin, a depletion from the 35% heterochromatin content of whole P. eremicus cells. This 26-30% heterochromatin content of the SS fraction still represents a substantial amount, however, considering the "low heterochromatin" cell lines used in previous studies contained only 15% heterochromatin.

These estimates of heterochromatin content based on histone modifications can be shown to be very realistic ones if they are compared to estimates of heterochromatin content derived from satellite DNA content, discussed previously. This comparison is made in Table 18, where it can be seen that the RS fraction is estimated to contain 41.7% heterochromatin by satellite analysis and 39-44% heterochromatin by the

TABLE 18

RELATIONSHIP OF SATELLITE DNA CONTENT, HISTONE MODIFICATIONS AND CONSTITUTIVE HETEROCHROMATIN CONTENT OF P. EREMICUS CELLS AND CHROMATIN FRACTIONS

Sample	Heterochromatin Content by Satellite Analysis*	Heterochromatin Content by Histone Modification+
RS Fraction	41.7%	39-44%
Whole Cells	35.0%	35%
SS Fraction	26.9%	26-30%

*Based on percentage contribution of satellite DNA (ρ = 1.703 g/cc) to total DNA determined by CsCl buoyant density gradient analysis (Fig. 20, Table 16).

+Based on the relationship between the proportion of MHP-H2A to total H2A and the proportion of unacetylated H4 versus constitutive heterochromatin content shown in Fig. 25.

analysis of histone modifications. The SS fraction is estimated to contain 26.9% heterochromatin by satellite analysis and 26-30% hetero chromatin by analysis of histone modifications. The similarity of these independent estimates of heterochromatin content is very striking, suggesting that the analysis of subtle, biochemical modifications of histones can be a very powerful tool in the study of chromatin.

Thus, the chromatin in nuclei from P. eremicus cells can be fractionated by hydrodynamic shear and steep sucrose gradient centrifugation into 2 fractions, differing in their content of constitutive heterochromatin. The basis of this fractionation is still unclear, however. Because the slowly sedimenting fraction is found to contain a substantial amount of this heterochromatin, the basis of this fractionation can not be entirely due to differential resistance of condensed and extended chromatin to hydrodynamic shear. Sedimentation in sucrose gradients of the sheared chromatin into two distinct peaks may be explained by chromatin conformation or by differential association of the DNA in this chromatin with protein or RNA. The demonstration that the RS and SS chromatin fractions contain different amounts of protein and RNA relative to their DNA content supports the latter suggestion.

PART IV. SUMMARY AND CONCLUSIONS

The overall intent of the studies presented in this dissertation was to examine the role of biochemical modifications of the histone proteins in constitutive heterochromatin. Reversible modifications of chromatin proteins, in particular the histones, may be involved in the modulations of chromatin structure which are necessary for controlling gene expression, gene replication and gene segregation. The hypothesis to be tested here was that certain specific histone modifications may occur in constitutive heterochromatin which could be responsible for the condensed and genetically inactive nature of this chromatin.

This hypothesis was tested in two ways. First by a comparative study of histone modifications in cell lines from the deer mouse genus, Peromyscus, differing in their genomic content of constitutive heterochromatin. Definite differences in the content of certain histone modifications were found in these cell lines, corresponding with their content of constitutive heterochromatin. The high heterochromatin cell line contained a greater proportion of a more hydrophobic H2A subfraction, compared to histone H2A from a low heterochromatin cell line. Furthermore, this more hydrophobic (MHP) H2A variant was found to be two times more phosphorylated than the other, less hydrophobic (LHP) H2A variant.

The high heterochromatin cell line was also found to contain a greater proportion of unacetylated forms of histones H2B, H3 and H4 when compared to low heterochromatin cell lines. Thus, acetylation of H2B, H3 and H4 was found to be inversely proportional to constitutive heterochromatin content in the Peromyscus cell lines. This relationship was observed even when histones were hyperacetylated by treatment of

cell cultures with sodium butyrate, although a significant amount of previously unacetylated nucleosomal histones became acetylated by this procedure. These results show that modifications such as histone H2A structural variation, H2A phosphorylation, and nucleosomal histone acetylation are somehow related to constitutive heterochromatin and provide further support to the concepts that H2A phosphorylation is related to higher order chromatin structure (Gurley et al., 1978b) and histone acetylation is related to genetic activity of chromatin (Allfrey, 1977).

Secondly, the hypothesis concerning a relationship between histone modifications and constitutive heterochromatin was tested directly by examining histone modifications in fractions of hetero- and euchromatin from the high heterochromatin cell line, Peromyscus eremicus. Although the scheme employed to fractionate chromatin into these two types of chromatin did not completely separate constitutive heterochromatin from euchromatin, two chromatin fractions were produced which differed in their content of heterochromatin and histone modifications. The same relationships between modification of the histones and content of heterochromatin that were found when comparing histone modifications in cells with different heterochromatin contents were observed in these fractions, demonstrating, again, a correlation between a larger proportion of the more phosphorylated, MHP-H2A variant, a larger proportion of unacetylated arginine-rich histones (H3 and H4) and an enrichment of constitutive heterochromatin.

Further, the heterochromatin content of these chromatin fractions could be determined using the histone modification data as a basis for calculation. It was found that, using measurements of two independent

histone modifications, the predicted heterochromatin contents were very close to those estimated by satellite DNA analysis. Thus, again, the same histone modifications were shown to be related to constitutive heterochromatin content.

Therefore, all of the observations of this work indicate that (1) heterochromatin contains a greater amount of the MHP-H2A variant than euchromatin, (2) this H2A is more phosphorylated than the LHP-H2A variant, and (3) heterochromatin contains a lesser amount of nucleosomal histone acetylation than euchromatin.

This investigation of histone modifications in the Peromyscus system has led to some interesting conclusions about the structure of constitutive heterochromatin. It is reported here that constitutive heterochromatin content is correlated with the relative amount of the more phosphorylated, MHP-H2A variant in Peromyscus cells. This suggests that the contribution of this H2A variant to nucleosome structure may somehow confer an overall increase in chromatin condensation. The amino acid substitutions in nucleosomal histone variants of mammalian cells occur mainly in the hydrophobic region of these proteins. Since these are likely to form the contact regions between histones in the nucleosome octomer, hybrid nucleosome cores may differ in stability and closeness of their histone-histone interactions (Thomas, 1978). These associations may also affect the higher order structure of chromatin. Gurley et al. (1978b) suggested that H2A phosphorylation may play a role in modulating chromatin structure to form condensed heterochromatin through bridging between nucleosomes of adjacent solenoids, perhaps with nonhistone proteins. The results reported here demonstrate that it is the MHP-H2A variant that is most likely to be involved in this

condensation, since it is two times more phosphorylated than the LHP-H2A variant. These intersolenoidal bridges may form a major part of the proposed higher structure "loop"-type organization of heterochromatin, mentioned previously (see I.1). The recent finding by Martinson et al. (1979) that histone H2A is conjugated to the nonhistone protein, ubiquitin, in some nucleosome cores suggests that H2A may be capable of participating in such a bridging relationship in condensed chromatin.

Thus, although the exact nature of the association is not known, it appears that the more phosphorylated, MHP-H2A variant is somehow involved in the structure of heterochromatin. It was discussed earlier (II.2.4) that there is a correlation between histone H2A variants and differentiation in developing systems. This observation is consistent with the idea that these nucleosomal histone variations are involved in the modulation of chromatin structure necessary for differential gene expression during development. In a similar way, these nucleosomal variants are probably involved in the modulation of chromatin structure leading to the condensation of heterochromatin. Due to the relatively unchanging nature of the constitutive heterochromatin content characteristic of a particular genome, however, the histone H2A variant composition of constitutive heterochromatin probably represents a more permanent example of nucleosomal changes leading to higher order structural changes in chromatin.

I have also found that the extent of arginine-rich histone acetylation in Peromyscus cells is inversely correlated with the heterochromatin content of these cells. Thus, relative amounts of unacetylated H3, H4 and H2B were found to be proportional to the amount of constitutive heterochromatin in the genome. The relationship of

these unmodified histones to the highly condensed structure of constitutive heterochromatin may simply be a lack of accessibility of these core histones to acetylase activity because they become deeply buried within chromatin coils when it is highly condensed. Two lines of evidence indicate that inaccessibility to enzymes is probably not the cause for this lack of acetylation, however. Treatment of cells with sodium butyrate demonstrated that a substantial proportion of previously unacetylated histones were available to be acetylated under hyperacetylating conditions where histone deacetylase is inhibited. Also, the observation that histone H2A phosphorylation is higher in constitutive heterochromatin (Gurley et al., 1978c) suggests that histones are accessible to phosphorylating enzymes in condensed chromatin.

A more interesting possibility, however, is that histone acetylation activates gene transcription. The enrichment of multiacetylated forms of arginine-rich histones in transcriptionally active chromatin is now well known (Allfrey, 1977; Davie and Candido, 1978; Levy-Wilson et al., 1979). These findings suggest that histone acetylation is an important factor in the modulation of chromatin structure necessary for gene transcription. This alteration in chromatin structure would most probably occur at the nucleosomal level, causing changes in the interactions between parts of the nucleosome. A model for the structure of the nucleosome, proposed by Weintraub et al. (1976) provides a mechanism by which this might occur. In this model DNA is coiled about two symmetrically paired histone tetramers [$(H2A\text{-}H3\text{-}H4\text{-}H2B)_2$]. The model proposed that the nucleosome contains a two fold axis of symmetry, along which it can open up into two half-nucleosomes, thus allowing the DNA to

be exposed for gene transcription and gene replication without removal of the histones. Furthermore, acetylation of the histones may favor this open state, by weakening the contacts between the tetramers and DNA. Thus, if histone acetylation is an important factor in the modulation of genetic transcription, the observed correlation between constitutive heterochromatin and unacetylated histones in Peromyscus cells reflects the results of deacetylation associated with suppression of genetic activity, instead of a passive absence of acetylation as a result of inaccessibility.

The results of the experiments presented in this dissertation raise several interesting questions. Are the histone modifications observed in Peromyscus cells specific to constitutive heterochromatin, or are they a necessary requirement for condensed facultative and differentiation-associated condensed inactive chromatin, as well? Would other organisms rich in constitutive heterochromatin demonstrate these same specific histone modifications? Does hyperacetylation through the use of sodium butyrate decrease the amount of heterochromatin bandable by the C-band technique? If rearrangements were made in the P. eremicus genome in it's heterochromatic component, would these histone modifications change? The answers to these questions could help us extend our understanding of the nature of chromatin inactivation and condensation through heterochromatization.

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