STUDIES ON THE CHROMATIN OF THE CELLULAR SLIME MOLD *DICTYOSTELIUM DISCOIDEUM*

Thesis by

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To my parents

with love and gratitude

Subtitle:

All you ever wanted to know about slime mold chromatin, but were afraid to ask

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ABSTRACT

This thesis concerns the chromatin of Dictyostelium discoideum which has been characterized chemically, physically, and biochemically. The chromatin was carefully purified by differential centrifugation using nuclease and protease inhibitors followed by a brief, controlled Staphylococcal nuclease digestion. It has a chemical composition in terms of mass of 1.0 part DNA to 0.18 part RNA to 0.98 part histone to 1.02 part nonhistone protein. Its ultraviolet absorption spectrum is typical of chromatins from other eukaryotes.

The histones have been extracted from chromatin with 0.4 N H_2SO_4 and characterized on three gel systems. Three are equivalent to histones in higher eukaryotes and have the following molecular weights: H1 is 20,000; H3 is 14,900, and H4 is 12,000. Another one is not found in higher eukaryotes, but may be present in lower eukaryotes other than Dictyostelium. It is termed H6 and has a molecular weight of 15,800. There are no equivalents of the higher eukaryotic histones H2a and H2b.

The slime mold histones were also analyzed by ion-exchange chromatography. H1 elutes with the weakly bound proteins, while H3, H4, and H6 elute with the tightly bound peak. No proteins elute with calf thymus histones H1, H2a, or H2b indicating that slime mold H1 is less basic than calf H1 and confirming that H6 is not equivalent to H2a or H2b.

The chromatin may be separated into two fractions by shearing briefly with Staph. nuclease, centrifuging, reshearing the insoluble pellet, and centrifuging again. The first supernatant contains 5% of the total DNA

and is the first sheared chromatin fraction. The second supernatant contains 80% of the DNA. Both fractions have the same histone compositions, but differ in their mass ratios of nonhistone chromosomal proteins (NHCP). The NHCP are characterized on gels and differences are pointed out.

A subset of NHCP are extracted and tentatively identified as actin-like and myosin-like proteins on the basis of solubility and molecular weight. The actin-like protein accounts for approximately 5% of the total NHCP mass.

The basic unit of structure in the chromatin is the nucleosome. In Dictyostelium it is 98.6 Å in diameter and has a sedimentation coefficient of 11.5S. It contains 187 b.p. of DNA consisting of a 137 b.p. core and a 50 b.p. linker. Approximately 50% of the chromatin is protected from Staph. nuclease digestion, but this decreases when protease activity is not inhibited. When the chromatin is melted, four transitions are observed at 54.5°, 66.7°, 74.9°, and 79.7°. The structure of Dictyostelium chromatin is very similar to that seen in higher eukaryotes.

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A Review of both the Regulation of Gene Expression and the Life Cycle of *Dictyostelium discoideum*

This thesis concerns the chromosomal proteins of Dictyostelium discoideum and their role in chromatin structure. Its purpose is to better understand the regulation of gene expression at the level of transcription. The slime mold is an appropriate subject because of its simple developmental cycle, small genome size, and large fraction of expressed single-copy DNA (Firtel, 1972). Before discussing the specific subjects of this research, I would like to review some related work on the regulation of gene expression and the life cycle of Dictyostelium. Each chapter following this review will concern itself with other specialized work on that particular topic.

Each eukaryotic cell contains enough DNA within its nucleus to code for tens of thousands of average proteins. However, not all of this information is expressed as proteins in any one cell type. Use of this information must be restricted to the requirements of a particular moment and place, and this restriction may occur at several levels. First, the DNA may be repositioned, amplified, removed, altered, or masked to change the amount of information available for transcription (Perlman et al., 1976; Gall, 1969; Tobler et al., 1972; Farrace et al., 1976; Tonegawa, 1976; Pellegrini et al., 1977; Bonner et al., 1973). Second, the RNA may be processed to remove specific molecules or portions of them (Lewin, 1975; Derman et al., 1976; Bastos and Aviv, 1977; Hames and Perry, 1977; Davidson et al., 1977). Third, the translation of the RNA may be altered by either storing some RNA molecules or accelerating their use (Gurdon et al., 1973; Nemer et al., 1975; Lodish, 1971). Several lines of evidence support the first level of restriction.

The Entire Genome is Not Expressed at one Time in One Cell

The number of genes expressed as mRNA in higher eukaryote s been estimated and Galau et al. (1976) found that 20,000 - 30,000 genes are expressed in the sea urchin from oogenesis to the feeding pluteus stage. The total sea urchin genome could code for many more proteins, but only a smaller number are needed. Galau et al. found that during development of the sea urchin different sets of structural genes are expressed at various stages. Using the gastrula as a reference stage, they proved that other stages expressed both similar and different mRNA sequences. All of the tissues studied contained a common set of 1,000 - 1,500 genes that are called "housekeeping genes." These genes must be expressed in all cell types for survival, but the remaining genes are only needed at certain times and in some cells.

If all of the DNA in the Dictyostelium genome coded for average proteins, there would be enough for 45,000 proteins. This is a far greater number than the slime mold appears to need during its cycle of existence. A bacterium, like E. coli, is only slightly less complicated than the slime mold. It has enough DNA for 4,500 average proteins, but only 500 to 1,000 have actually been mapped (Hood et al., 1975). Estimates have been made of the actual number of expressed RNA sequences in mRNA for the slime mold. Firtel (1972) found that 56% of the single copy DNA is expressed in the portions of the life cycle which can be studied and this is equivalent to between 10,000 and 20,000 average proteins (Lodish et al., 1973). Total cellular RNA was used for this measurement. If the primary transcripts differed greatly in size from mRNA, the estimate of the number of genes would be affected, but the

hnRNA of slime mold is only 25% larger than the mRNA (Firtel and Lodish, 1973). Different fractions of the single copy DNA are expressed at different stages of development, but the complexity of the RNA sequences in common between the tested stages of development is equivalent to 18.8% of the single copy DNA. These would be "housekeeping genes." Therefore, 37.2% of the expressed DNA must be in regulated genes that are not always required. The slime mold mRNA molecules also contain an oligo-deoxyadenosine-5'-phosphate sequence near their 3' termini, which may signal the end of the transcription unit. There are 14,000 to 15,000 corresponding oligo-thymidine-5'-phosphate sequences in the slime mold genome (Firtel et al., 1976). This number correlates well with the first estimate for expressed proteins.

Regulation is Not Due to Loss of DNA

Information is not usually lost from the cell because in several cases tissues and even entire organisms with many cell types can be regenerated from a single cell or a transplanted nucleus of that organism. A carrot cell can be grown in tissue culture until it develops into a whole carrot with all of its organs (Steward et al.,1970), but these are undifferentiated somatic cells. There are two cases of regeneration in differentiated animal cells. One is Wolffian regeneration of the eye lens from iris cells (Yamada, 1967). The second is the regeneration of an adult amphibian after the transfer of a somatic cell nucleus to an enucleate egg (Gurdon, 1974). From seven to eight percent of the intestinal epithelial cell or skin cell nuclei that were transferred could support tadpole development to a stage containing functioning muscle, nerve, lens, heart, blood, etc. cells. This would be impossible if portions of the

genome had been irreversibly eliminated from the original transplante nuclei. Therefore, the development of both intestinal and skin cells does not require any loss of information, since this information can be reused later.

During Dictyostelium development a slug is formed containing two cell types in a constant ratio. If the slug is cut into several fractions, the cells of each fraction will regulate with time to give the normal ratio of stalk and spore cells (Raper, 1940). Again none of the information is lost from either cell type.

Template Active Chromatin

The synthesis of RNA can be localized to certain sites in the chromatin (Gall and Pardue, 1969), which can be thought of as a linear array of genes with only certain ones being expressed. The regions which are being expressed are termed template-active, since the DNA there is acting as a template for RNA. The inherent functional difference of the active regions is RNA synthesis and it must be reflected as either a structural and/or a compositional difference in the chromatin. Weintraub and Groudine (1976) have found that globin genes from red blood cell nuclei are sensitive to DNase I digestion, but not ones in fibroblast nuclei. Since the red blood cells are actively making globin, their globin genes should be in template-active regions. The authors believe that there is a structural difference in these genes that makes them sensitive to the enzyme, but this is not necessarily due to the presence or absence of nucleosomes. Lacy and Axel (1975) and Kuo et al. (1976) have shown that cDNA prepared to total mRNA hybridizes almost identically to both total and monomer DNA. Nucleosomes must be present in template active regions for monomer DNA

to contain transcribed genes. The difference in template-active regions may arise from three possibilities: 1) a temporary structural difference in the nucleosome; 2) a lower density of nucleosomes along the DNA; or 3) the association of specific acidic, nonhistone proteins. Weintraub et al. (1975) found that four different histones can exist as a tetramer, and they believe that two associated tetramers in the nu-body can separate exposing the DNA for transcription. This would create an altered conformation. Marushige and Bonner (1971) and Gottesfeld et al. (1975) have noted a somewhat lowered histone to DNA ratio in template active chromatin implying that there are fewer nucleosomes in these regions. However, these missing histones may have been artifactually degraded or have become associated with other regions of the DNA. Foe et al (1976) have seen a lowered density of nucleosomes in regions of the chromatin underlying nascent ribosomal RNP (ribonucleoprotein) fibers. Gottesfeld et al. (1975) have detected differences in the complement of the NHCP associated with template active chromatin. None of these lines of evidence have absolutely established the nature of template active chromatin.

A Model for the Regulation of Gene Expression

Since only a portion of the information in the genome is expressed, a mechanism must act to regulate this expression. Britten and Davidson (1969) have proposed a model for the regulation of gene expression, which follows the lines of one proven in prokaryotes (Jacob and Monod, 1961) and could regulate specific sets of genes. Both spatial and temporal alterations in the pattern of gene expression during development can be accommodated. The model contains five elements: 1) a sensor gene, which

detects an external signal and activates the integrator gene(s); 2) one or more integrator genes, which produce the internal signal or activator for a battery of genes; 3) the activator (either RNA or protein) which binds to several receptor genes; 4) one or more receptor genes, which lie adjacent to producer genes and respond to activators from one or more sensors by activating the producer gene; and 5) the producer gene, which finally yields the RNA molecule needed by the cell and acts in concert with the other producers of the gene battery. The regulation of gene expression is mediated by the regulator molecules. These molecules must contain considerable information within their structure, since they must interact specifically with their target receptor genes. The two most likely candidates for these activators are proteins and RNA molecules.

In prokaryotes several cases of protein activators and deactivators are well characterized. Both the lac (Gilbert and Müller-Hill, 1966; Riggs et al., 1968) and the lambda (Chadwick et al., 1970) receptor proteins have been shown to bind to specific DNA operator sequences and to regulate transcription of the adjacent structural genes. Catabolite-regulating protein (CRP) binds strongly to DNA and this binding is enhanced by cyclic AMP (Riggs et al., 1971). CRP and cAMP bind to a promoter site adjacent to the operator and together with RNA polymerase, they form a proper initiation complex for transcription of the structural, or producer, genes. Restriction endonucleases also bind to specific, short sequences of DNA (Smith and Wilcox, 1970).

Eukaryotes also contain DNA binding proteins. Rat liver DNA methylase forms a tight complex with DNA at 37°C (Drahovsky and Morris, 1971). A

specific estradiol-receptor protein binds to sites in the chromatin and seems to affect gene expression (Yamamoto and Alberts, 1975; Rosen et al., 1973). The nonhistone chromosomal proteins (NHCP) of the nucleus contain species which specifically bind DNA (Sevall et al., 1975; Wang et al., 1976). Park et al. (1976) have shown that NHCP from S-phase HeLa chromatin allow E. coli RNA polymerase to transcribe histone genes in G 1-phase chromatin. The histone genes were not transcribed without the addition of S-phase NHCP and increasing doses of NHCP yielded increased transcription of the histone genes. This kind of a molecule could easily be the activator that Britten and Davidson envisioned.

The other candidate for an activator within the nucleus is RNA. Large amounts of RNA are synthesized and degraded without ever leaving the nucleus (Lewin, 1975). Two possible roles have been proposed for this heterogeneous, nuclear RNA (hnRNA). It might be a precursor to messenger RNA (mRNA) and/or a gene activator (Britten and Davidson, 1969; Dickson and Robertson, 1976; Morgan and Wells, 1968; Sivolap and Bonner, 1971). A sequence of 17 nucleotides defines a unique region in the human genome (Britten and Roberts, 1969). Small RNA's could specifically detect the regulator sequences. However, a role for these RNA's has not been unambiguously defined as yet.

The activators needed for gene regulation would be present in very low levels. One E. coli cell contains one operator site per lac operon and approximately ten molecules of repressor protein (Gilbert and Müller-Hill, 1966). The techniques now available can not detect a small number of protein molecules per genome. The minimum detectable number is about 4×10^3 per eukaryotic genome (Garrard et al., 1974). The situation is not much better with RNA unless a specific probe is available.

The Life Cycle of *Dictyostelium discoideum*

The slime mold leads a two-sided life with a vegetative and a developmental phase. During the vegetative phase it exists as individual amoebae, but when the food supply is exhausted, they begin emitting cAMP as a signal to initiate the developmental phase. About 10^5 cells stream towards a center and form a slug, which searches for light and avoids its own ammonia wastes. The cells in the slug develop into prestalk cells in the anterior portion and prespore cells in the posterior. Finally the slug settles and the spore cells crawl up a thin tendril of stalk cells into the air. The spores are scattered to germinate when food is available (for review see Loomis, 1975).

The development of *Dictyostelium* is a good example of gene regulation on the morphological level, as already mentioned, and also on the biochemical level. A number of enzyme activities appear only at specific stages of development in the slime mold. After they have served their purpose they disappear. Some examples are N-acetyl glucosaminidase, trehalose-phosphate synthetase, and UDP-glucose pyrophosphorylase. Their appearance is susceptible to inhibitors of RNA synthesis even before the enzyme activities appear (Loomis, 1969). Each enzyme is susceptible during a different period. Some event during development must signal the approaching need for a specific enzyme activity. The necessary gene is activated and a messenger RNA is synthesized. If RNA synthesis is inhibited, the enzyme activity does not appear and development cannot proceed. The synthesis of an enzyme can also be reinduced after the stage in development when it would normally appear. Newell *et al.* (1972) found that slime mold cells can quickly regain their proper place in

development after they have been disrupted. Cells that are disrupted at the 12 hour stage and replated require only two hours to reach the 12 hour stage again. However, they must repeat many of the same gene regulation steps. Synthesis of UDPG pyrophosphorylase is reinitiated and rises to a higher level than normal. Sussman and Newell (1972) suggested that the new level represented a quantum jump in synthesis. It is also sensitive to RNA inhibitors.

The following three chapters are the beginning of the study of gene regulation in Dictyostelium at the level of chromatin. The first chapter deals with the purification of chromatin and the characterization of slime mold histones. The second describes the nonhistone chromosomal proteins, which contain a subset of contractile-like proteins. The third dissects the structure of slime mold chromatin.

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I. The Purification and the Histones of *Dictyostelium discoideum* Chromatin

INTRODUCTION

Gene regulation has been studied for many years in prokaryotes and eukaryotes. One approach has been to isolate the genetic apparatus - DNA, RNA and protein - disassemble it and study its parts separately and together. In prokaryotes this method has yielded information about operator and promotor sites (Beckwith and Zipser, 1970), the lac repressor protein (Riggs and Bourgeois, 1968), the CRP activating protein (Zubay et al., 1970), and the interaction of RNA polymerase in this system (Chambon, 1975). However, one is confronted with a much more complex system in eukaryotes. Only a small fraction of the genome is expressed, and eukaryotic cells differentiate into specialized cells by defined, temporal pathways. Histones (Lacy and Axel, 1975; Kuo et al., 1976; Gottesfeld et al., 1975), non-histones (Park et al., 1976), and RNA (Britten and Davidson, 1969) combine with DNA in eukaryotic chromatin and each may be involved with gene regulation. Clearly, a simple, model system would be easy to study. A number of simple eukaryotes have been studied, such as Saccharomyces (Wintersberger et al., 1973), Euglena (Netrawali, 1970), Neurospora (Noll, 1976), Physarum (Mohberg and Rusch, 1969) and Dictyostelium (Coukell and Walker, 1973; Charlesworth and Parish, 1975). Dictyostelium offers the advantages of both a small genetic apparatus and a simple developmental cycle. In the past problems of purification and yield have plaqued the study of Dictyostelium chromatin. During the writing of this report another paper appeared (Pederson, 1977),

which describes a procedure for obtaining highly purified chromatin, but with a consequent reduction in yield. We have endeavored to develop a method of purifying chromatin in high yield. This chromatin has been characterized by chemical and physical methods. Its histones have been extracted and identified by both gel electrophoresis and ion-exchange chromatography.

Methods

Organism and growth conditions. Dictyostelium discoideum, strain Ax-3, was grown at 22°C on a rotary shaker in a sterile liquid medium termed HL5 by Cocucci and Sussman (1970). The cells have a generation time of about 12 hrs and were grown to a density of 0.8×10^7 cells/ml before harvesting. To prevent loss of developmental ability after repeated serial passages the cells must be cloned at monthly intervals on nutrient agar with Klebsiella aerogenes. A clone with a normal fruiting body is then reinoculated with a fired wire loop diped in HL5 into medium containing 50 µg/ml streptomycin sulfate to prevent growth of any contaminating bacteria. Cells were harvested in a large swinging bucket rotor at 700 xg in one liter bottles and washed once with cold 0.4% NaCl.

Isolation of nuclei and chromatin. Nuclei are isolated by a modification of the method of Firtel and Lodish (1973). The harvested and washed cells are suspended at a density of 2×10^8 cells/ml in cold 0.37 M sucrose, 40 mM KCl, 20 mM phosphate pH 7.6, 0.1 mM EGTA,[1] 1 mM PMSF, 0.5% nonidet P-40 (NP-40) with 0.5 mM $MgAc_2$ (acetate) added after the EGTA is dissolved. This suspension is shaken by hand for 45 sec to lyse the cells. Observation under phase contrast microscopy reveals that 100% of the cells are lysed. The solution is then pelleted at 1,000 xg for five min in a swinging bucket rotor. This procedure is repeated once more except that the $MgAc_2$ is increased to 2 mM, the pellet is resuspended with a glass teflon homogenizer to shear off cytoplasmic material adhering to the nuclei, and the suspension is sedimented at 2 000 xg for 5 min. The nuclei are further purified by resuspending with the homogenizer in the same buffer but containing 1 M sucrose, 2 mM $MgAc_2$, and 0.1% NP-40. They are centrifuged at 5,000 xg for 10 min. At this point most of the nuclei are intact and hardly any cytoplasmic contamination remains attached to them.

Chromatin is next purified from these nuclei by a modification of the procedure of Bonner et al. (1968). The nuclei are resuspended in 0.05 M NaCl, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF H8 (SEEP buffer), homogenized and pelleted at 4,000 xg for 15 min in a fixed angle rotor.

This is repeated once. The nuclei have begun to lose their integrity at this point. They are next suspended in 10 mM Tris, 0.1 mM EGTA, 1 mM PMSF pH 8 and centrifuged at 12,000 xg for 10 min. This is repeated once. The chromatin is then washed with the same buffer but without the EGTA and finally with the nuclease shearing buffer: 5 mM Tris·acetate, pH 7.8, 20 mM ammonium acetate, 0.4 mM $CaCl_2$, 0.2 mM EDTA, and 1 mM PMSF (Noll et al., 1975). This material is then sheared with the nuclease to yield the chromatin.

Nuclease Shearing. The chromatin is suspended in the nuclease buffer at 50 A_{260} units/ml (determined in 0.1 N NaOH) and cleaved with Staphylococcal nuclease (Worthington, micrococcal, 3.1.4.7) for 1 min at 23°C using 1 unit of enzyme per 3 A_{260} of chromatin. The reaction is halted by adding EGTA to 1 mM and cooling to 0°C. The insoluble material is then removed by sedimentation at 10,000 xg for 10 min. The insoluble chromatin is again resuspended and $CaCl_2$ added to compensate for the EGTA remaining in the volume of the pellet. It is sheared again (second sheared) for 4 min with nuclease at 1 unit per four A_{260} units of chromatin. The reaction is stopped and the insoluble material pelleted and discarded. The supernatants contain two fractions (first and second sheared) of chromatin. Although it is usually used at this stage for purification of proteins

or DNA, the chromatin can be further purified by pelleting at 100,000 xg for 90 min.

Assays for chemical composition. The method of Burton (1956) was used to determine the DNA content of sheared chromatin. To determine RNA content aliquots were made 0.3 N in KOH, digested 2 hrs at 37°C, and precipitated by titrating to pH 1 with 3 N $HClO_4$. The supernatant was assayed for RNA by the orcinol method (Dische and Schwartz, 1937). Basic proteins were extracted with 0.4 N H_2SO_4. The samples were stirred for 30 min and then sedimented at 100,000 xg for 2 hrs. The supernatants contained the basic proteins. To determine protein content these were dialyzed against two or three changes of 0.08 N NaOH and assayed by the method of Lowry et al. (1951). The pellet was assayed by the same method for acidic proteins after dissolving in 0.08 N NaOH and dialyzing. Total proteins were assayed by either this method or by the method of Brumhall et al. (1969). Hexose was assayed by the anthrone method of Hassid and Abraham (1957). Total dry weight was determined gravimetrically after drying measured volumes in covered weighing vessels at 110°C. The ultraviolet spectral data were recorded by a Varian 635 spectrophotometer connected to a Tektronix 31 calculator. The buffer was 10 mM Tris pH 8 and its contribution was automatically subtracted by the calculator.

Ion exchange chromatography. The procedure for separating the histones on BioRex 70 with a guanidinium chloride (GuCl) gradient has been described by Bonner et al. (1968).

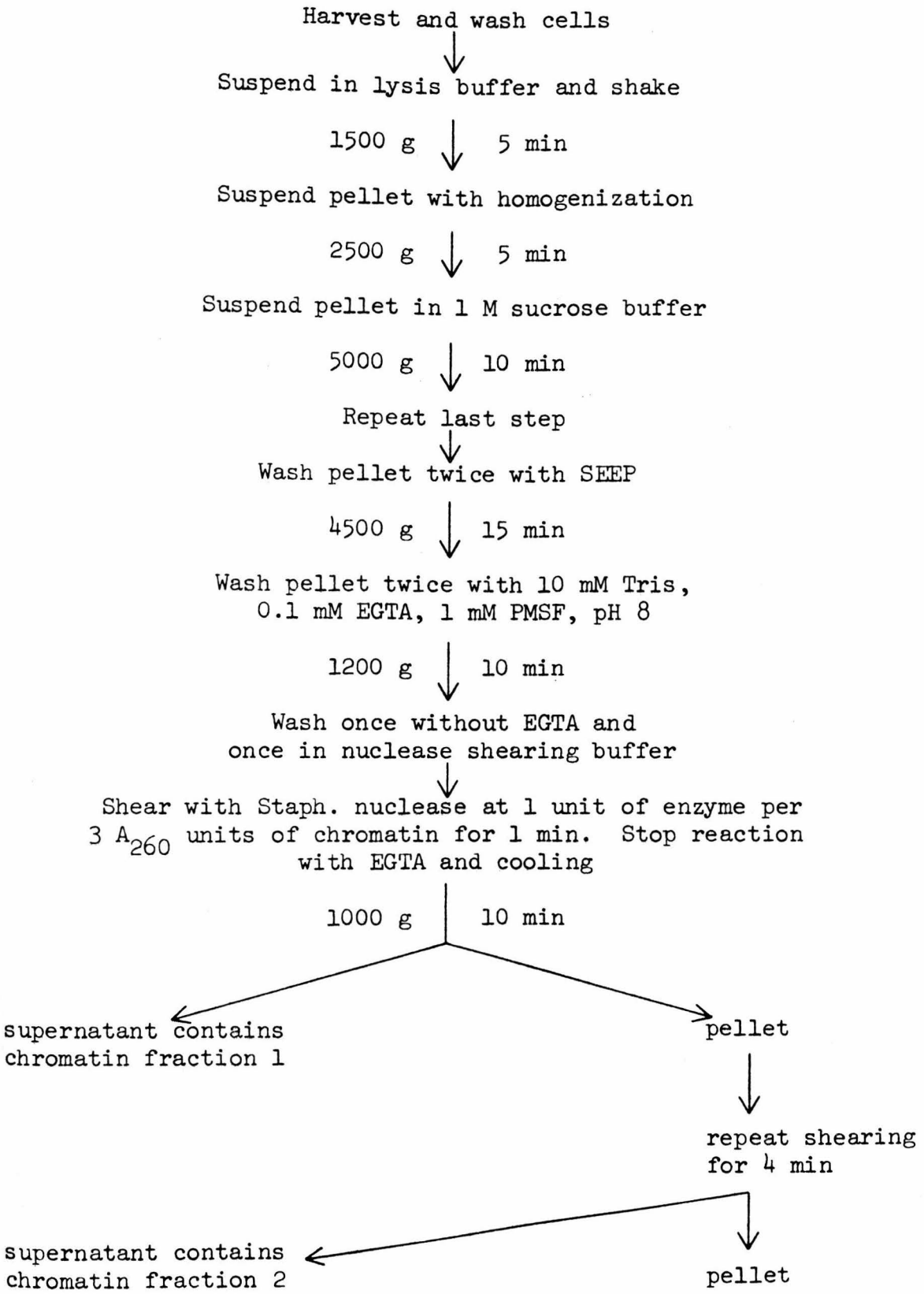
Gel electrophoresis. Basic proteins were analyzed on several gel systems including an acid-urea system (Panyim and Chalkley, 1969), a high pH system (Panyim and Chalkley, 1971), and a neutral pH system (ibid.). The proteins prepared by acid extraction (above) were either dialyzed extensively against 1% acetic acid and lyopholized or precipitated with four volumes of ethanol at -20°C for at least 12 hrs. Calf histone, BSA, cytochrome C, ovalbumin, and IgG were used as standards in some of these systems.

Results and Discussion

The purification procedure has three successive sections: isolation of intact nuclei; washing the chromatin from lysed nuclei; and shearing the chromatin to a soluble size. A flow diagram is shown in Figure 1. Each section is unique and has a primary objective.

Isolation of intact nuclei. This is complicated by a high lipid to DNA ratio in the rather small nuclei, the presence of nucleases and proteases released from the lysosomes, and the high polysaccharide content of slime mold cells. The lipid to DNA ratio is reduced by

FIGURE 1: Flow chart for the purification of Dictyostelium discoideum chromatin.


Harvest and wash cells
Suspend in lysis buffer and shake
1500 g
5 min
Suspend pellet with homogenization
2500 g
5 min
Suspend pellet in 1 M sucrose buffer
5000 g
10 min
Repeat last step
Wash pellet twice with SEEP
4500 g
15 min
Wash pellet twice with 10 mM Tris, 0.1 mM EGTA, 1 mM PMSF, pH 8
1200 g
10 min
Wash once without EGTA and once in nuclease shearing buffer
Shear with Staph. nuclease at 1 unit of enzyme per 3 A_{260} units of chromatin for 1 min. Stop reaction with EGTA and cooling
1000 g
10 min
supernatant contains chromatin fraction 1
pellet
repeat shearing for 4 min
supernatant contains chromatin fraction 2
pellet

lysing the cells with a nonionic detergent like nonidet (NP-40, Shell, Inc.). This strips off the plasma membrane and at least part of the outer nuclear membrane (Hancock, 1974). Nuclear membranes can occasionally be seen in preparations of nuclei by electron microscopy (pictures not shown). Increasing the temperature of the lysis medium reduces the concentration of NP-40 required to lyse the cells, but also increases the activity of degradative enzymes. Therefore, higher concentrations of NP-40 and lower temperatures have been chosen (all procedures are carried out at 0-4°C except where noted).

Since Dictyostelium possesses only seven chromosomes and its total haploid DNA content is only eleven times that of E. coli (Firtel and Bonner, 1972), a few internal breaks may produce chromatin too small to be pelleted and purified. Therefore, nuclease activity must be controlled. The common classes of nucleases require either a low pH (∿5) or the presence of calcium ions for activity. Dictyostelium contains both general types: DNase I and II (see Table I). By altering either the pH or the divalent metal ion concentration these two activities can be partially separated. DNase II has a pH optimum of 4.6 and requires no metal ions. It has ten-fold less activity at pH 7 and almost none at pH 8. On the other hand, DNase I requires divalent metal ions, especially calcium. Therefore, a pH of 8 and EGTA are used to reduce these common nuclease

TABLE I: DNAase Activity of Dictyostelium

Type of DNAase Assayed	Acid Soluble CPM[a]	Percentage
DNAase II at pH optimum of 4.6	13 475	41.0%
DNAase II at pH 7 with EDTA	506	1.5%
DNAase I at pH optimum of 7 with Mg^{2+}	1 430	4.3%
DNAase I at pH 8 with Mg^{2+}	670	2.0%
Control minus enzyme	36	0.1%

[a]A total of 33,000 CPM of DNA is added to 300 µl of chromatin (3 A_{260} units/ml) in the appropriate buffer and incubated for 1 hour at 37°C. The reaction is stopped by adding 0.5 ml of 7% PCA.

activities. However, since nuclei only remain intact when some divalent metal ion is present, magnesium ions are added. It has been known for some time that magnesium and other divalent metal ions will precipitate chromatin. This phenomenon may give the nucleus a rigid structure.

Another source of enzymatic difficulties is protease activity. Many common proteases, such as trypsin and a rat chromosomal protease (Chong et al., 1974), which contain a serine amino acid at their active site, may be inhibited with phenylmethylsulfonyl fluoride (PMSF). When less than 1 mM PMSF is used, degradation products from protease activity appear on gels of both histones and nonhistones.

The next problem to overcome is polysaccharide contamination. The polysaccharide is primarily glycogen and may be reduced in amount by growing the cells to mid-exponential phase (a cell count of 0.8 to 0.9 x 10^7 cells/ml), instead of stationary phase (White and Sussman, 1963). Some glycogen and associated packaging proteins will still co-purify with the chromatin. These will be removed later during the shearing and can be partially removed by washing with isotonic and hypotonic solutions, in the following sections.

Lysis of the nuclei. Lysis of the nuclei in SEEP buffer is facilitated by removing any divalent metal ions with EDTA and EGTA

which also reduce any nuclease activity that depends on these ions. The adventiously bound RNA in the form of ribosomes and ribonucleoprotein (RNP) are dissociated and removed by washing with SEEP buffer.

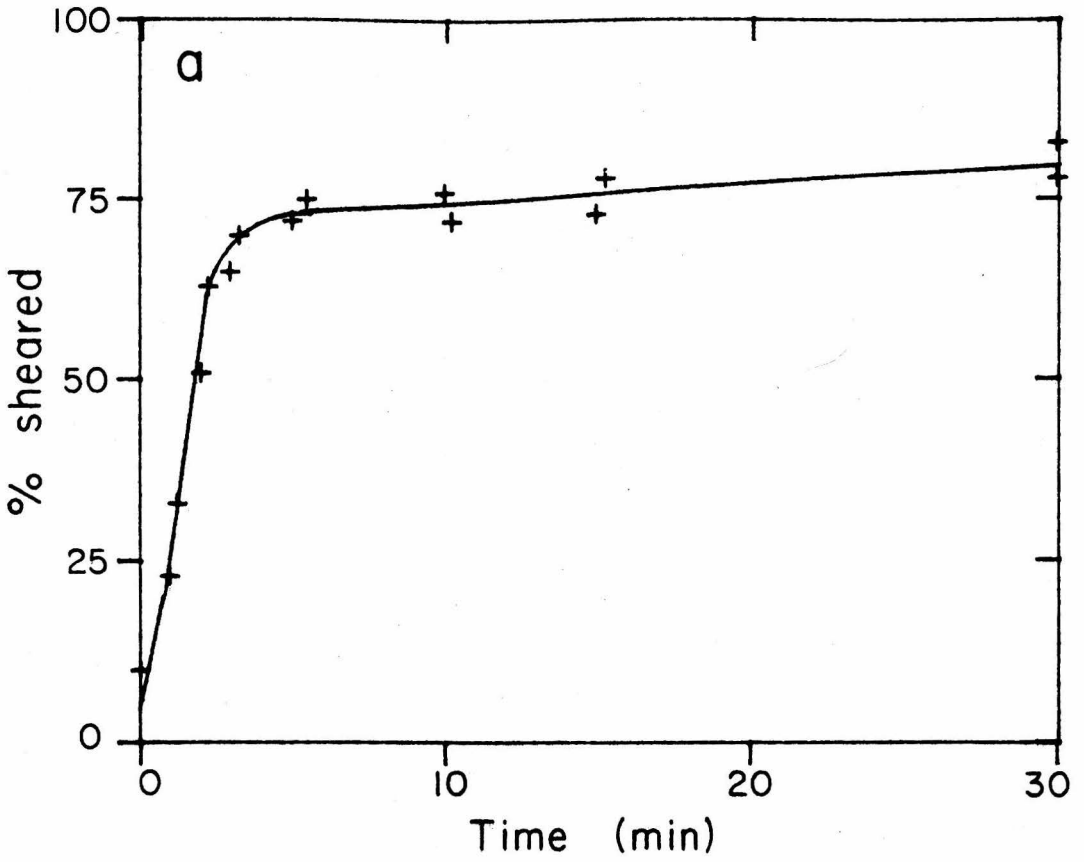
The lysed nuclei (crude chromatin) are repeatedly washed in a low ionic strength buffer to remove the nucleoplasm and any loosely bound protein. Again EGTA and PMSF remain in the buffer to reduce nuclease and protease activities, respectively. The crude chromatin is washed a limited number of times and suspended in nuclease shearing buffer, since prolonged washing will eventually remove a great deal of the chromosomal protein (Murphy and Bonner, 1975).

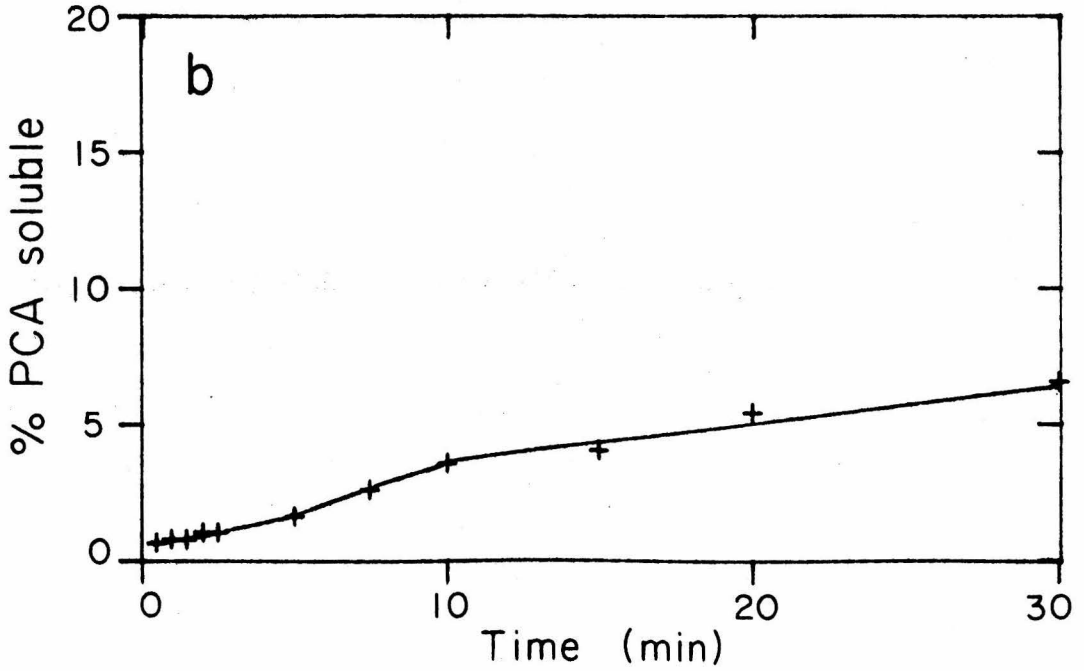
Nuclease shearing and characterization of chromatin. Mechanical shearing cannot separate polysaccharides and nonchromosomal proteins from chromatin and also disrupts the native structure of the chromatin (Noll et al., 1975). We have chosen nuclease shearing as the best method of solubilizing the chromatin while preserving its structure and removing contaminants. The chromatin may be sheared with either high or low levels of nuclease. In this case Staphylococcal nuclease (Worthington) is used. If only one unit of enzyme is used for three alkaline OD_{260} units of chromatin, long pieces are generated that are soluble in low ionic strength buffer but not in perchloric acid (Fig. 2a and b, respectively). Between 80 and 90% of the DNA is

FIGURE 2: Nuclease shearing of Dictyostelium chromatin.

a. Fragments of chromatin soluble in a low ionic strength buffer are produced by nuclease shearing. The chromatin is labeled with ^{3}H-thymidine and then digested with nuclease. The reaction is stopped by adding EGTA to 1 mM and cooling to 0°C. The samples are sedimented at 10 000 x g for 10 min and the supernatants adjusted to 1% SDS and are counted in a Beckman liquid scintillation counter using Aquasol 2 scintillation fluid (Amersham/Searle). Total radioactivity was determined by adjusting an aliquot of totally digested chromatin to 1% SDS and counting.

b. Only a small percentage of the DNA is rendered acid soluble during the digestion. The chromatin is sheared as in a., but the aliquots are precipitated with 7% perchloric acid and sedimented. The supernatants are again counted after neutralizing. Total radioactivity was determined by digesting aliquots in 7% PCA at 70°C for 15 min and counting after neutralizing.


a
100
75
50
25
0
% sheared
0
10
20
30
Time (min)


b
20
15
10
5
0
% PCA soluble
0
10
20
30
Time (min)

released, while only 5 to 10% of the polysaccharide and nonchromosomal protein are released. The remaining pellet is 50% of the original mass before shearing and 30% of the original alkaline OD_{260} units. The pellet is 70% protein, 25% polysaccharide and 5% DNA.

The chromatin may either be sheared once completely, or twice in partial successive stages. If the insoluble, crude chromatin is exposed briefly to the nuclease, centrifuged to remove the supernatant, and sheared again, the chromatin can be separated into two fractions (first and second sheared). The first nuclease treatment releases 3-7% of the DNA into the supernatant. This DNA has a hyperchromicity of 13% (about 30% double-stranded) and 95% of the DNA is longer than 1000 b.p. (base pairs) on electrophoresis gels (data not shown). The second sheared fraction (75-80%) has a hyperchromicity of 28.7% (about 90% double-stranded). The length of the DNA in fraction two is also greater than 1000 b.p. From these results fraction two is much more representative of total chromatin. A typical ultraviolet absorption spectrum of this chromatin is shown in Figure 3. The A 230/260 ratio is greater than that of higher eukaryotes, but otherwise it is similar.

The chemical compositions of total, sheared chromatin and the two separated fractions are shown in Table II. The histone to DNA ratio is 0.98 for the entire genome and it is adjusted for a slightly greater (12%) reactivity of histones in the Lowry protein reaction.

FIGURE 3: Ultraviolet absorption spectrum of Dictyostelium chromatin.

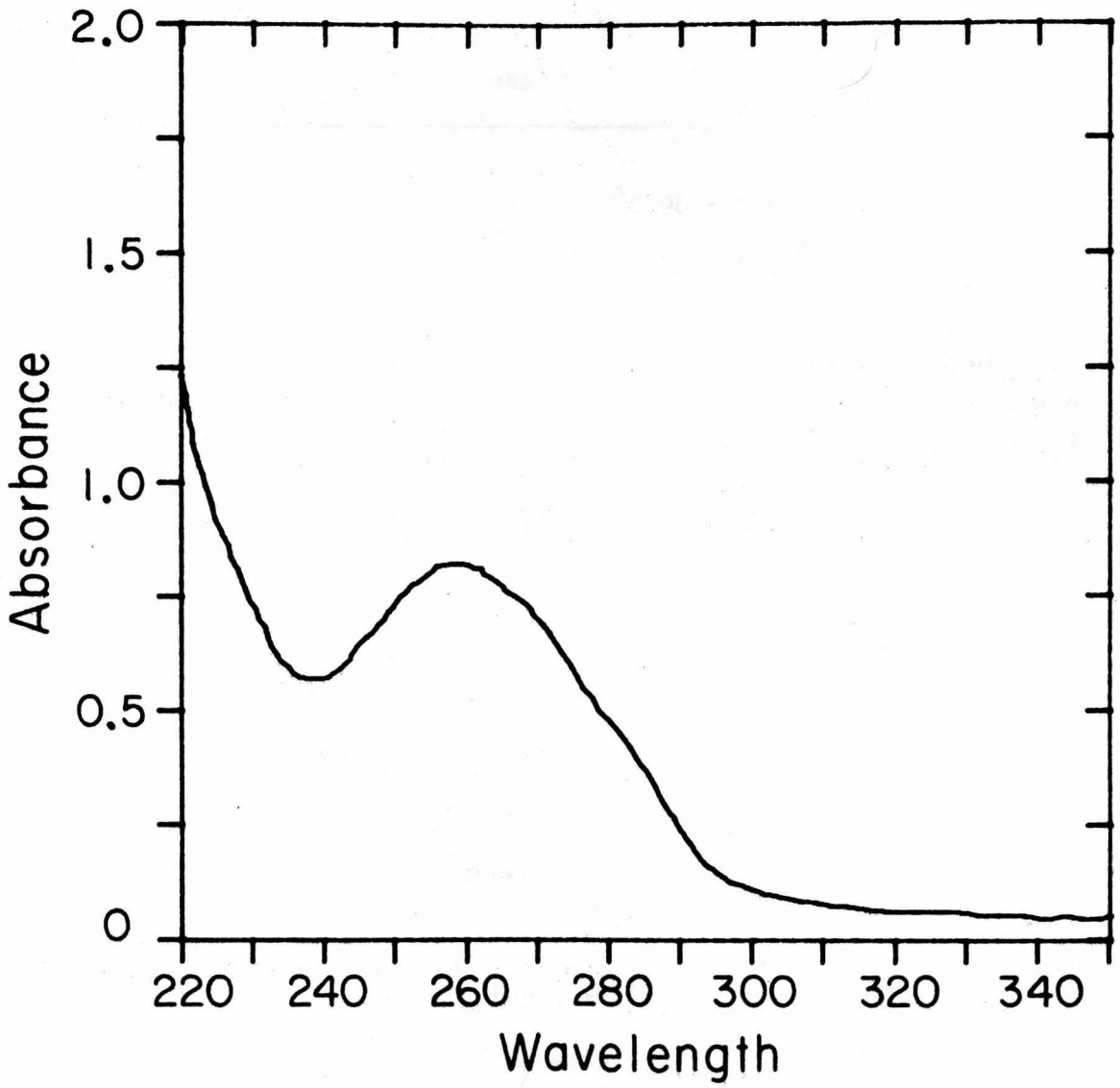

2.0
1.5
1.0
0.5
0
Absorbance
220
240
260
280
300
320
340
Wavelength

TABLE II: Chemical Composition of Dictyostelium Chromatin

Fraction	% Total DNA	Mass Ratios DNA : RNA : Histone : Nonhistone[e]			
Total Chromatin[a]		1	0.18 + 0.1	0.98 + 0.093	1.02 + 0.27
First sheared[b]	3-7	1	-	1.06 + 0.11[d]	10.3
Second sheared[c]	75-80	1	-	1.02 + 0.014	1.01

[a]The data for total chromatin are compiled from preparations which were separated into first and second sheared fractions and from ones which were sheared once totally.

[b]The crude chromatin is sheared for 1 min. at 22°C and centrifuged. The supernatant is the first sheared fraction.

[c]The pellet from the first shearing is again sheared for 4 min., centrifuged and washed. The supernatants are the second sheared fraction.

[d]This ratio is calculated after subtracting the contribution from nonchromosomal protein in the acid extraction of the first fraction. This can be done from an analysis of a gel scan of these

proteins, providing one assumes equal molar staining of the protein bands. In this case the histones make up 38 percent of the scan. It is likely that the basic histones bind more than an equal amount of stain. Therefore, 1.06 is an upper limit to the histone:DNA ratio for fraction one.

[e]A major difference in the chemical composition of these two fractions occurs in their nonhistone to DNA ratios. For the first fraction the observed ratio is 11.1, while for the second it is 1.08. Some of the nonhistones are loosely bound to the chromatin of the first sheared fraction and are extracted with the histones by acid. These may be contaminants and may give the high ratio. The two fractions contain 7.4 percent and 6.6 percent contamination, respectively, according to the ^{14}C-labeled protein experiment. Subtracting this contribution would alter the protein ratios to give the ones reported above.

The nonhistone to DNA ratio is 1.02 for the entire genome, but there is a difference in the nonhistone content of the first and second sheared fractions (Table II).

The final yield of chromatin amounts to between 70 and 80% of the expected yield of chromatin from these cells (6×10^{-14} g DNA/cell or 0.08 mg DNA/g wet, packed cells). The slime mold is haploid and contains between 0.056 and 0.06 pg (Firtel and Bonner, 1972; Sussman and Rayner, 1971) of DNA per nucleus. In addition, while growing exponentially, approximately 40% of the cells will be in the G2 phase (diploid) of the cell cycle (Sussman and Rayner, 1971). The average yield per cell is therefore raised by a factor of 1.4.

Assaying the purity of the chromatin. The purity of this chromatin has been assayed by the following four methods: the activity of a cytoplasmic enzyme, the absence of mitochondrial DNA, contamination from ^{14}C-labeled proteins, and the quantity of the RNA isolated with the chromatin. A typical cytoplasmic enzyme is lactate dehydrogenase. The data for the activity of lactate dehydrogenase in the whole cell lysate and in the purified chromatin indicate that less than 0.1% of the total activity is isolated with the chromatin. This is equivalent to a 14-fold reduction in specific activity (see upper section of Table III).

TABLE III: Contamination of Chromatin

Assay	Specific Activity	Contamination (%)[a]
Lactate Dehydrogenase Activity[b]		
homogenate	69.5 units/µg	100
total chromatin	5.0 units/µg	7.2
Binding of Labeled Crude Cytoplasm[c]		
homogenate	0.76 CPM/µg	100
first sheared fraction chromatin	0.19 CPM/µg	25.0
second sheared fraction chromatin	0.11 CPM/µg	14.5
Binding of Labeled Pure Cytoplasm[c]		
homogenate	3.32 CPM/µg	100
first sheared fraction chromatin	0.247 CPM/µg	7.4
second sheared fraction chromatin	0.218 CPM/µg	6.6

[a]Contamination is the percent of the total protein, which can be attributed to nonchromosomal origin.

[b]This enzyme is assayed by the oxidation of NADH to NAD^+. One unit is equivalent to one micromole of NADH oxidized per min.

[c]The specific activity of the protein is determined for the labeled homogenate and for chromatin prepared after mixing unlabeled muclei with a labeled supernatant. The proteins are precipitated with hot trichloroacetic acid. Contamination is equivalent to 100 times the specific activity of the fraction divided by the specific activity of the homogenate.

Slime mold cells contain a great many mitochondria. Twenty-eight percent of the whole cell DNA is mitochondrial (Firtel and Bonner, 1972). When whole cell DNA is sheared with EcoRI restriction enzyme, three distinct mitochondrial DNA bands are present on gels (Firtel, et al., 1976). Nuclear DNA isolated by the procedure described here does not contain any visible mitochondrial bands. If mitochondrial DNA is present but invisible in these gels, it would amount to less than 5% contamination of the chromatin.

Contamination was also determined using ^{14}C-labeled proteins. In this experiment one batch of cells was grown with ^{14}C-amino acids and one without. The nuclei were isolated from both as usual. No precautions were taken to further purify the cytoplasm. The labeled cytoplasm was then mixed with the unlabeled nuclei for a few minutes at 0-4°C. The nuclei were next pelleted, chromatin prepared as usual, and sheared with the nuclease. From the middle section of Table III it is apparent that there is considerable contamination. The first sheared fraction is especially high with 25%, while the second sheared fraction has 15%. These values are an upper limit for contamination, since no steps were taken to remove any protein contributed from lysed nuclei, mitochondrial, or whole cells in the supernatant. When the lysate is purified by adding 4 M NaCl until the solution is 0.2 M and centrifuging at 20,000 xg for 30 min,

all but about 7% of the contamination is removed (lower section of Table III). Escaped nucleoproteins will pellet under these conditions (Bonner et al., 1968) and not artifactually contribute to the contamination. The value of 7% is probably a lower limit of contamination and correlates well with the 14-fold reduction in specific activity of lactate dehydrogenase.

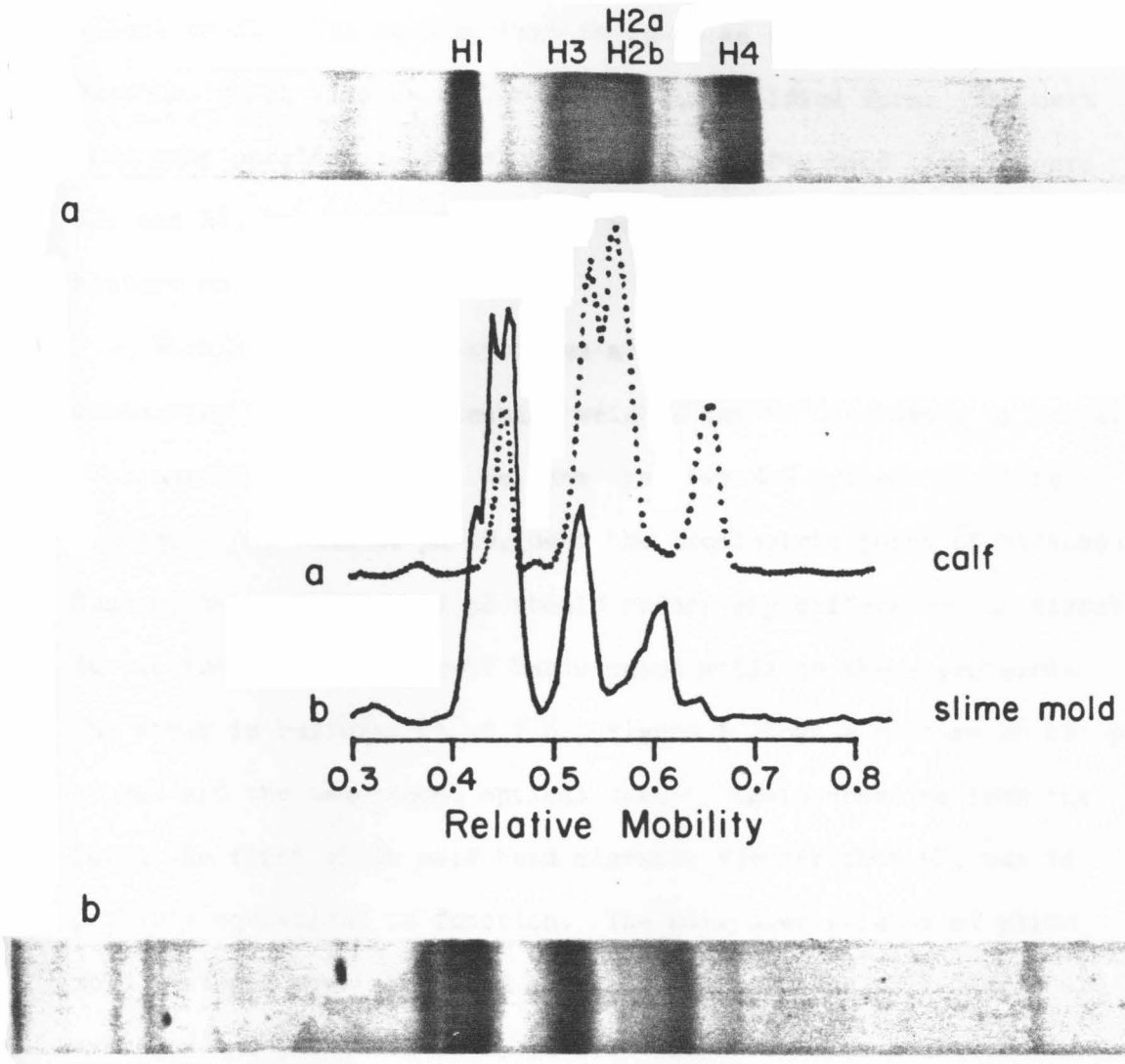
The final assay of purity is the quantity of RNA isolated with the chromatin. Large amounts of RNA, especially ribosomal RNA, would indicate contamination. The RNA to DNA ratio of the chromatin is only 0.18, and it compares well to the ratios in other eukaryotes (Rizzo, 1976; Elgin and Bonner, 1972). The range of the RNA to DNA ratio varied from 0.022 to 0.28 and depended on the number of times that the chromatin is washed.

Histones analyzed on acrylamide gels. To further characterize the basic proteins, or histones, of Dictyostelium they were analyzed by gel electrophoresis on several systems. The classical system uses a low pH (3.2) and urea in a high percentage of acrylamide. The proteins are separated on the basis of charge and molecular weight. Five histone bands are found in mammals (DeLange and Smith, 1971). Dictyostelium also has five bands, but in different positions as seen in Figure 4. Starting at the left of the gels, there is a

FIGURE 4: pH 3.2, acid-urea gels of histones.

a. Calf histone gel with its associated scan (····). The histones are labeled above their bands. Histones H2a and H2b appear as a single band, although they can be resolved into two bands on longer gels.

b. Dictyostelium histone gel with its associated scan (——). The relative mobility is on a scale of 0.0 to 1.0. The portions of the gels not shown in the scan have almost zero background. The bands lie opposite their peaks in the scans.


H1
H3
H2a
H2b
H4
a
a
b
calf
slime mold
0.3
0.4
0.5
0.6
0.7
0.8
Relative Mobility
b

triplet near the position of H1. Other data (see below) indicate that there are actually two polypeptides here and one is probably modified producing the third band. The one furthest left is equivalent to H1. The doublet next to it is an unusual slime mold histone, which will be called H6′ and its modified form. The next band runs parallel to H3 of mammals. The fifth band lies between H2a and H4, but there is no exact equivalence between it and a mammalian histone on these gels.

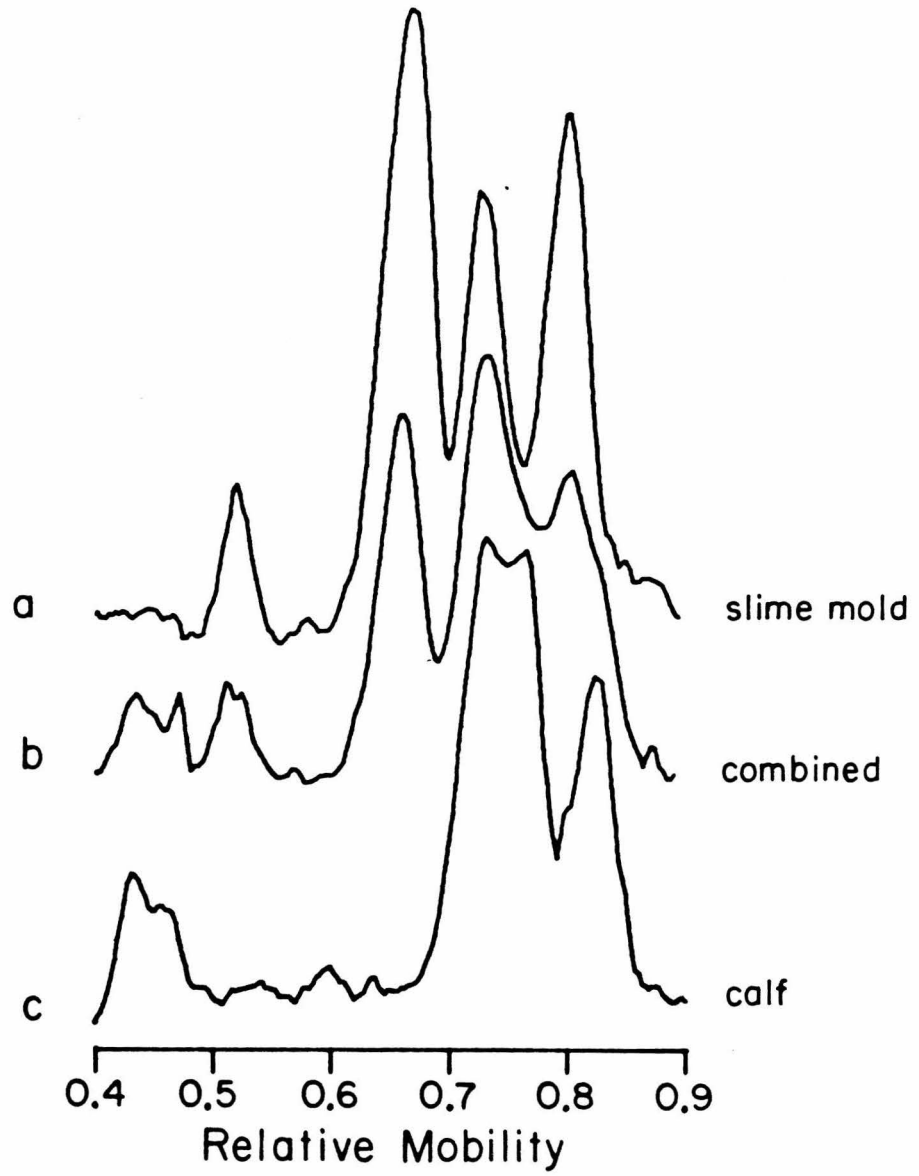
When the slime mold histones are electrophoresed on gels containing SDS, their molecular weights can be determined by comparison with mammalian histones. We have used two SDS systems for this purpose. One runs at pH 10, near the isoelectric point of histones. Running the gels at this pH should reduce any differences in migration due to the high fraction of basic amino acids in these proteins. The other is buffered at pH 7.6. Figure 5 shows a picture of the pH 10 gel and the associated optical scans. Again starting from the left, the first slime mold band migrates further than H1, but is probably equivalent in function. The molecular weights of slime mold histones are determined on these gels and the pH 7.6 gels are averaged and presented in Table IV. The next slime mold band is the unusual H6′, which has no equivalent in higher eukaryotes. The last two slime mold bands migrate with H3 and H4, respectively.

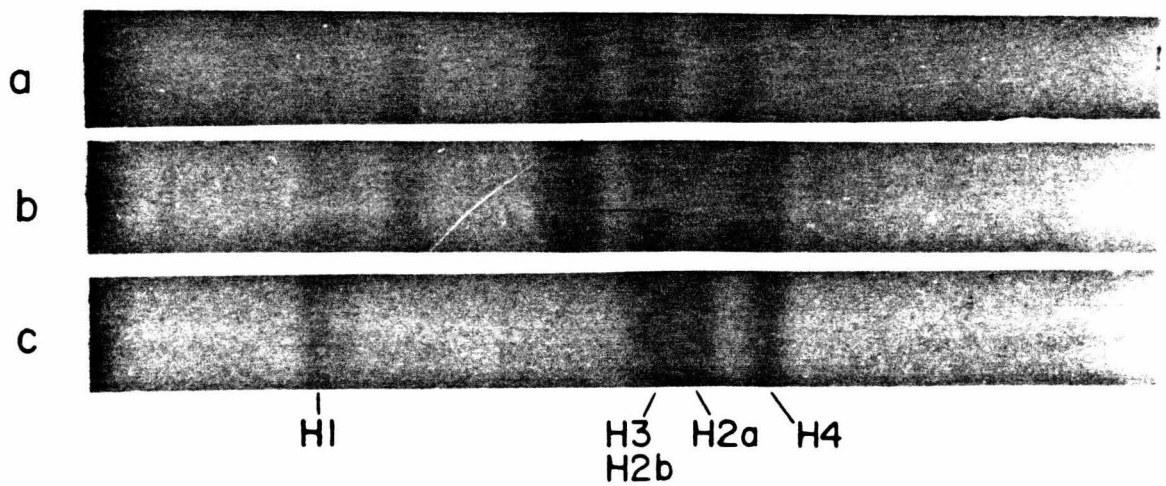
FIGURE 5: pH 10, SDS-urea of histones.

a. Dictyostelium histones are shown with their scan and slab gel lane aligned.

b. Dictyostelium and calf histones were run in the same slab gel lane to show their exact register.

c. The scan and gel lane of the calf histones are shown with the histones labeled below the lane. Here histones H3 and H2b migrate in parallel (Panyim and Chalkley, 1971).


a
slime mold
b
combined
c
calf
0.4
0.5
0.6
0.7
0.8
0.9
Relative Mobility


a
b
c
H1
H3
H2b
H2a
H4

The slime mold H4′ migrates slightly less than the calf H4 probably indicating a higher molecular weight.

Although some of the slime mold histones are similar to those in higher eukaryotes, their exact homology can only be determined by knowing either their primary structure or specific function. Therefore, a prime is added to the current histone nomenclature to indicate that they are histone-like. The bands are labeled H1′, H3′, H4′ and H6′. The histone H6′ migrates in a position similar to that of H5 in duck erythrocytes (Tobin and Seligy, 1975). Histone H5 serves a unique function in erythrocyte chromatin. Since it is unlikely that they have the same function in these different cell types, they are not labeled the same.

The pH 7.6 gels shed additional light on the complexity of slime mold histones. Five bands can be distinguished on these gels (Fig. 6) but a different five than are seen on the acid-urea gels (Fig. 4). Again starting from the left slime mold H1′ is a rather broad band. This may be due to unresolved subspecies of H1′ or to limited proteolysis. Histone H1 has previously been shown to contain subspecies in higher eukaryotes (Panyim and Chalkley, 1969). Next is the unusual slime mold H6′ still migrating between H1′ and H3′, but as a single band - not the doublet seen in Figure 5. However, the next band, H3′, has become a doublet, which it was not at pH 10 or pH 3.2. The last band is H4′,

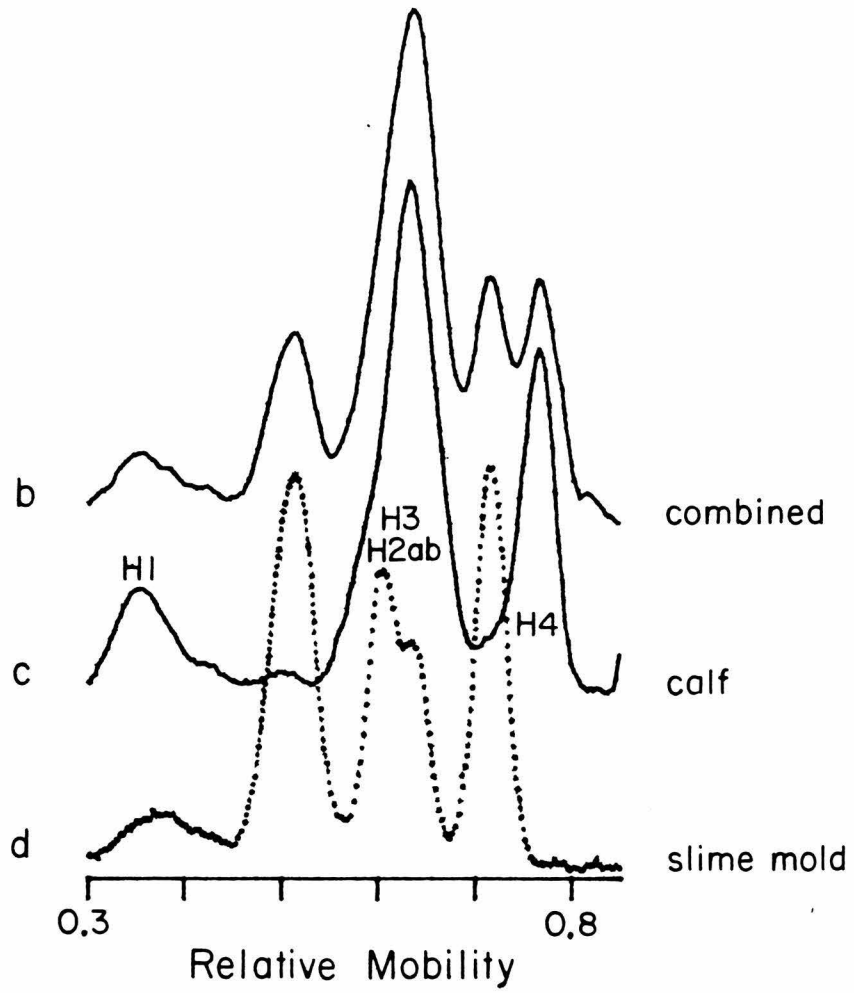
FIGURE 6: pH 7.6 SDS-urea gels of histones.

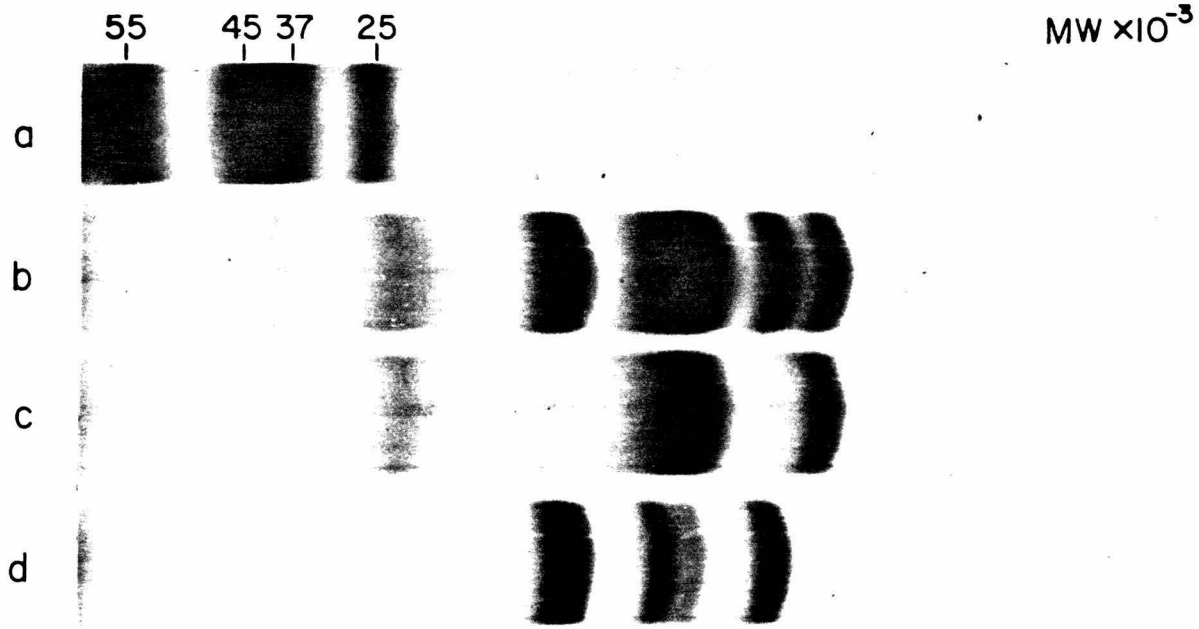
a. Molecular weight markers are used to determine the size of any nonhistone contaminants in the histones. They are IgG (55,000 and 25,000 daltons for the heavy and light chains, respectively), ovalbumin (45,000), and alcohol dehydrogenase (yeast, 37,000). They do not migrate accurately with respect to histones because of their vastly different isoelectric points. No scan is shown of this gel.

b. A scan and gel of a mixture of calf and Dictyostelium histones illustrates their alignment (——).

c. A scan and gel of calf histones. The scan is labeled with the names of the peaks. Histones H3, H2a and H2b have migrated as a single broad peak (——).

d. A scan and gel of Dictyostelium histones (....).


b
c
d
H1
H3
H2ab
H4
combined
calf
slime mold
0.3
0.8
Relative Mobility


55
45 37
25
MW ×10⁻³
a
b
c
d

which still migrates a shorter distance than calf H4. Molecular weight standards are included with these gels (Fig. 6a). Histones do not migrate as expected when compared to proteins with lower isoelectric points. These standards indicate the approximate molecular weights of nonhistones on these gels. There is a very faint contaminant in the slime mold histones with a molecular weight of 45×10^3.

The relative number of molecules of each histone in the slime mold genome may be estimated from scans of the gels. A series of pH 7.6 gels was run with carefully determined amounts of histone. They were all stained and carefully destained to a constant background. A computer was used to analyze the scans and fit Gaussian curves to them (Wallace et al., 1977). The results are plotted in Figure 7 for slime mold histones. Assuming that slime mold histones stain equally with respect to mass (Fambrough et al., 1968), the weight fraction of each histone may be estimated from the scans (see Table IV). From this fraction the number of molecules of each per haploid genome can be calculated. H3′ and H4′ are approximately equal in number, but there are 1.3 H6′ molecules for each H4′ and only 0.25 H1′ molecules for each H4′.

Histones analyzed by ion-exchange chromatography. To further confirm the identity of slime mold histones we analyzed them on a

FIGURE 7: The staining of Dictyostelium histones. The scans of a series of gels were fit with Gaussian components by a computer. The total absorbance is the sum of the absorbances in a Gaussian peak multiplied by the increment of each point along the abcissa and by one thousand. This value is plotted versus the total mass of protein loaded on the gel. The various lines represent a least-squares fit to the data for the following histones: H6′ (□), H4′ (⊙), H3′ (◇) and H1′ (+). The total absorbance may be converted to the stain extinction coefficient per μg of protein by multiplying each value by the following factor:

(length of the gel) x (fraction of the gel cylinder or slab observed by the spectrophotometer) x $\left(\frac{1}{1000 \times \mu g \text{ protein}}\right)$.

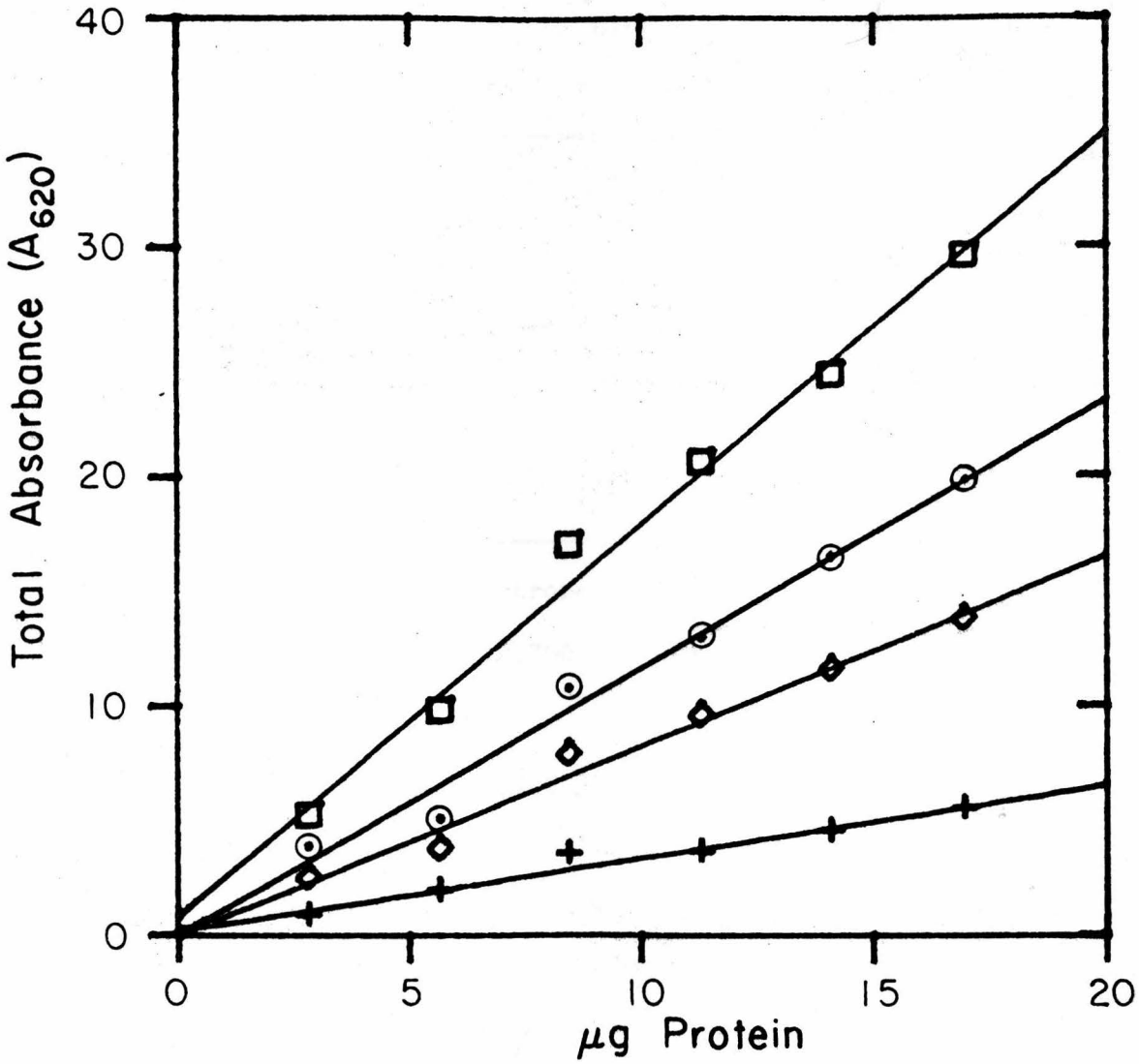

40
30
20
10
0
Total Absorbance (A_{620})
0
5
10
15
20
μg Protein

TABLE IV. Histone Stoichiometry of Dictyostelium

Histone	Molecular[a] Weight	(Molecular Weight of Calf Histones)	Weight Fraction	Molecules per[b] Haploid Genome	Molar Fraction
H1′	20,000	(22,130)[c]	0.10	1.6×10^5	0.07
H6′	15,800	-	0.4	8.3×10^5	0.38
H3′	14,900	(15,324)	0.26	5.6×10^5	0.26
H4′	12,000	(11,282)	0.24	6.5×10^5	0.29
H2a	--	(14,004)			
H2b	--	(13,774)			

[a]The molecular weight of cytochrome C on these gels is 11,600, while its actual molecular weight is 11,700. These estimates have less than a 5% relative standard deviation.

[b]The genome size of Dictyostelium discoideum is 3.3×10^{10} daltons (Firtel, 1972; Sussman and Rayner, 1971).

[c]The molecular weights of the equivalent calf thymus histones are in parentheses (Fasman et al., 1977; Elgin and Weintraub, 1975).

BioRex 70 column using a guanidinium chloride (GuCl) gradient. Li and Bonner (1971) have found that the histones can be separated into species apparently by basic charge. The slime mold histones are also separated into species by this method. Their pattern of elution from this column is compared to that of mammalian histones in Figure 8. This profile compares two separate runs, but labeled mammalian histones gave similar results when run in parallel with slime mold histones. No slime mold histone co-chromatographs with either H1, H2a or H2b. The slime mold proteins elute with the unbound and with the tightly bound proteins. The peaks were pooled and run on pH 7.6 electrophoresis gels. The results are shown in Figure 9. The slime mold H1′ is not bound to the column in eight percent GuCl. This may simply be due to its lower molecular weight than mammalian H1. A small amount of the other histones also elute with H1′. This is probably due to overloading of the column and proteolysis of the histones. The remaining slime mold histones are tightly bound and only eluted by 35% GuCl. This supports the gel results, which indicated that the slime mold has both histone H3′ and H4′. Finally, the unusual H6′ is tightly bound indicating that it is more basic than either mammalian H1, H2a or H2b.

FIGURE 8: Ion-exchange chromatography profile of histones. The histones are applied to the column in 8% GuCl. An 8-15% GuCl gradient is used to elute some of the histones. Then a 35% GuCl step elutes the remaining histones. The samples are precipitated with 1.1 M trichloracetic acid for 15 min and read to 400 angstroms. (+) GuCl percentage; (□) calf histones; (◇) Dictyostelium histones. The calf histone peaks are labeled.

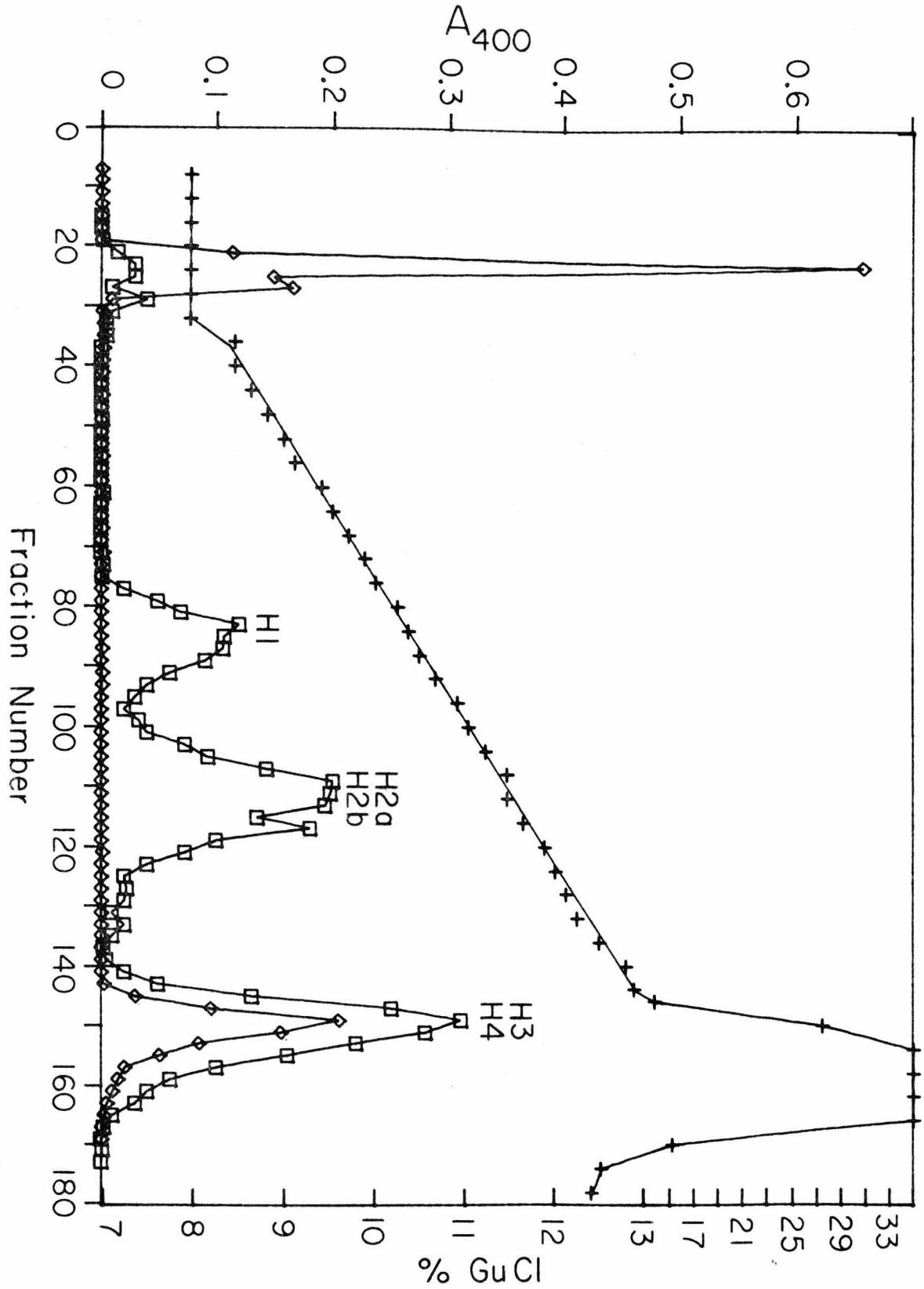
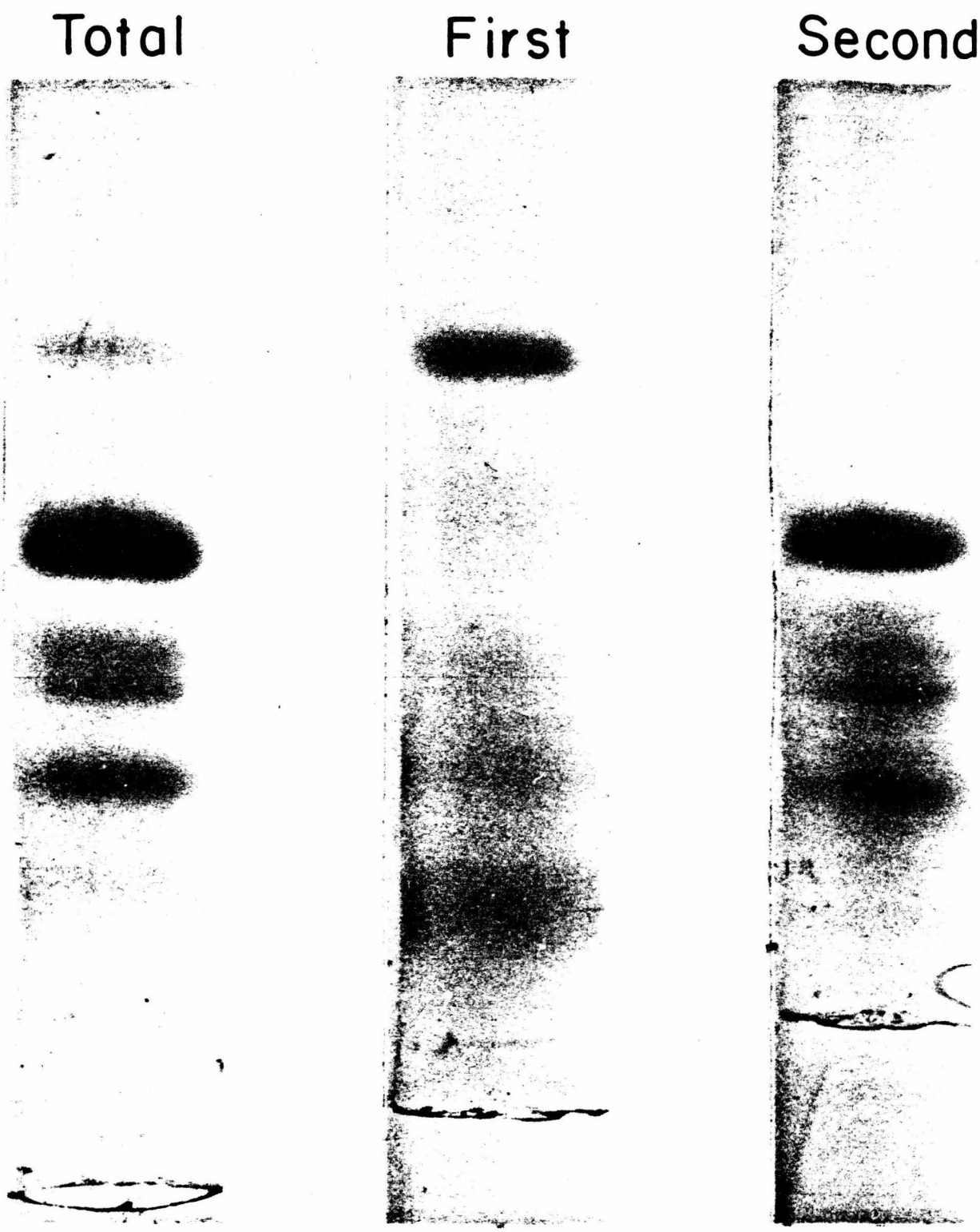

A400
0
0.1
0.2
0.3
0.4
0.5
0.6
Fraction Number
0
20
40
60
80
100
120
140
160
180
H1
H2a
H2b
H3
H4
% GuCl
7
8
9
10
11
12
13
17
21
25
29
33

FIGURE 9: Dictyostelium histone peaks from ion-exchange column. The peaks were pooled, precipitated with trichloracetic acid, washed with acetone, dissolved in sample buffer and run on pH 7.6 SDS-urea gels.

a. Total Dictyostelium histones.

b. First peak from the ion-exchange column.

c. Second peak from the column.


Total
First
Second
a
b
c

Further Discussion

Some of the results reported here require additional discussion including the purification of chromatin, the character of slime mold histones, and the results of other research on this topic.

There is no universal criterion for the chemical composition of purified nuclei and chromatin. Obviously, the chemical composition would vary with organism, cell type, and stage in the cell growth cycles. However, upper limits for an acceptable composition can be established from the large amount of available data (Bonner et al., 1968; Stein and Borun, 1972; Elgin and Bonner, 1970; Itzhaki and Cooper, 1973). We suggest that the following mass ratios should be an upper limit for purified chromatin: RNA:DNA $\leq$ 0.3 and protein:DNA $\leq$ 3.0. The DNA should account for approximately 70% or greater of the UV absorbance at 260 nm. The procedure followed in this paper does yield purified chromatin by the above criteria. The RNA:DNA ratio is 0.18 and this small amount may be nascent RNA. The total protein to DNA ratio is 2.0 to 1. This protein consists of 48% basic protein, or histone, and 52% acidic, or nonhistone protein.

The purification of Dictyostelium chromatin involves several problems: cell lysis, inhibition of nucleases and proteases, and removal of contaminating polysaccharide, RNA, and protein. These amoeboid cells are difficult to lyse by any mechanical method without

also damaging the nuclei. Low levels of detergent (<.1%) will lyse the cells at 22°C (Coukell and Walker, 1973; Charlesworth and Parish, 1975). However, nucleases and proteases are very active at this temperature and reduce the yield to less than half. Using 0.5% detergent at 0-4°C, the cells lyse within one min. The use of EGTA, EDTA and pH 8 to inhibit DNases has already been mentioned. In addition, the action of proteases has a two-fold effect: proteins are degraded and stretches of DNA are exposed to nuclease attack (Bakke and Bonner, in preparation). If less than 1 mM PMSF is used during the isolation, degradation of the histone can be detected on gels. The RNA:DNA ratio for Dictyostelium chromatin is fairly high as compared to that of other higher eukaryotes, but a larger part of the genome is also being expressed; i.e., a three- to six-fold greater fraction of the DNA is expressed as RNA (Firtel, 1972). The axenic slime mold cells have a very high level of polysaccharide (Ashworth and Watts, 1970), which sediments with the chromatin as does a substantial amount of nonchromosomal protein. The polysaccharide and protein are removed by solubilizing the chromatin with nuclease shearing. These results indicate that 7% of the chromosomal proteins are contaminants from the cytoplasm.

On the basis of the results presented here, Dictyostelium has four histones. These are compared to mammalian histones in Table V

according to molar ratio in the nucleus and basic charge. Basic charge is approximated by the percentage of GuCl required to elute the histone from BioRex 70. There are three anomalies to be considered: the doublet of H3′ on the pH 7.6 gels; the higher apparent molecular weight of H4′; and the doublet of H6′ on the pH 3.2 gels. If the doublet of H3′ were actually two polypeptides, the most likely candidates besides H3′ would be H2a or H2b. In this case one band should chromatograph differently on BioRex 70. Instead, they both elute with mammalian H3. Also the doublet should continue to appear either on the pH 10 or pH 3.2 gels. Therefore, the H3′ doublet must be due to modified variant of H3′. Dictyostelium H4′ appears to have a higher molecular weight than calf H4. This may be the case, since the two species are only distantly related. An alternate explanation is that slime mold H4′ is highly modified, which may cause it to migrate differently on gels. Finally, the H6′ doublet does not appear on the pH 7.6 or pH 10 gels. Rather, they chromatograph as one species. It also does not consistently appear on the pH 3.2 gel. There was some evidence of proteolytic degradation in this preparation and the doublet may be due to that. The presence of histone H6′ in Dictyostelium is somewhat surprising. The ion-exchange chromatography results indicate that it is more basic than either H2a or H2b. However, since the other histones are present and Dictyostelium has a normal nucleosome structure

TABLE V: Comparison of Histones

	Mammalian		Dictyostelium	
Histone	Molar Ratio[a]	% GuCl to Elute	Molar Ratio	% GuCl to Elute
H1	0.292	10	0.25	8
H2a	0.627	11.3	not present	-
H2b	1.09	11.3	not present	-
H3	1.14	35	0.86	35
H4	1.0	35	1.0	35
H6	not present	-	1.3	35

[a]Molar ratios are given relative to H4.

(Bakke and Bonner, in preparation), H6′ probably replaces H2a and H2b. Histone H6′ appears to be present on histone gels from other laboratories in Dictyostelium (Coukell and Walker, 1973, Charlesworth and Parish, 1975), in Physarum polycephalum (Mohberg and Rusch, 1969), and possibly in Saccharomyces cerevisiae (Franco et al., 1974), and Euglena gracilis (Netrawali, 1970). Histone H1′ has a lower molecular weight than its analogue in higher eukaryotes. Both Noll (1976) and Morris (1976) have suggested that either the length of H1 or the availability of its basic residues for interaction with the DNA phosphates may determine the length of the DNA linker in the nucleosome. Dictyostelium has a shorter linker than higher eukaryotes (Bakke and Bonner, in preparation).

An interesting implication for chromatin structure arises from the histone composition of Dictyostelium and other lower eukaryotes (Mohberg and Rusch, 1969). The histones approximately equal the DNA in mass as they do in higher eukaryotes. Since the fraction of DNA being expressed as RNA is several fold higher in the lower eukaryotes (Firtel, 1972), and since there is no decrease in the histone mass ratio, histone must be present in regions of the chromatin that are being transcribed. Therefore, the primary role of histones may be structural and the differences between transcribed and non-transcribed regions of the chromatin may be mainly due to differences in this structure.

Quantitation of the individual histones requires knowledge of each histone's ability to bind dye. The results for total slime mold histone in Figure 8 indicate that dye binding is linear in the concentration range used, is not cooperative, and is constant for each histone species. However, these results do not reveal whether the amount of dye in a peak is due to the number of protein molecules there or to the number of dye-binding sites per molecule. Fambrough et al. (1968) found that equal masses of individual histones bound equal quantities of Amido Schwarz dye, implying that the number of dye molecules bound is proportional to the length of the polypeptide chain. The basic amino acids would be the only other likely dye-binding sites. All of the mammalian histones except H1 have approximately the same proportion and number of basic amino acids. Since Dictyostelium histones, except H1′, have similar basic charges, as reflected in their ion exchange behavior, this conclusion probably also holds for them.

Models of chromatin structure (Kornberg, 1974) suggest that histones H2a, H2b, H3 and H4 are present in equimolar amounts in higher euakryotes. If a similar model applies to Dictyostelium chromatin, the molar ratios of H6′ to H3′ to H4′ would be 2, 1 and 1, respectively. Since the observed quantity of H6′ is lower than this, there may have been a selective loss of H6′ due to the extraction or proteolysis.

Charlesburg and Parish (1975) and Coukell and Walker (1973) have isolated basic proteins from slime mold nuclei. They were forced to use an extended extraction procedure because nuclei containing large amounts of protein cannot be simply extracted by dilute acid (Bonner et al., 1968). Both laboratories report more than four histone bands. The extra bands may be either modified histones, basic cytoplasmic proteins, or the products of proteolysis. Approximately 80% of the area under the gel scans of Charlesworth and Parish lies in bands corresponding to those reported here. Coukell and Walker found a histone to DNA ratio of 1.06, which is very similar to our results. However, after carefully purifying the chromatin we find much smaller quantities of RNA and total protein than either of these groups report. They assayed the chemical composition of whole nuclei instead of chromatin, and this may account for the difference.

Several lines of investigation can be pursued to further understand the phylogenetic relationship of Dictyostelium to other eukaryotes. The amino acid composition and primary structure of the histones, especially H4′, would help place this organism. Also, the internal structure of the slime mold nucleosomes will be of interest since it apparently has histone H6′ instead of H2a and H2b.

Note Added in Proof:

Another paper on Dictyostelium chromatin appeared while this paper was being written (Pederson, 1977). The author reports a chemical composition, ultraviolet absorption spectrum, and nuclear appearance very similar to those reported here.

Acknowledgements

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II. The Nonhistone Chromosomal Proteins of *Dictyostelium discoideum*

INTRODUCTION

Several non-histone proteins have been identified as regulators of gene expression in prokaryotes including: the lac repressor protein (15), the λ repressor protein (18), and the catabolite-regulating protein (26). Proteins with a similar function have been searched for in higher eukaryotes. There are hormone receptor "proteins" that bind to DNA and may activate specific genes (21,25). A subset of non-histone chromosomal proteins (NHCP), which specifically bind to rat DNA, can be isolated (20). Nonhistones from S-phase HeLa cells apparently cause the activation of the histone genes in G1-phase chromatin (16). To investigate the changes in the nonhistone protein components during development, LeStourgeon and Rusch (12) have studied the acellular slime mold Physarum polycephalum and found several specific nonhistones which appear or disappear during development.

The non-histone chromosomal proteins of the cellular slime mold Dictyostelium discoideum have been quantitated by protein assay and characterized by gel electrophoresis in this report. To further dissect this complex group of proteins, a subset has been extracted and tentatively identified as contractile-like proteins.

Chromatin from Dictyostelium nuclei is purified and sheared by the methods of Bakke and Bonner [1]. Briefly, the cells were lysed in 0.37 M sucrose, 40 mM KCl, 20 mM phosphate, pH 7.6, 0.5 mM $MgAc_2$ (acetate), 0.1 mM EGTA (ethyleneglycol-tetraacetic acid), 1 mM PMSF (phenyl-methanesulfonyl fluoride), 0.5% nonidet P-40 (NP-40, Shell) by shaking and then centrifuged. The nuclei were washed first with the lysis buffer, then with 0.05 M NaCl, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF, pH 8, and finally with 10 mM Tris, 0.1 mM EGTA, 1 mM PMSF, pH 8. The crude chromatin is suspended in 5 mM Tris-acetate, pH 7.8, 20 mM ammonium acetate, 0.4 mM $CaCl_2$, 0.2 mM EDTA, and 1 mM PMSF, sheared with Staphylococcal nuclease (Worthington, micrococcal, 3.1.4.7), centrifuged, and the insoluble chromatin sheared and centrifuged again to yield two fractions (first and second sheared). Both fractions of chromatin are then extracted with 0.4 N H_2SO_4 at 10 A_{260} units/ml and the insoluble DNA and nonhistone chromosomal proteins (NHCP) are sedimented at 100,000 x g for one h. Both pellets are redissolved in their original volume of 0.1 M Tris, pH 8.2, 0.01 M EDTA, 0.14 M 2-mercaptoethanol and extracted twice with an equal volume of buffer-saturated phenol (23). The clear phenol phases are pooled and precipitated with four volumes of 100% ethanol (phenol soluble NHCP). The cloudy interfaces contain the phenol insoluble NHCP. They are pooled and precipitated with four volumes of 100% ethanol. The precipitates are collected by centrifugation and dissolved in 0.25 M sucrose, 0.01 M phosphate, pH 7, and 1.5% SDS.

---

[1] In preparation.

Aliquots are dialyzed to 0.08 N NaOH before assaying for protein by the Lowry method (13). The samples are then dialyzed to the sucrose buffer with 0.1% SDS and afterwards 2-mercaptoethanol is added to 1% and bromphenol blue tracking dye to 0.002%.

A subset of NHCP, including the contractile proteins, is extracted by the following method. Total, sheared chromatin is mixed slowly with 0.4 volume of 3.5 M KCl, 17.5 mM 2-mercaptoethanol, 35 mM Tris, pH 7 and stirred at 0°C for 2 h. The DNA and some proteins are sedimented at 150,000 x g for 24 h. The supernatant is dialyzed against 100 volumes of 0.025 M KCl, 20 mM Tris, pH 7, 10 mM 2-mercaptoethanol, and 15 mM EDTA and the resulting precipitate is collected by centrifugation at 5000 x g for 30 min. It is treated as above for electrophoresis.

Electrophoresis is carried out by the method of Laemmli (10) using slab gels. They are stained with Coomassie blue by the method of Fairbanks et al. (4) and scanned with a Gilford 2000 spectrophotometer. The data were analyzed by computer.

All protein concentrations are determined by the method of Lowry et al. (13).

RESULTS

The purification of Dictyostelium discoideum chromatin and the characteristics of its histones have been described elsewhere (Bakke and Bonner [1]). The nonhistone chromosomal proteins can also be purified and divided into several classes. The first class is liberated along with 5% of the DNA when the chromatin is sheared with Staphylococcal nuclease for 1 min (first sheared chromatin fraction). All of the proteins in

this class are phenol soluble. The second and third classes remain with the bulk of the DNA, which is solubilized by a second shearing for 4 min (second sheared chromatin fraction). The second and third classes differ by their phenol solubility. The phenol-soluble proteins constitute 40% of the nonhistones in the second sheared fraction, while 60% of the nonhistones remain phenol-insoluble after this treatment. The displays of these proteins are pictured in Fig. 1 along with the associated scans. There are 56 different, visible polypeptide species on these three gels. This can be compared to rat liver chromatin which contains 115 resolvable species of nonhistones (6). Although many of the bands are common to all three classes, a few are unique or present in different proportions. These are minor bands and several are marked by broken lines in Fig. 1. The three classes also differ in the relative amount of protein in different molecular weight regions. A far greater percentage of the phenol-insoluble protein from the second sheared chromatin fraction lies above a molecular weight of 50,000. Table I compares the three protein classes on the basis of the amount of protein above 50,000 daltons and on the basis of the presence or absence of six unique bands. Each fraction contains a small amount of histone contamination near the end of the gel and also a major protein with a molecular weight of 46,400.

This major protein plus another with a molecular weight of 200,000 are isolated as described in Methods. The results of this procedure are shown in Fig. 2. This same procedure is used to extract contractile proteins from muscle tissue and from the chromatin of higher eukaryotes (22,3,8,9). On the basis of their solubility and mobility on gels these proteins appear to be actin-like and myosin-like. The actin-like protein

FIGURE 1: Scans and polyacrylamide gels of Dictyostelium nonhistone chromosomal proteins. The upper scan and gel are the phenol insoluble proteins of the second sheared fraction, i.e., the bulk of the chromatin. The middle scan and gel are the phenol soluble proteins of the second sheared fraction. The lower scan and gel are the total phenol soluble proteins of fraction one, i.e., all the proteins in the earliest nuclease sheared chromatin. Each gel slot contains 100 µg of protein.

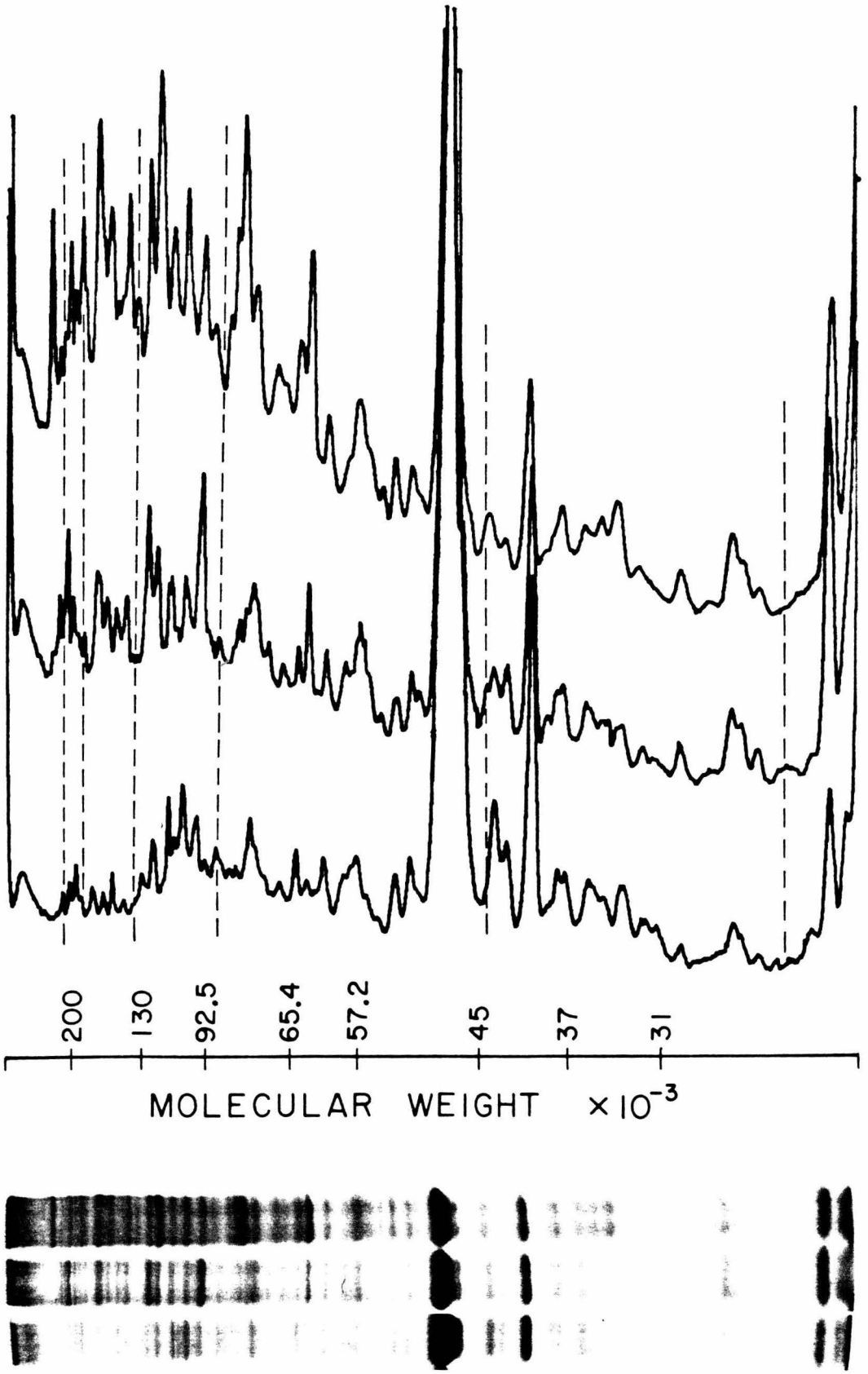

200
130
92.5
65.4
57.2
45
37
31
MOLECULAR WEIGHT × 10⁻³

Table I

Differences Between Nonhistone Chromosomal Protein Classes

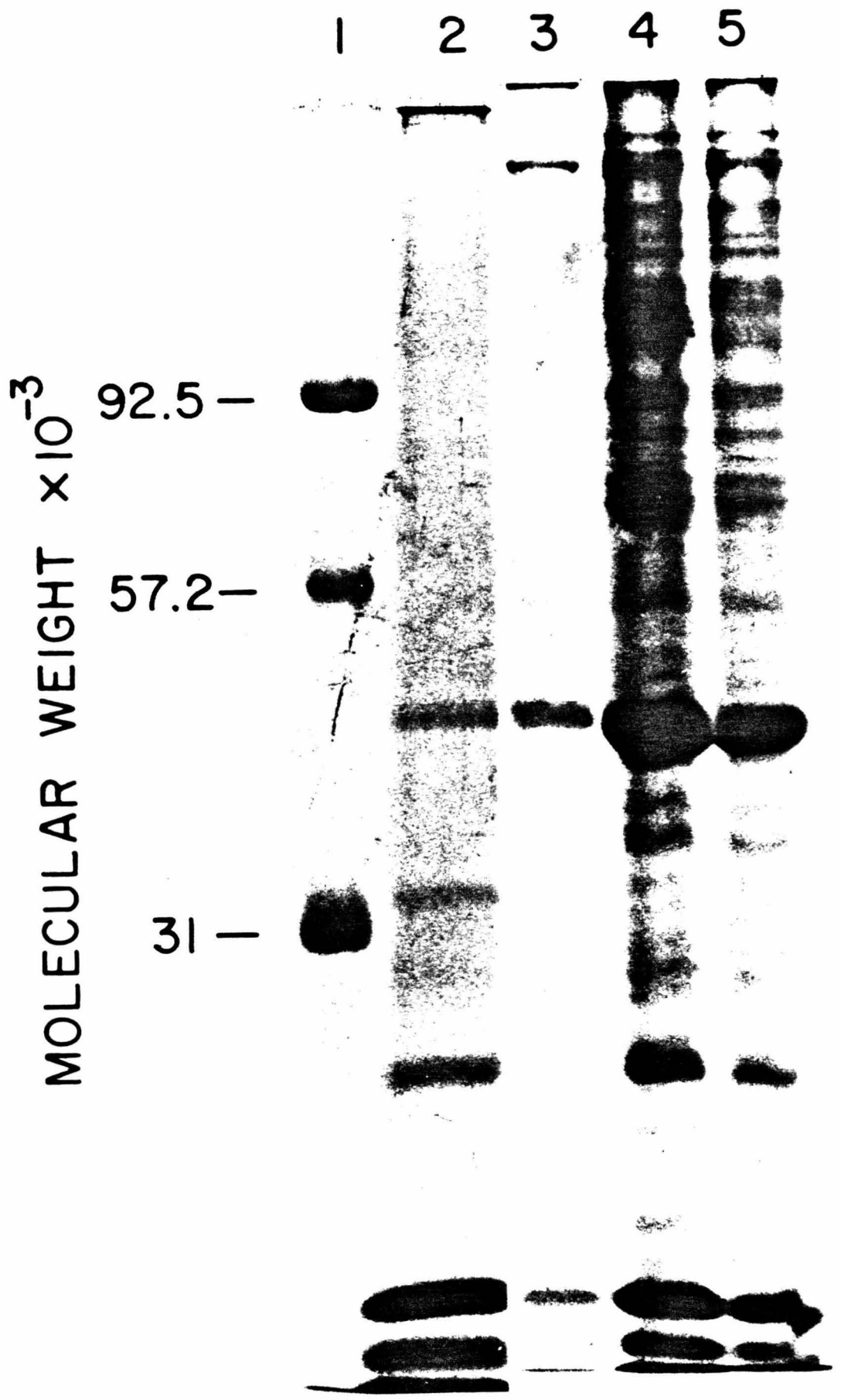
	Nonhistone class		
	First sheared phenol soluble	Second sheared phenol soluble	Second sheared phenol soluble
% of total NHCP*	21%	31%	48%
% above 50,000 Mol. Wt.‡	41%	56%	72%
Molecular Weight§ of Specific Proteins	Qualitative Presence or Absence in NHCP Class		
219,000	+	+	-
188,000	-	-	+
134,000	-	-	+
86,300	+	+	-
43,700	-	+	-
20,300	-	+	-

*Determined by the method of Lowry et al. (13).

‡Determined by integrating gel scans.

§These six minor polypeptides account for less than 2% of the total gel scan area and are not common to all three protein classes. A + indicates the presence of that particular band, while a - indicates that it is absent or present in a relatively low quantity.

FIGURE 2: Slab polyacrylamide gels of the chromosomal proteins of Dictyostelium. From left to right: (1) molecular weight standards; (2) acid soluble (0.4 N H_2SO_4) chromosomal proteins - 50 µg; (3) low ionic strength (0.025 M KCl) precipitate of chromosomal proteins, including the actin- and myosin-like proteins - 25 µg; (5) total chromosomal proteins - 110 µg.


1
2
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5
MOLECULAR WEIGHT ×10⁻³
92.5
57.2
31

is an especially large component in each NHCP fraction, but actin is also a large component of the cell. When the contaminating pellet, which remains after the removal of the sheared, soluble chromatin, is analyzed on gels, its major proteins have the mobility of actin and myosin. However, it has been found that this method of chromatin purification yields a product with 7% contamination (Bakke and Bonner[1]). If all of this contamination was due solely to actin, it would not account for the levels of actin-like protein found in the NHCP fractions and in the total chromosomal proteins.

The amount of protein in a gel band is related to the quantity of dye bound to the band and is determined by integrating the area under the gel scans. Each gel in Fig. 1 contains 100 micrograms of protein as determined by the method of Lowry et al. (13). The extinction coefficients per microgram of protein stained under these conditions are given in Table II. The gels with a greater proportion of bands above a molecular weight of 50,000 also have a higher extinction coefficient. However, the difference between the gels is less than a factor of two. Two possible explanations for this phenomenon are either the dye molecules interact along longer peptides causing more dye to bind, or the peptides bind more dye cooperatively creating a higher background. To check the second possibility equal masses of each protein class were precipitated on filters and stained. The ratios of bound stain were similar to those seen on the gels indicating that denser gel backgrounds would not explain the differences between the extinction coefficients.

Table II

Staining Characteristics of Nonhistone Chromosomal Proteins

	Extinction* Coefficient per microgram protein	% of actin-like protein in fraction Before subtracting contamination	% of actin-like protein in fraction and After‡ subtracting contamination
Total chromosomal protein including histone	2.098	11	4.3
Nonhistone protein fraction			
First sheared phenol soluble	2.828	29%	22%
Second sheared phenol soluble	3.89	15	8.6
Second sheared phenol insoluble	5.095	9	2.2

*The gels are scanned at 590 nm. The optical density of the total amount of stain bound by each gel can be calculated by integrating the area under each scan and multiplying by a correction factor for the portion of the gel actually observed by the spectrophotometer. Since slab gels were used, no correction is required for the shape of the gel.

‡If all of the contamination is due to actin-like protein, this is the minimum quantity of actin-like protein remaining.

DISCUSSION

The nonhistones are a fascinating group of proteins, but functions for only a few of them are known, and are primarily enzymatic. Roles in gene regulation, chromatin structure, and mitotic and meiotic division have been suggested for the remaining proteins (1,16,11,19). Dictyostelium is well suited to studying these proteins because of its simple developmental cycle, small genome size, and large fraction of expressed genes (5). Since it is simple, it may not require the complex regulation that higher eukaryotes do. This is reflected in the lower total number of visible non-histone chromosomal protein bands. Dictyostelium only contains half the number found in mammals.

In the three classes of nonhistones isolated here there are a number of unique bands. The differences between the first and second sheared chromatin fractions may reflect real functions. Testing this hypothesis must await further characterization of these chromatin fractions to determine if they differ structurally or functionally, and the purification of active proteins.

Two of the nonhistone chromosomal proteins have been tentatively identified as contractile-like proteins. They have the solubility characteristics and molecular weights of actin and myosin. However, cytoplasmic actin and myosin co-purify with the chromatin. They are probably in the form of actomyosin filaments. These filaments are formed by the following reactions (7,14):

$$n\text{Globular-actin} + n\text{ATP} + n\text{Me}^{2+} \rightleftharpoons \text{Filamentous actin} \cdot n\text{ADP} \cdot n\text{Me}^{2+} + n\text{P}_i$$

$$\text{F - actin} + \text{myosin} + \text{ATP} + \text{Me}^{2+} \rightleftharpoons \text{actomyosin} \cdot \text{Me}^{2+}\ \text{ADP} + \text{P}_i.$$

This reaction takes place in the presence of ATP and divalent metal ions

(Me^{2+}, either Mg^{2+} or Ca^{2+}). It requires the presence of thiol groups in both the actin and myosin molecules. To reduce the contamination these reactions are reversed by adding EGTA, reducing the level of Mg^{2+} concentration used in purifying the nuclei, using an inorganic phosphate buffer, and by not adding reducing agents to stabilize enzymes.

The percentage of actin-like protein has been approximated by integrating the gel scans, but this calculation requires some knowledge about the staining characteristics of the proteins. The extinction coefficients indicate that higher molecular weight proteins bind more stain. Correcting for this factor would increase the percentage of actin-like protein because it has a lower molecular weight than the majority of the NHCP. Since about 7% of the protein isolated with the chromatin is nonchromosomal, it is important to determine if all of the actin-like protein could be a contaminant. The third column of Table II is calculated by assuming that all of the contamination is due to actin-like protein and subtracting it from the percentage of actin-like protein in the NHCP. Although the exact quantity cannot be determined without knowing the extinction coefficient for each individual NHCP, the actin-like protein is a major protein in Dictyostelium chromatin.

The myosin-like protein is a minor band by comparison with the actin-like protein. Low ratios of myosin-like to actin-like protein are observed in the chromatin of higher eukaryotes and in other non-muscle actomyosins (2,3,11). However, the low ratio in slime mold chromatin could also be in part an artifact due to selective extraction of the myosin during purification or to proteolytic activity, which is insensitive to PMSF (3).

Several tests are warranted to confirm the identity of the actin-like and myosin-like proteins. Actin will polymerize and depolymerize, actin and myosin superprecipitate, myosin has an ATPase activity and actin the unusual amino acid, 3-methyl histidine.

SUMMARY

Dictyostelium discoideum contains a complex group of non-histone chromosomal proteins on electrophoresis gels. There are 56 visible bands, which have been fractionated and compared qualitatively and quantitatively. Two of the major non-histones have been selectively extracted. On the basis of their solubility and molecular weight they are probably actin- and myosin-like proteins.

Note added in proof:

While this paper was being written another paper appeared describing the presence of nonhistone chromosomal proteins with the molecular weight of actin and myosin in Dictyostelium discoideum (17).

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III. Chromatin Structure in the Cellular Slime Mold *Dictyostelium discoideum*

INTRODUCTION

Olins and Olins (1) first described the basic unit of chromatin structure, or nucleosome (also called the nu body or psi particle). By carefully spreading the chromatin they saw 70 Å beads strung together in long chains, which were linked by thin 15 Å, DNA-like threads. The appearance of the linker is somewhat unstable since it is determined by salt concentration (2,3) and the presence of histone H1 (4).

Baldwin et al. (5) determined the position of the elements in the simple monomer nucleosome of calf thymus using neutron diffraction. There is an inner protein core 64 Å in diameter, surrounded by a 20 Å thick sheath of DNA making the entire structure 104 Å in diameter. Kornberg and Thomas (6) suggested that the protein core consisted of two each of four histones (H2a, H2b, H3 and H4) on the basis of cross-linking experiments and X-ray patterns. D'Anna and Isenberg (7) demonstrated that the histones bind to each other as specific pairs in solution with the tightest binding between H3 and H4. These two formed a tetramer (i.e., homotypic) to which two molecules each of H2a and H2b bound less tightly. On the other hand, Weintraub et al. (8) found that a tetramer of one each of H2a, H2b, H3 and H4 (i.e., heterotypic) could exist in high salt without DNA and Altenburger et al. (9) showed that DNase II cleaves chromatin at 100 base pair (b.p.) intervals under certain ionic conditions. These observations suggested that the monomer might be divided into two equal halves.

In chromatin the DNA helix wraps around the histone core. The length of the monomeric DNA varies widely from 154 to 241 base pairs, depending on the organism and tissue (10). Aspergillus has a monomer length of 154 base pairs (11), while mammals have longer pieces of DNA varying from 188 to 207 base pairs (12). Chicken erthrocytes have a longer repeat length (212 b.p.) than chicken liver cells (200 b.p.) and this difference apparently is due to variations in the length of the linker, which is determined by the presence of H1 or H5 histone (13). The presence of a linker is revealed when monomers are extensively digested. This produces a stable core length of DNA, which is about 140 base pairs in all organisms that have been investigated. The DNA between the cores is the linker and is covered by either H1 or its equivalent (14). Histones more basic than H1, such as H5, may require longer linkers.

Lower eukaryotes, such as Dictyostelium discoideum, are well suited for studying chromatin structure and gene expression. Since the slime mold has a large fraction of template active chromatin, any structural differences in the active and inactive chromatin should be observable. One aspect of chromatin structure is confirmed in the following study. Dictyostelium has a less basic histone H1 than higher eukaryotes and also has a shorter length of linker DNA in the nucleosome. In addition, the presence of four, instead of the usual five histones, does not effect the basic chromatin structure.

METHODS

Growth and Labeling of Cells. A variant of Dictyostelium discoideum (Ax 3) is grown in an axenic culture (15) on a rotary shaker. The DNA is labeled with ^{3}H-thymidine for the nuclease digestion studies. The cells are first grown to 1 x 10^{7} cells/ml. They are harvested and washed by centrifugation. They are then suspended at 1 x 10^{8} cells/ml in 20 mM KCl, 2.5 mM $MgCl_2 \cdot 6H_2O$, 50 mM phosphate buffer, pH 6.5, and 4 μC/ml of ^{3}H-thymidine and shaken for 8 hrs. At the end of this time they are transferred to a minimal growth media containing 10 g/l glucose, 10 g/l proteose peptone (Difco), 0.5 g/l yeast extract, 5 mM PB pH 6.6, 0.02 mg/l biotin, 0.005 mg/l cyanocobalamine, 0.2 mg/l folic acid, 0.4 mg/l lipoic acid, 0.5 mg/l riboflavin, and 0.5 mg/l thiamine (16). The yeast extract is lowered to remove competing unlabeled thymidine. The vitamins are added to compensate for the low level of yeast extract. The cells are diluted ten-fold to 1 x 10^{7} cells/ml in the minimal media and shaken an additional 10 hrs. They are then harvested for use.

Preparation of Nuclei for Electron Microscopy. Slime mold cells are suspended in 0.1 M sucrose, 10 mM Tris pH 7, and 0.4% Nonidet P-40 (NP-40, Shell) at 2 x 10^{8} cells/ml and are lysed by shaking for 45 sec. The suspension is centrifuged at 800 xg for 5 min. The supernatant is discarded and the pellet is gently resuspended in the buffer and centrifuged. The nuclei are resuspended and adjusted to a concentration of 0.5 x 10^{8} nuclei/ml. They are diluted one hundred-fold to five hundred-fold in 1 mM EDTA pH 7.5 and allowed to swell for 15 min. Carbon coated,

copper grids are placed in a small well (17). The sample is layered over a 0.1 M sucrose cushion in the well and covered with a coverslip. The nuclei are centrifuged at 2000 xg for 5 min. The grids are removed, rinsed with 1% photoflo (Kodak) and stained with 0.5% uranyl acetate for 30 sec. The uranyl acetate is carefully washed away using a clean set of forceps and the grids are air-dried. Finally, the grids are rotary shadowed with platinum and palladium (80/20) and viewed in a Phillips 300 electron microscope. The particles seen in the film are measured with a Hewlett-Packard digitizer.

Nuclease Digestion. Labeled nuclei are prepared by first lysing cells in 0.37 M sucrose, 40 mM KCl, 20 mM phosphate buffer pH 7.5, 0.5 mM $MgAc_2$ (acetate), 0.1 mM EGTA (ethyleneglycolbis-(β-aminoethyl ether)-N,N'-tetraacetic acid), 1.5 mM PMSF (phenylmethylsulfonyl fluoride), and 0.5% NP-40 at 2 x 10^8 cells/ml. The nuclei are pelleted at 1500 xg for 5 min. They are homogenized in the same buffer but with 2.5 mM $MgAc_2$ and 0.2% NP-40 and centrifuged at 2500 xg for 7 min. The nuclei are then homogenized in a 1 M sucrose buffer without NP-40 and centrifuged at 5000 xg for 10 min. They are resuspended in this buffer before use on the same day. An aliquot is removed and pelleted at 5000 xg for 10 min. The nuclei are washed once in the nuclease shearing buffer (5 mM Tris Ac pH 7.8, 20 mM NH_4 Ac, 0.4 mM $CaCl_2$, 0.2 mM EDTA) and resuspended in it at 10 alkaline A_{260} units/ml. The samples are then digested with Staphylococcal nuclease (Worthington) under the conditions described in the figures. Small aliquots are removed at the designated

times and precipitated in cold, 7% PCA (perchloric acid). The pellets are washed with PCA again and the supernatants are pooled for liquid scintillation counting in Aquasol (Amersham/Searle). After all the aliquots are removed, the remaining sample is made 1% in SDS and aliquots removed to determine the total radioactivity. All vials contain an equivalent amount of SDS.

Gel Electrophoresis. The DNA is extracted from digested nuclei with phenol and chloroform. It is then ethanol precipitated and dissolved in one-tenth strength gel buffer with 10% glycerol. The gel buffer is 89 mM Tris, 89 mM boric acid and 2.5 mM EDTA (ethylenediaminetetraacetic acid). The gels are run according to Peacock and Dingman (18) with 2% acrylamide and 0.5% agarose, but at 100 volts and without cooling.

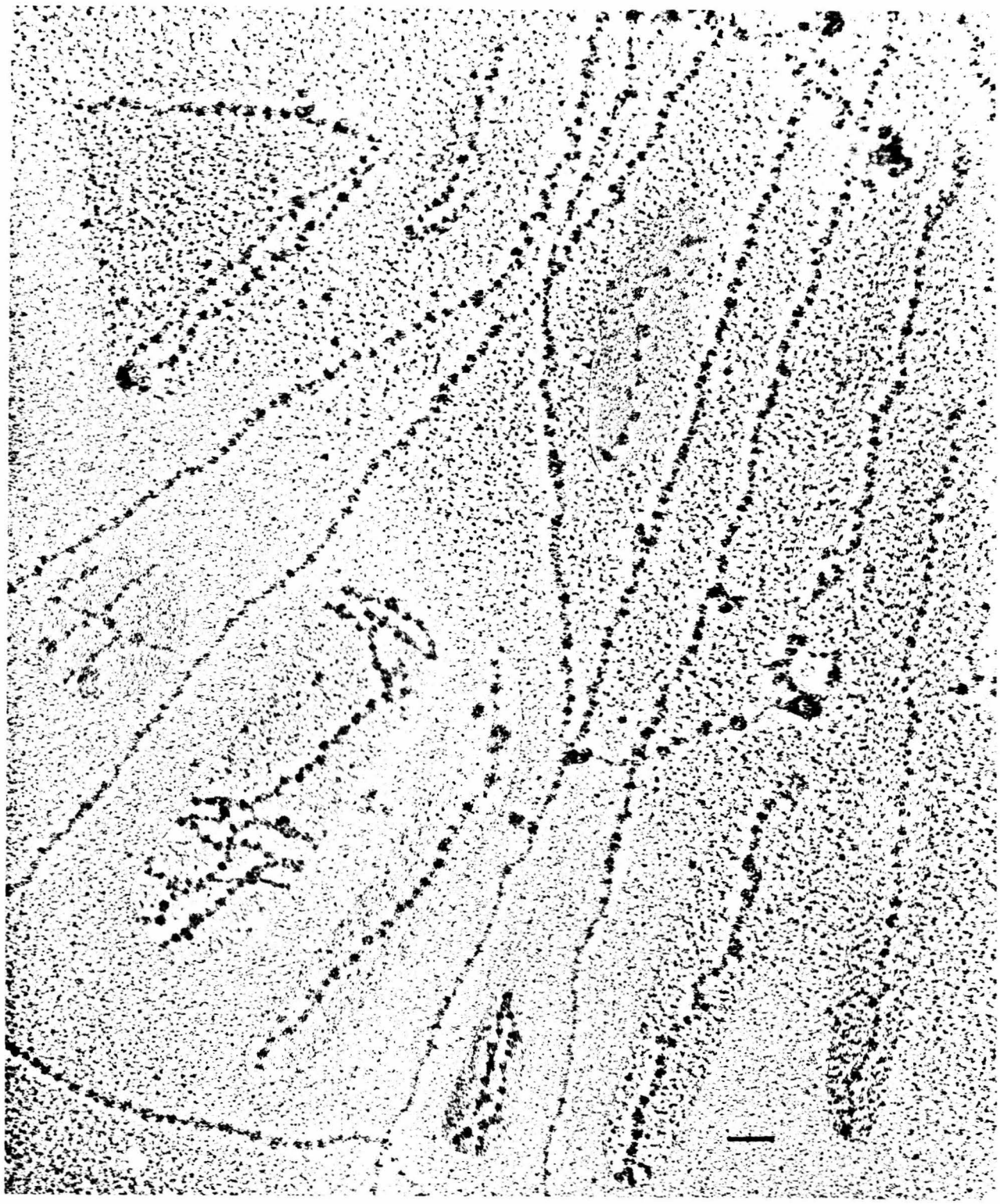
Sucrose Gradient Centrifugation. Isokinetic sucrose gradients are formed according to Noll (19) and centrifuged in a Beckman SW41 rotor at an average rpm of 33,400 for 12 hrs. The gradient characteristics are as follows: top concentration, 5.1% sucrose (w/v); reservoir concentration, 29.84% (w/v); mixing volume, 9.82 ml; minimum radius, 7.785 cm; maximum radius, 14.55 cm; sample volume, 0.5 ml; temperature, 4°; and particle density, 1.45 g/cm^3. The gradients are pumped through an Isco flow cell and the A_{260} recorded. The samples contain labeled, digested rat liver nuclei as marker nucleosomes. Fractions are collected and counted in Aquasol with a Beckman scintillation counter.

Melting Chromtin Unlabeled chromatin is purified from nuclei prepared as above. The nuclei are further washed with 0.05 M NaCl, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF, pH 8, twice and with 10 mM Tris, 0.1 mM EGTA, 1 mM PMSF, pH 8, twice. The resulting chromatin is suspended in nuclease shearing buffer and lightly sheared with Staphylococcal nuclease to solubilize it in the buffer. This supernatant is dialyzed against several changes of 0.25 mM EDTA pH 8 and then melted in a Gilford 2400 spectrophotometer using a thermo-programmer and thermal cuvette. The data are analyzed by a computer and fit to Gaussian components (20).

RESULTS

Electron Microscopy of Dictyostelium Nucleosomes. Nucleosomes may be observed by spreading them on grids as described in the Methods. The solutions used must have a low ionic strength to allow swelling of the chromatin. Otherwise it will remain condensed and nucleosomes will not be visible. In addition platinum-palladium shadowing greatly facilitates the microscopy by enlarging the nucleosomes and making them more dense. Fig. 1 is a mosaic of nucleosomes seen on several grids. They are found extending from nuclei, but the majority of the chromatin in the nucleus is still condensed. The nucleosomes are found in long strings and may be next to each other or with thin filaments connecting them. These filaments are assumed to be DNA. In unshadowed pictures the DNA filaments have an average diameter of 30.9 ±3.5 angstroms (Å). The shadowed DNA has a diameter of 51.4 ±9.3 Å. From X-ray crystallography

Fig. 1. A mosaic of several pictures of Dictyostelium chromatin taken with an electron microscope. The bar line is 0.1 micron.



work B-form DNA is found to have a diameter of 20 Å (21). These figures are used to correct the measured diameters of the nucleosomes to the corresponding real values minus the effects of preparation. The diameters of all roughly circular particles are measured in several planes intersecting their centers and an average taken. These averages are plotted as a histogram in Fig. 2. This distribution has an average and standard deviation of 98.6 ±15.2 Å (N = 500) for the diameter of Dictyostelium nucleosomes. The length of the filaments between the nucleosomes is measured where they are present and the average is 170 Å or 50 b.p. In the majority of cases no filament is visible. To further study the structure of chromatin, it is perturbed by the use of enzymes.

Nuclease Digestions of Dictyostelium Nuclei. Labeled, purified Dictyostelium nuclei are digested with Staphylococcal nuclease for various lengths of time and the percent acid solubility determined. Fig. 3 illustrates the results under several different digestion conditions. The nuclei are purified in the presence of PMSF, a serine protease inhibitor, and, when PMSF is added to the digestion buffer, along with the nuclease, acid soluble DNA is released as shown in Fig. 3a. The digestion begins to plateau at 50 to 55%, but there is a gradual increase to 70% between 30 and 60 min of digestion. If PMSF is removed from the nuclease buffer, digestion continues to 75% solubility as in Fig. 3b, indicating an increased sensitivity of the DNA. The relative initial rate of this reaction with respect to the control digest with PMSF (rate = 1.0) is 1.1. This is calculated by comparing the products of the first-

Fig. 2. A histogram of the corrected diameters of Dictyostelium nucleosomes (N = 500).

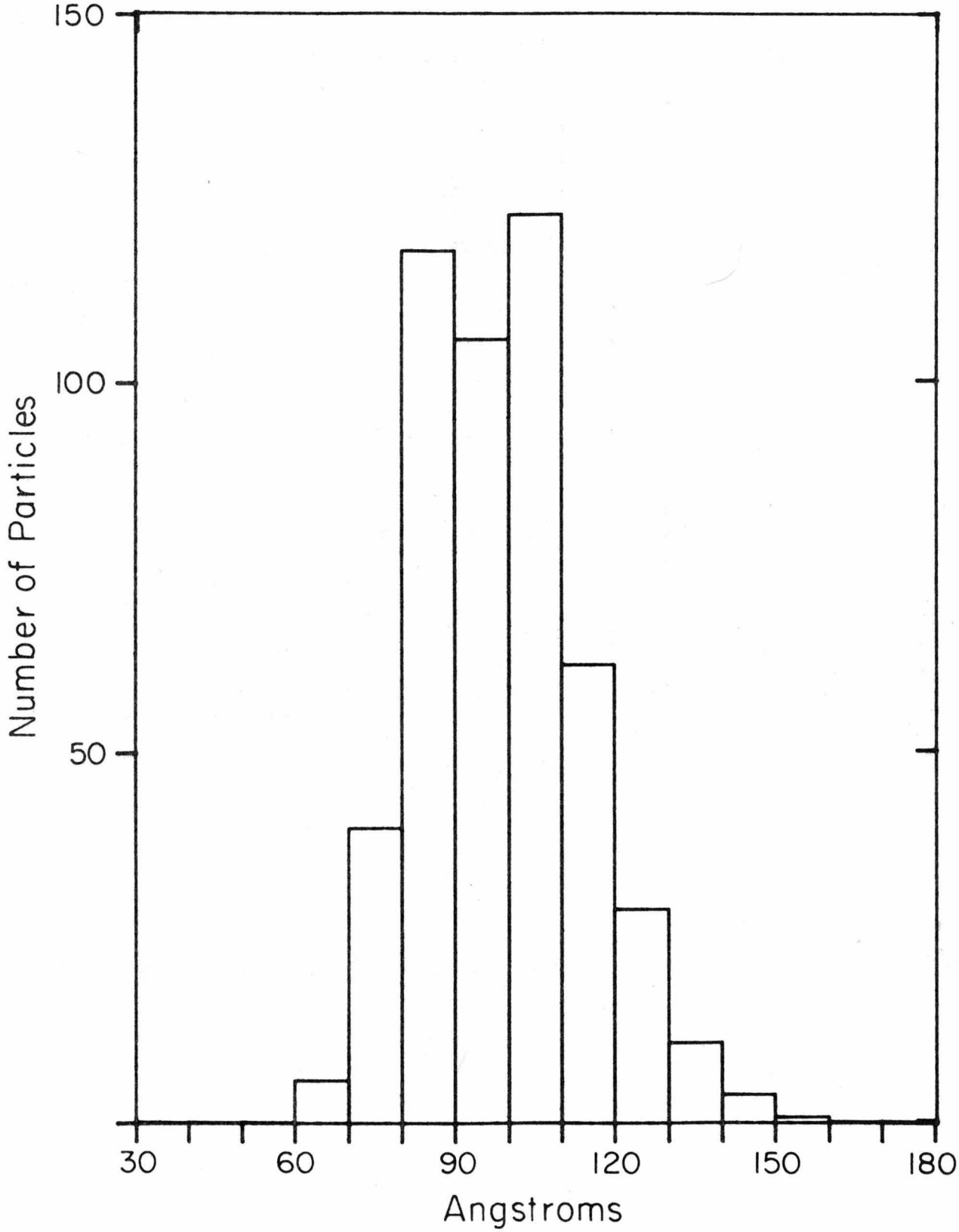
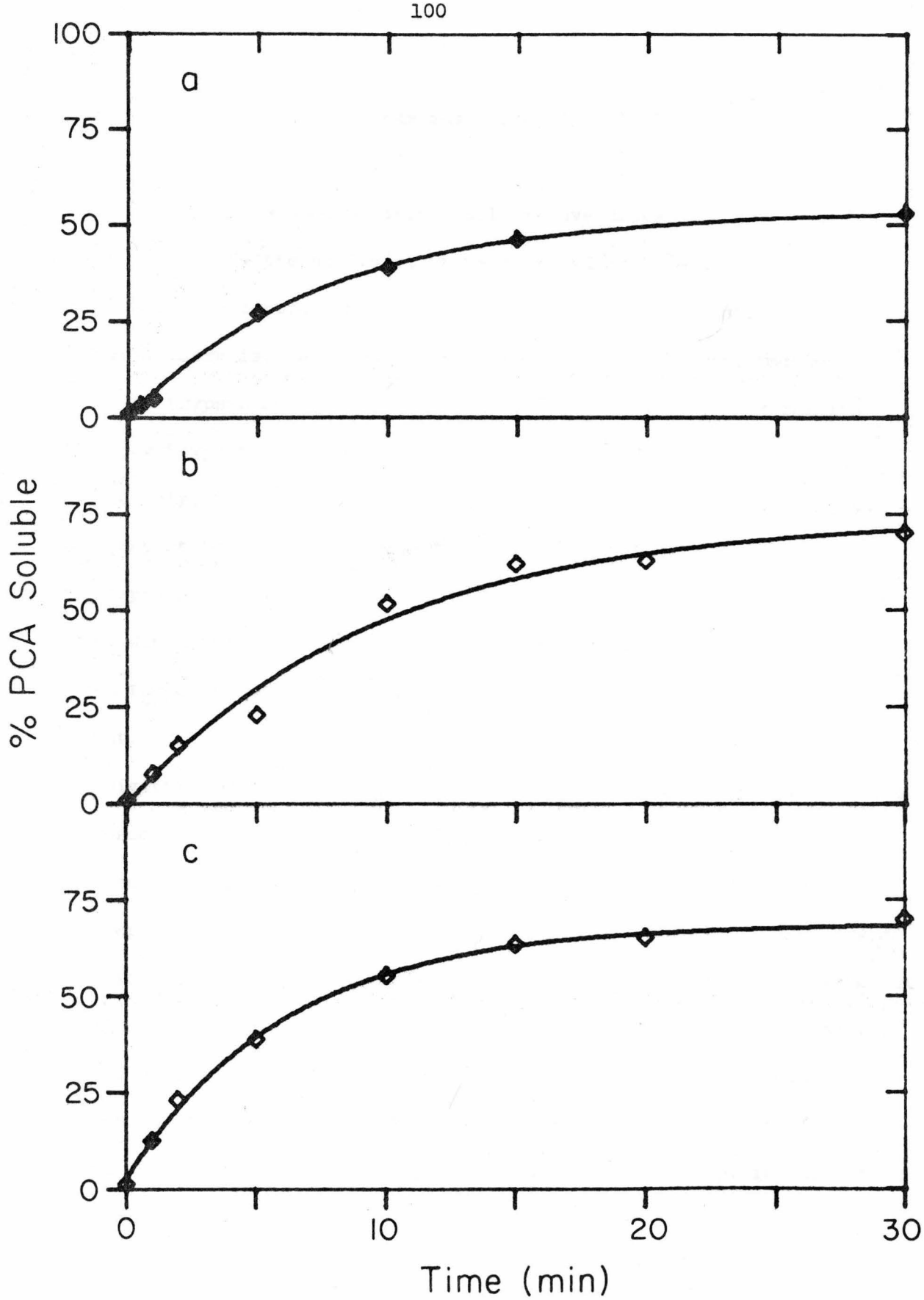

150
100
50
Number of Particles
30
60
90
120
150
180
Angstroms

Fig. 3. Digestion of Dictyostelium nuclei with Staphylococcal nuclease. (a) Nuclei are digested at 10 A_{260} units/ml (alkaline) and 100 units of nuclease/ml in the presence of 1 mM PMSF. (b) Same conditions as "a", except no PMSF. (c) Nuclei are digested as above with 10 µg/ml trypsin added and without PMSF.

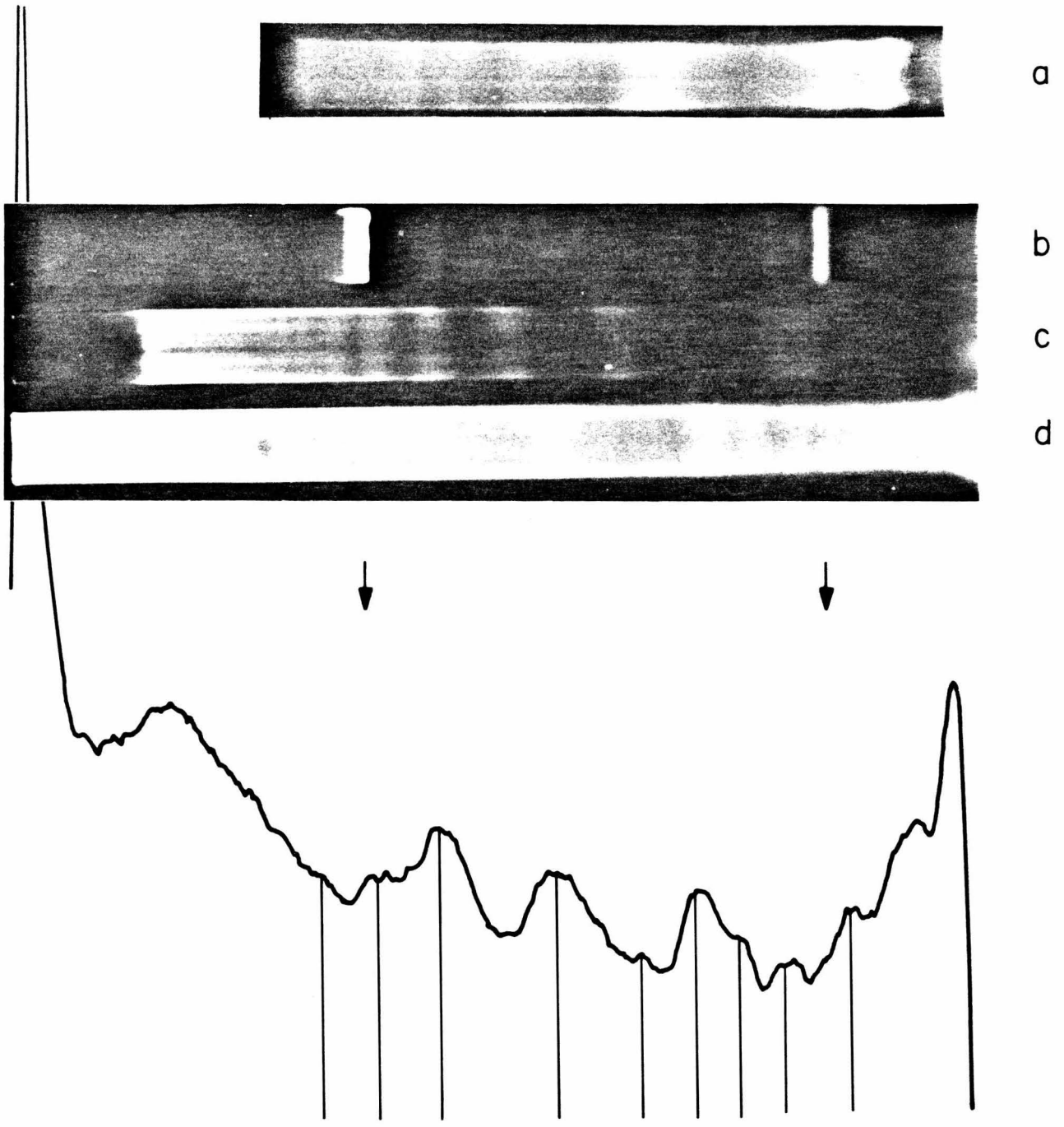

a
b
c
100
75
50
25
0
% PCA Soluble
0
10
20
30
Time (min)

order rate constant and the maximal percentage digested for the two reactions.

Since an endogenous protease could remove proteins exposing additional lengths of DNA to the nuclease, trypsin was added to mimic this activity. At a trypsin concentration of 10 µg/ml and a DNA concentration of 350 µg/ml there is one trypsin molecule for every 1250 base pairs of DNA. The trypsin has an activity of 89.4 units/mg (22). The results of adding trypsin are shown in Fig. 3c. The acid solubility increases more rapidly, but it still plateaus at 75%. The relative initial rate with respect to the control digest is 1.6. When PMSF is added along with trypsin, the kinetics return to the original rate in Fig. 3a. The nuclei demonstrate a higher level of nuclease sensitivity after being stored for 8 hrs at 0°, similar to Fig. 3b, even when PMSF is continually present.

Digestion may also be used to determine the length of the DNA in the nucleosome and the hydrodynamic size of the nucleosome. Nuclei are purified and digested with Staphylococcal nuclease. Purified DNA is electrophoresed along with markers, which are used to prepare a standard curve for determining the size of the monomer repeat (Fig. 4). The monomer DNA size is measured by two methods: the length of the higher multimers divided by their multiplicity (12), and the difference in length between adjacent multimers (14). In the first case the length of monomer DNA is 187 ±8 base pairs (b.p.), and it is 190 b.p. in the latter. The nucleosome bands on gels are fairly broad indicating some heterogeneity. However, some monomer DNA is apparently digested to a more constant,

Fig. 4. Two percent acrylamide with 0.5% agarose gels of DNA. Electrophoresis is from left to right. (a) An early preparation of Dictyostelium nucleosomal DNA sheared with Staph. nuclease, which was only electrophoresed with rat liver nucleosomal DNA. It is approximately aligned with the other gels. (b) Hind II restriction fragments of ØX174 DNA. The arrows mark the positions of fragments R1 (1049 b.p.) and R9 (161 b.p.). Nine markers are visible when the picture is overexposed. (c) DNA from rat liver nuclei sheared with Staph. nuclease. (d) DNA from Dictyostelium nuclei sheared with Staph. nuclease. The optical scan is from the negative of gel d. The vertical lines mark the positions of the following lengths of DNA in base pairs from left to right: 1160, 967, 755, 483, 349, 275, 225, 184 and 136. Gels b, c and d are adjacent lanes in one slab gel.


a
b
c
d

basic size producing a sharper DNA band called the core (11). For slime mold it is 137 b.p. The DNA that is removed from the monomer, leaving the core, is the linker DNA, which is generally covered by histone H1 or H5 (13,14). Its length is determined either by measuring the half-width of a multimer band, or by finding the difference between the monomer and core length of DNA. This is 48 b.p. and 50 b.p., respectively, in Dictyostelium and correlates well with the length of the filaments between nucleosomes seen in the electron microscope (50 b.p.). The DNA gels (Fig. 4) show some unusual complexity in the case of Dictyostelium. In the position of the putative dimer, a doublet is found with DNA lengths of 225 and 275 b.p. Below the doublet there are five additional peaks at 184, 137, 110, 96 and 78 b.p.

The products of a nuclease digestion may also be analyzed on isokinetic sucrose gradients. In Fig. 5 slime mold chromatin is centrifuged along with labeled rat liver markers. The sedimentation coefficient ($S_{20,w}$) for rat liver nucleosomes is 11.8S for the monomer and 16.3S for the dimer. Slime mold monomers are not significantly different at 11.5S, but the dimers are slightly smaller at 15.7S. This agrees with the results from the gel electrophoresis, which indicate that Dictyostelium nucleosomes contain a shorter length of DNA.

Melting of Dictyostelium Chromatin. Basic proteins stabilize DNA toward melting and the structure of chromatin may be elucidated by melting it (20,23,24). Slime mold chromatin is slowly melted in a low ionic strength buffer and the resulting data are differentiated in Fig.6. Fig. 6a

Fig. 5. Centrifugation of nucleosomes on isokinetic sucrose gradients. Dictyostelium nuclei digested with Staphylococcal nuclease are layered on top of sucrose gradients along with labeled rat liver nucleosomes. The solid line is the A_{260} profile of slime mold nucleosomes. ^{14}C-labeled rat liver nucleosomes are used as markers in the same gradient (□ -- □). The arrow marks the position of an 11.8S particle. Centrifugation is from left to right.

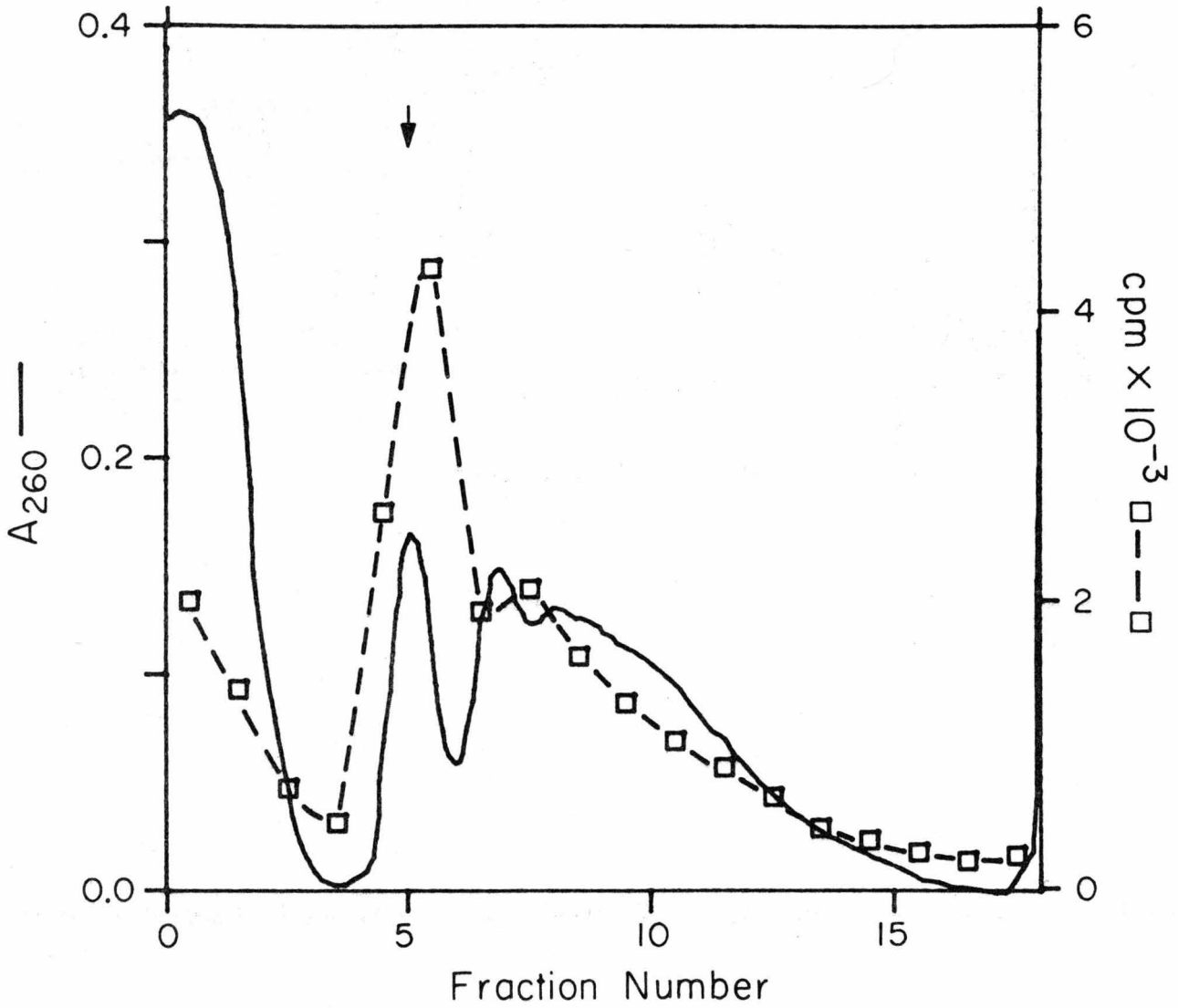
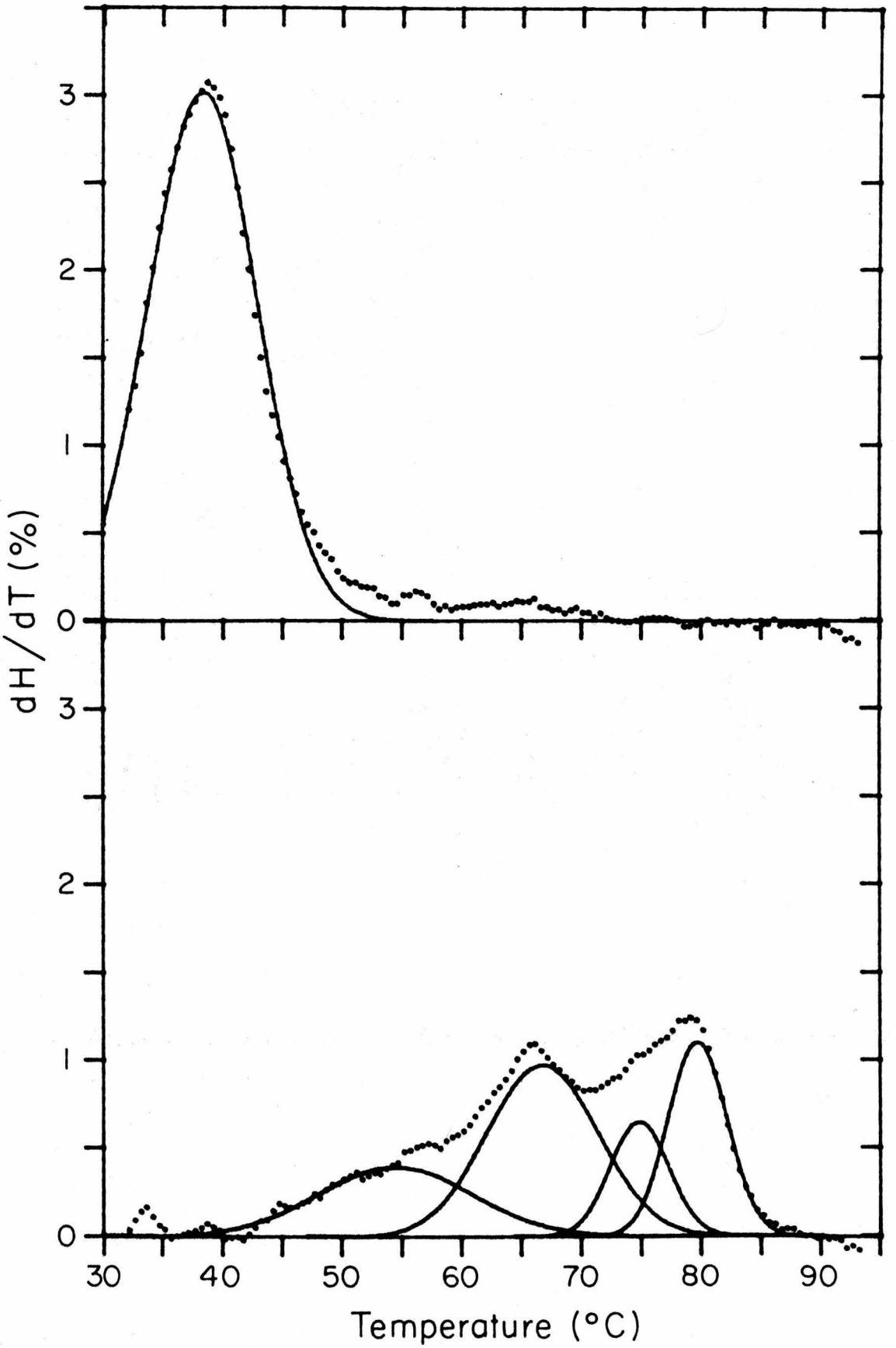

0.4
0.2
0.0
A_{260} ——
0
5
10
15
Fraction Number
6
4
2
0
cpm X 10^{-3} □—□

Fig. 6. Derivative profiles of melting curves for samples in 0.25 mM EDTA, pH 8. The plots are normalized to the same beginning optical density. The ordinant scale has units of change in hyperchromicity per degree Celcius (dH/dT). (a) Dictyostelium nuclear DNA (···). Total hyperchromicity is 36%. (b) Dictyostelium chromatin (···). Total hyperchromicity is 29%. The solid lines are the fitted Gaussian components.


dH/dT (%)
3
2
1
0
3
2
1
0
30
40
50
60
70
80
90
Temperature (°C)

shows the derivative for pure Dictyostelium nuclear DNA. It is plotted in terms of change in hyperchromicity divided by change in Celcius degrees on that ordinant so that different melts may be compared. Fig. 6b is the equivalent derivative profile for slime mold chromatin, and four major Gaussian components may be fitted to this curve. The results of this fit and a comparison of a similar fit for rat liver chromatin are listed in Table I. It is apparent that Dictyostelium chromatin also has four components, but they are different in proportion. The components may be compared between rat and slime mold by considering ones with similar differences between their Tm and the Tm of pure, homologous DNA. The lowest melting component (Tm1) of Dictyostelium has a Tm only 16.0° higher than pure DNA, while the equivalent component in rat shows a difference of 21.2° in the Tms. The second, third and fourth components of both are similar with respect to their Tm differences, but they have different proportions. The second slime mold component (Tm2) is especially pronounced, while it is small in the rat. It seems that a larger fraction of the slime mold chromatin is poorly stabilized by its nucleoproteins.

DISCUSSION

Two conclusions may be drawn from this investigation of chromatin structure. First, the basic unit of structure in Dictyostelium chromatin is the nucleosome and its size is very similar to that found in higher eukaryotes. Second, endogenous proteases may drastically affect the chromatin structure. The measured diameter of the nucleosome is 98.6 Å

Table I

Summary of melting transitions from

Fig. 6 and Wallace et al. (1977)

	Tm1*	%	Tm2	%	Tm3	%	Tm4	%
Dictyostelium								
Chromatin	54.4	22.6	66.7	39.7	74.9	14.0	79.7	23.7
DNA	38.4	100						
Rat Liver								
Chromatin	62.0	29.1	71.1	18.7	77.1	23.4	83.4	28.8
DNA	40.8	100						

*Temperatures are in degrees Celcius. Transitions are numbered in order of increasing temperature. Percentages refer to the fraction of the total hyperchromicity of each sample in a given transition.

and is very close to the 104 Å measured in calf thymus (5). Its core size of 137 b.p. has been found to be nearly identical in all organisms. These similarities are somewhat surprising in view of the fact that Dictyostelium has only four histones instead of the usual five, and one of the four is completely different. Apparently, the histones alone do not define the nucleosomes. This correlates with the results of Griffith (25), who found that under the correct salt concentrations free DNA would form nucleosome-like structures. Perhaps this nature of the DNA is responsible for the uniformity in core size.

A DNA linker is attached to the core producing the monomer length of DNA and is associated with histone H1 (14). Dictyostelium has a shorter linker than higher eukaryotes, which may be a general characteristic of lower eukaryotes where H1 is less basic than in mammals (11,26). The number of basic residues in the H1-equivalent molecule may define the length of the linker. Histone H5 contains 37% basic residues and produces a linker of 72 b.p. in chicken erthyrocytes, while H1 has 30% basic residues and yields a 60 b.p. linker in chicken liver cells (27,28, 13). Dictyostelium H1 is less basic than mammalian H1 (Bakke and Bonner, in preparation) and the linker is only 50 b.p. long.

The visibility of the linker may also depend on H1. When H1 is present, the chromatin is condensed with no visible DNA filaments connecting the nucleosomes. However, when H1 is removed, the fibers unfold into a string of beads separated by filaments (2-4). The conditions used in this study to spread the chromatin did not extract H1 and left most of the chromatin condensed with few visible linkers. This may partially

explain the lack of monomer length DNA on the gels, while other conditions may give a more definite monomer band.

The nuclease digestion pattern is complex. A doublet of 225 and 275 b.p. is found at the position of the dimer. These are both small for a dimer with a 187 b.p. repeat, but may be explained by combinations of a core plus two linkers (∿237 b.p.) and two adjacent cores with no linker (∿276 b.p.). Endogenous protease or nuclease activity might also generate this pattern. The digestion studies emphasize the effect of proteases, which must be taken into account in interpreting data.

An alternate explanation of the digestion pattern is that the monomer repeat length is approximately 245 b.p. and the doublet represents the monomer and an intermediate degradation product. Since the presence of the linker allows some variability in the predicted results, the data could also fit a long repeat length. Lengths of 218 b.p. in sea urchin gastrula, 241 b.p. in sea urchin sperm, and 220 b.p. in Stylonychia macronucleus are reported in the literature (29,30). However, fitting this long repeat size to the data for Dictyostelium could not explain the observed 190 b.p. differences in length between higher multimers, nor the 185 b.p. monomer length seen in earlier gels, which contain rapidly prepared DNA to reduce enzyme activity and are compared to rat liver nucleosome markers (Fig. 4, gel a).

A $\bar{M}w$ of 245,000 for a nucleosome can be calculated using the Svedberg equation, a sedimentation coefficient of 11.5S, a diffusion coefficient of 3.44×10^{-7} cm^2/sec, and an assumed density of 1.45 g/cm^3 (31,32). It is very close to the predicted value for a combination of

eight histones and 187 b.p. of DNA. With this $\bar{M}w$ and the 98 Å diameter mentioned above, the nucleosome is apparently a disc 40 Å thick. This calculation is consistent with the disc-shaped nucleosomes observed by electron microscopy (33) and X-ray crystallography (34).

The melting of chromatin reveals additional details about its structure. Dictyostelium chromatin has four transitions as seen in higher eukaryotes (20). The two at the highest temperatures are due to the melting of nucleosomes. The lower one of these is due to monomers, while the higher one is due to multimers. The lower temperature transitions below these two are very broad and are due to the combined effects of histone H1 and basic nonhistone proteins. Since the digestion studies have shown that protease activity can quickly alter chromatin structure, any differences may be artifactual. Further studies must take this into account. However, it is still apparent that the chromatin is heterogeneous in its melting structure and that this fact may be used to dissectit. Further research may be able to correlate this with several observations: 1) the Staphylococcal nuclease digestion pattern of Dictyostelium chromatin on gels is very heterogeneous; 2) Dictyostelium contains a large fraction of expressed DNA; and 3) it contains large, ribosomal DNA units. Some of the heterogeneity in the melting transitions and in the DNA gel patterns may be due to the template active and the ribosomal DNA.

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