Isolation, Identification, and Characterization of Histones from Plasmodia of the True Slime Mold *Physarum polycephalum* Using Extraction with Guanidine Hydrochloride†

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**ABSTRACT:** Histones from plasmodia of the true slime mold *Physarum polycephalum* have been prepared free of slime by an approach to histone isolation that uses extraction of nuclei with 40% guanidine hydrochloride and chromatography of the extract on Bio-Rex 70. This procedure followed by chromatography or electrophoresis has been used to obtain pure fractions of histones from *Physarum* microplasmodia. *Physarum* microplasmodia have five major histone fractions, and we show by amino acid analysis, apparent molecular weight on three gel systems containing sodium dodecyl sulfate, mobility on gels containing Triton X-100, and other characterizations that these fractions are analogous to mammalian histones H1, H2A, H2B, H3, and H4. Significant differences between *Physarum* and mammalian histones are noted, with histone H1 showing by far the greatest variation. Histones H1 and H4 from *Physarum* microplasmodia have similar, but not identical, products of partial chymotryptic digestion compared with those of calf thymus histones H1 and H4. Labeling experiments, in vivo, showed that histone H1 is the major phosphorylated histone and approximately 15 separate phosphopeptides are present in a tryptic digest of *Physarum* histone H1. The core histones from *Physarum*, histones H2A, H2B, H3, and H4, are rapidly acetylated; histone H4 shows five subfractions, analogous to the five subfractions of mammalian histone H4 (containing zero to four acetyllysine residues per molecule); histone H3 has a more complex pattern that we interpret as zero to four acetyllysine residues on each of two sequence variants of histone H3; histones H2A and H2B show less heterogeneity. Overall, the data show that *Physarum* microplasmodia have a set of histones that is closely analogous to mammalian histones.

*Physarum polycephalum* is a true slime mold whose value as a model system for biochemical studies was first fully recognized and exploited by Dr. H. P. Rusch (Rusch, 1970). In the last 10 years, it has been used extensively, and two books have recently been compiled describing *Physarum* and its use in research (Dove & Rusch, 1980; Aldrich & Daniel, 1982). Its principal advantages are (i) the naturally synchronous mitotic cycle (e.g., Evans et al. (1982)) and (ii) the microplasmodium containing the ribosomal genes (e.g., Matthews & Bradbury (1982)). There are also some studies of differentiation that used the various stages of the life cycle. We, and others, have exploited the synchronous cell cycle and the microplasmodium to obtain correlations between structural features of chromatin and changes in chromosome function, such as the correlation of histone H1 phosphorylation with chromosome condensation (Matthews, 1980), the correlation of tetraacetylated histone H4 with transcription (Chahal et al., 1980), and the correlation of sensitivity to micrococcal nuclease and nucleosome instability with transcription (Johnson et al., 1978a). Prior et al. (1980) have used *Physarum* to assemble fluorescent-labeled histone H3 into fully active nucleosomes. *Physarum* histones were first isolated and characterized by Mohberg & Rusch (1969, 1970), who found that acid extraction of *Physarum* nuclei released large amounts of polysaccharide that prevented further analysis. They introduced the use of 1 M CaCl₂ for extraction of nuclei, followed by precipitation of proteins from the extract and then acid extraction of the precipitate. This method gives a preparation of total histone that is suitable for analysis by acrylamide gel electrophoresis or fractionation on gel filtration columns (Corbett et al., 1977). Mohberg and Rusch identified seven histone fractions, bands 1, 2a, 2b, 3, 4, 5, and 6. The major components were bands 1, 3, 4, and 6, and bands 1 and 6 were identified as equivalent to mammalian histones H1 and H4. Band 4 was shown to contain two components. The amino acid composition of H1 was determined and has been confirmed in several laboratories (Matthews & Bradbury, 1982; Fischer & Laemmli, 1980; Cruikshank & Walker, 1981; Coté et al., 1982). While we have found the CaCl₂ extraction method very useful for studies of histones H1 and H4, it has been criticized by others (Fischer & Laemmli, 1980; Gurley et al., 1978), and we have found variable yields of histone H3.

In this paper, we describe an alternative extraction procedure, based on a suggestion by Dr. R. Braun (unpublished results), that is much faster, gives less contamination with polysaccharide, gives reproducible high yields of all histone fractions, and inhibits potential activity of modifying enzymes such as phosphatases and deacetylases. This method has been used to isolate and characterize each of the five histone fractions from *Physarum* plasmodia, thus providing a more solid basis for ongoing studies of histone function with the *Physarum* model system.

**Materials and Methods**

*Physarum Culture.* *Physarum polycephalum*, strain M3C, was cultured as microplasmodia in shaking flasks or as macroplasmodia on filter papers in petri dishes, essentially as described previously (Daniel & Baldwin, 1964; Guttes & Guttes, 1964). The semidefined growth medium described by Daniel & Baldwin (1964) was used, with a final hematin concentration of 0.5 mg/200 mL. Microplasmodia were
A 20-mL aliquot of the filtrate (total volume of 200 mL) was underlayered with 10 mL of 1 M sucrose buffer (homogenizing thin-layer chromatography). Microplasmodia were harvested by allowing them to settle for 30 s and then pouring off the growth medium. The microplasmodia were washed 1–3 times in cold distilled water by swirling and centrifugation (500g, 5 min) and then used for preparing nuclei. Microplasmodia were harvested, still on their filter paper, by shock-freezing in liquid nitrogen.

For labeling with $^{32}$P, microplasmodia were grown for at least 24 h in semidefined medium from which the KH$_2$PO$_4$ had been omitted (Bradbury et al., 1973). A total of 5 mCi of $^{32}$PO$_4$$^+$ (New England Nuclear, “carrier free” in dilute HCl) was added, and the culture was allowed to grow for a further 48 h. Labeled cultures were harvested as for unlabeled cultures except that a Plexiglas screen was used to protect us from radiation, and radioactive waste was disposed of separately. In some experiments, 5 volumes of unlabeled carrier nuclei were added to labeled nuclei.

For labeling with $^3$Hacetate, microplasmodia were cultured as described previously (Guttes & Guttes, 1964), and the time of the second mitosis after fusion (M2) was determined. Twenty minutes after metaphase 2, the filter paper with the microplasmodium was removed from the growth medium, allowed to drain for a few seconds, and then placed on 1 mL of semidefined growth medium in a clean petri dish. This medium contained 8 mCi of $^3$Hacetate (New England Nuclear, 2–3 Ci/mmol) per mL. Culture was continued for 3 min and the plasmodium harvested by dropping the filter paper and plasmodium into liquid nitrogen.

Isolation of Nuclei. Nuclei were isolated from microplasmodia or macroplasmodia as described by Mohberg & Rusch (1971). All steps were carried out in a 4°C cold room or refrigerated centrifuge or on ice. Ten microplasmodia or up to 20 mL of washed microplasmodia were suspended and homogenized in 200 mL of homogenizing medium [0.25 M sucrose-0.01 M CaCl$_2$-0.01 M Tris-HCl$^+$$^-$$^-$0.1% (w/v) Triton X-100-1 mM phenylmethylsulfonyl fluoride, pH 7.1] by blending for 30 s at high speed on a Waring blender in a 1-L cup. Single microplasmodia were homogenized in a Potter homogenizer (Waterborg & Matthews, 1982). The foam was allowed to settle for 10 min and the homogenate centrifuged (50g, 5 min) or collected by syphoning. The supernatant was filtered through two milk filters and the filtrate centrifuged (1500g, 10 min). The pellet was resuspended in 80 mL of homogenizing medium with a syringe and a gauge-18 needle and centrifuged again. The pellet was washed once in homogenizing medium. The concentration of nuclei was determined by counting in a hemocytometer. In some experiments with microplasmodia, a modified homogenizing medium [0.03 M NaCl$^-$1 mM KCl-5 mM MgCl$_2$-0.1% (w/v) Triton X-100-0.01 M Tris-HCl$^-$1 mM phenylmethylsulfonyl fluoride, pH 7.1] (Waterborg & Matthews, 1982) was used.

In some experiments, nuclei were purified by centrifuging through 1 M sucrose (Mohberg & Rusch, 1971) as follows. A 20-mL aliquot of the filtrate (total volume of 200 mL) was underlayered with 10 mL of 1 M sucrose buffer (homogenizing medium with the sucrose concentration raised to 1 M) and centrifuged (50g, 10 min). Aggregated nuclei and unbroken plasmodia were removed by taking 3 mL from the bottom of the centrifuge tube. The “gradients” were centrifuged again (1000g, 10 min), and ten pellets were resuspended in a total of 80 mL of homogenizing medium with a syringe as described above. The underlayering and centrifugation procedure was repeated once.

Isolation of Physarum Histones. (A) CaCl$_2$ Methods. In early experiments, histones were prepared as described by Mohberg & Rusch (1969). The procedure was later modified to avoid the Cl$_4$COOH precipitation step by acidifying the extract and then desalting it on Sephadex G-25 (Matthews et al., 1979). Most of the data described in this paper were obtained by a Gdn-HCl method.

(B) Gdn-HCl Methods. Guanidine hydrochloride was purchased from Sigma (water-soluble grade) and used directly. A total of 10$^8$–10$^9$ nuclei was resuspended in 2–3 volumes of 40% GP buffer [40% (w/v) guanidine hydrochloride–50 mM KH$_2$PO$_4$–50 mM K$_2$HPO$_4$ adjusted to pH 6.8 with KOH] and homogenized with a syringe fitted with an 18-gauge needle. The very viscous suspension was then sonicated (3 × 20 s) in a sonicator (Heat-Systems Ultrasonics, Inc.) with an exponential microtip at an amplitude of 3.5 (115 W) while cooling in an ice bath. The solution became much less viscous, and unbroken nuclei and nuclear debris were removed by centrifugation (30000g, 10 min). HCl was added to the supernatant to a final concentration of 0.25 N and the solution allowed to stand on ice for 15–30 min. The solution was centrifuged (30000g, 30 min) to remove acidic components that precipitated as a yellow-orange pellet. The supernatant was diluted with 100 mM phosphate buffer to make its refractive index the same as 5% GP buffer (5% guanidine hydrochloride–100 mM potassium phosphate, pH 6.8) and the pH adjusted to 6.8 with KOH. A total of 1–10 mL of Bio-Rex 70 cation-exchange resin was equilibrated with 5% GP buffer and the slurry added to the supernatant from the previous step. The mixture was gently agitated at room temperature (22°C) overnight, and the resin was allowed to settle. The supernatant was discarded, and the resin was washed 2–3 times until the wash supernatant was clear. The resin was then packed into a chromatography column. Under these conditions, quantitative binding of all histones to the resin was observed. The column was eluted with 3–5 column volumes of 12% GP (12% guanidine hydrochloride–100 mM potassium phosphate, pH 6.8) at 1 cm/2–5 min and then washed with 3–5 column volumes of 40% GP. In some experiments, the 12% GP elution was omitted, and the column was eluted directly with 40% GP. The column eluant was collected into fractions and the protein peak located by its absorbance at 280 nm. For recovery of core histones, the peak fractions were pooled and dialyzed against 5% (v/v) acetic acid to remove the guanidine hydrochloride. Dialysis against either water or 0.02 N sulfuric acid gave similar results. The protein was finally recovered by dialysis into the acetone wash, presumably due to an effect of the guanidine hydrochloride. Histone H4 can be recovered by dialysis of the first acetone supernatant followed by lyophilization.
Gel Electrophoresis. Histones were routinely analyzed by electrophoresis in polyacrylamide slab gels by either the acid–urea–Triton system described by Bonner et al. (1980) with 8 M urea and 8 mM Triton X-100 or the NaDodSO₄ system described by Laemmli (1970). For comparative studies, the NaDodSO₄ systems described by Schreier et al. (1977) and by Panyim & Chalkley (1971) and the acid–urea system described by Panyim & Chalkley (1969) were also used. These systems are summarized in Table 1.

Histones were isolated by preparative gel electrophoresis with a procedure according to Wu et al. (1982). In outline, the method was as follows. The preparative gel was 30-cm long and up to 3-mm thick, and the acid–urea–Triton buffer system was employed (Bonner et al., 1980). Up to 5 mg of protein/cm² of gel cross section was loaded and electrophoresed. The gel was stained lightly with Coomassie Brilliant Blue R and destained. Histone bands were cut out and allowed to soak in 1 M acetic acid–50 mM NaOH–1% (w/v) cysteamine for at least 1 h. A 0.5% (w/v) tube gel of low-melting agarose in 1 M acetic acid–50 mM NaOH was prepared. The histone bands were placed on top of the agarose gel together with methyl green marker dye (0.2 mg/sample). The upper reservoir buffer (Table 1) was added and electrophoresis started. The histones were dissolved, eluted from the acrylamide gel slice, and concentrated by isotachophoresis in the system was employed (Bonner et al., 1980). Up to 3-mm long and up to 3-mm thick, and the acid-urea-Triton buffer system was used (Bonner et al., 1980). Up to 5 mg of protein/cm² of gel cross section was loaded and electrophoresed. The gel was stained lightly with Coomassie Brilliant Blue R and destained. Histone bands were cut out and allowed to soak in 1 M acetic acid-50 mM NaOH-1% (w/v) cysteamine for at least 1 h. A 0.5% (w/v) tube gel of low-melting agarose in 1 M acetic acid–50 mM NaOH was prepared. The histone bands were placed on top of the agarose gel together with methyl green marker dye (0.2 mg/sample). The upper reservoir buffer (Table 1) was added and electrophoresis started. The histones were dissolved, eluted from the acrylamide gel slice, and concentrated by isotachophoresis in the agarose gel. Electrophoresis was continued until the isotachophoresis stack reached a steady state, as judged by the sharpness of the dye bands. Coomassie Brilliant Blue moved as soon as the background started to darken; then it was washed several times in distilled water.

Fluorography of acid–urea–Triton gels was used to detect 3H-labeled histones. After electrophoresis, the gel was dehydrated by successive washes of 10 min each in 25%, 50%, and 100% (v/v) acetic acid and then equilibrated for 2 h in 0.02 M HCl-0.1% (w/v) cysteamine. The eluate was desalted by chromatography on a column of Sephadex G-25 (Pharmacia PD-10) eluted with 5% (v/v) acetic acid. This step also removed the methyl green marker dye. The excluded peak from the Sephadex column was lyophilized and stored.

Molecular Weight Determination. Gels containing sodium dodecyl sulfate were used for molecular weight determination. Calf thymus histones were used as molecular weight markers, and the molecular weight value assumed for each calf thymus histone is given in Table IV. The values were based on the amino acid sequences, except for H1 where values of 21000 and 22000 were used for the two major calf thymus components (Cole, 1977). For estimation of core histone molecular weights, the logarithm of the molecular weight of the calf thymus core histones was plotted as a function of migration distance. The slope of the minimum least-squares line through these points was calculated. The molecular weight of each Phasusum core histone was estimated from the semilog plot from a calibration line with the calculated slope and passing through the analogous calf thymus histone data point. The same procedure was used for Phasusum band 1 (histone H1) except that the calf thymus H1 data points were included when calculating the slope of the minimum least-squares line.

Chromatographic Methods. Phasusum histones, like mammalian histones, can be fractionated on gel filtration columns (Sommer & Chalkley, 1974; Von Holt & Brandt, 1977; Corbett et al., 1977; Chalal et al., 1980; Matthews & Bradbury, 1982; Coté et al., 1982). For high resolution, a column of Bio-Gel P-60, 200 cm long × 2.5-cm diameter, was used and eluted with 20 mM HCl–50 mM NaCl-0.02% NaN₃ at 10 mL/h. The sample (up to 5 mL) is loaded in 8 M deionized urea–0.02 M HCl-1% (w/v) β-mercaptoethanol. This column gives Phasusum histone H1, H2A, and H4 completely separated from other histones, but Phasusum histones H2B and H3 coelute (Matthews & Bradbury, 1982). For a more rapid separation, a smaller column (65 cm long
Table I: Summary of Gel Electrophoresis Systems

<table>
<thead>
<tr>
<th>mneumonic</th>
<th>ref</th>
<th>acrylamide: bis-(acrylamide) [% (w/v)]</th>
<th>detergent</th>
<th>urea (M)</th>
<th>buffer and other constituents</th>
<th>stacking gel</th>
<th>upper reservoir buffer; lower reservoir buffer</th>
<th>marker dye</th>
</tr>
</thead>
<tbody>
<tr>
<td>acid-urea-Triton</td>
<td>Bonner et al., 1980</td>
<td>15:0.1</td>
<td>Triton X-100 (8 mM)</td>
<td>8</td>
<td>1 M acetic acid, 50 mM NH₄OH</td>
<td>yes</td>
<td>0.1 M glycine-1 M acetic acid; 0.1 M glycine-1 M acetic acid</td>
<td>methylene blue</td>
</tr>
<tr>
<td>acid-urea-CTABa</td>
<td>Bonner et al., 1980</td>
<td>15:0.1</td>
<td>none</td>
<td>6</td>
<td>1 M acetic acid, 50 mM NH₄OH</td>
<td>yes</td>
<td>0.1 M glycine-1 M acetic acid-0.15% CTAB; 0.1 M glycine-1 M acetic acid</td>
<td>methylene blue</td>
</tr>
<tr>
<td>acid-urea (Bonner)</td>
<td>Bonner et al., 1980</td>
<td>15:0.1</td>
<td>none</td>
<td>8</td>
<td>1 M acetic acid, 50 mM NH₄OH</td>
<td>yes</td>
<td>0.1 M glycine-1 M acetic acid; 0.1 M glycine-1 M acetic acid</td>
<td>methylene blue</td>
</tr>
<tr>
<td>acid-urea (Panyim &amp; Chalkley) elution</td>
<td>Panyim &amp; Chalkley, 1969 West &amp; Bonner, 1980</td>
<td>15:0.5</td>
<td>none</td>
<td>2.5</td>
<td>0.9 M acetic acid</td>
<td>no</td>
<td>0.9 M acetic acid; 0.9 M acetic acid</td>
<td>methyl green</td>
</tr>
<tr>
<td>12.5% NaDodSO₄</td>
<td>Laemmli, 1970</td>
<td>12.5:0.34</td>
<td>NaDodSO₄ (0.1%)</td>
<td>none</td>
<td>0.375 M Tris, pH 8.8</td>
<td>yes</td>
<td>25 mM Tris-0.19 M glycine-0.1% NaDodSO₄; 25 mM Tris-0.19 M glycine-0.1% NaDodSO₄</td>
<td>bromophenol blue</td>
</tr>
<tr>
<td>15% NaDodSO₄ (pH 8.8)</td>
<td>Laemmli, 1970</td>
<td>15:0.26</td>
<td>NaDodSO₄ (0.1%)</td>
<td>none</td>
<td>0.375 M Tris, pH 8.8</td>
<td>yes</td>
<td>25 mM Tris-0.19 M glycine-0.1% NaDodSO₄; 25 mM Tris-0.19 M glycine-0.1% NaDodSO₄</td>
<td>bromophenol blue</td>
</tr>
<tr>
<td>15% NaDodSO₄ (pH 10)</td>
<td>Panyim &amp; Chalkley, 1971</td>
<td>15:0.1</td>
<td>NaDodSO₄ (0.0625%)</td>
<td>none</td>
<td>25 mM glycine, pH 10</td>
<td>no</td>
<td>25 mM glycine, pH 10-0.05% NaDodSO₄; 25 mM glycine, pH 10-0.05% NaDodSO₄</td>
<td>bromophenol blue</td>
</tr>
<tr>
<td>17.5% NaDodSO₄</td>
<td>Schreier et al., 1977</td>
<td>17.5:0.07</td>
<td>NaDodSO₄ (0.1%)</td>
<td>none</td>
<td>0.37 M Tris, pH 8.8</td>
<td>yes</td>
<td>0.025 M Tris-0.19 M glycine-0.1% NaDodSO₄, pH 8.8: 0.025 M Tris-0.19 M glycine-0.1% NaDodSO₄, pH 8.8</td>
<td>bromophenol blue</td>
</tr>
</tbody>
</table>

a CTAB, cetyltrimethylammonium bromide.

Table II: Molecular Weights of Physarum Histonesa

<table>
<thead>
<tr>
<th>Physarum histone</th>
<th>NaDodSO₄ gel systemb</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>average ± standard error (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>28 300</td>
<td>27 200</td>
<td>33 900</td>
<td>30 700</td>
<td>30 692 ± 1280 (10)</td>
<td></td>
</tr>
<tr>
<td>H2A</td>
<td>nd</td>
<td>18 400</td>
<td>19 500</td>
<td>19 300</td>
<td>19 056 ± 203 (11)</td>
<td></td>
</tr>
<tr>
<td>H3</td>
<td>nd</td>
<td>15 600</td>
<td>15 800</td>
<td>14 500</td>
<td>15 512 ± 134 (15)</td>
<td></td>
</tr>
<tr>
<td>H2B</td>
<td>nd</td>
<td>14 100</td>
<td>14 300</td>
<td>14 100</td>
<td>14 188 ± 87 (13)</td>
<td></td>
</tr>
<tr>
<td>H4</td>
<td>nd</td>
<td>11 440</td>
<td>11 600</td>
<td>11 500</td>
<td>11 542 ± 73 (11)</td>
<td></td>
</tr>
</tbody>
</table>

a The molecular weights of Physarum histones estimated using calf thymus histones for calibration as described under Materials and Methods. The molecular weights of the calf core histones were calculated from their known sequences: H4 = 11 236 (DeLange et al., 1969); H3 = 15 273 (DeLange et al., 1972); H2B = 13 774 (Iwai et al., 1970); H2A = 13 960 (Sautiere et al., 1974). The molecular weights of the two major H1 variants were assumed to be 22 000 and 21 000 (Cole, 1977). The standard error was derived from measurements on all gel systems, and the number of measurements for each histone is given in parentheses. nd = not determined. b Gel systems (see Table I): (1) 12.5% NaDodSO₄ (Laemmli, 1970); (2) 15% NaDodSO₄, pH 10 (Panyim & Chalkley, 1971); (3) 15% NaDodSO₄, pH 10 (Panyim & Chalkley, 1971); (4) 17.5% NaDodSO₄ (Schreier et al., 1977).
was necessary to release them by trypsin as follows: After 20 h at 110 °C in vacuo. The samples were dried over NaOH and run on a Durrum D-500 amino acid analyzer. Values were not corrected for hydrolytic losses. There were indications of the presence of small amounts of methionylglycine, but these were not quantitated. Cysteine was determined as cystic acid as follows: 5–10 nmol of protein or peptide was dissolved in 500 μL of formic acid, and 20 μL of H2O2 was added. The sample was incubated at 0 °C for 2 h and then diluted with 2 mL of water and lyophilized. It was then hydrolyzed and subjected to amino acid analysis as above.

**Edman Degradation of Physarum Histones H1 and H4.** Physarum histone H1 was further purified by chromatography on a Bio-Rex 70 column eluted with a gradient from 5% GP to 40% GP at a reduced speed of 1 mL/h. The purity was checked by gel electrophoresis. Physarum histone H4 was further purified by thin-layer chromatography in pyridine–butanol–acetic acid–water (5:7.5:1.5:6). A total of 10 nmol of Physarum histone H1 and H4 as well as 10 nmol of a reference protein of known sequence (glucagon) was then submitted to an Edman degradation cycle with the colored reagent 4-N,N-(dimethylamino)-5'-isothiocyanatoazobenzene as described by Chang et al. (1978). The reference protein showed the expected N-terminal sequence, whereas neither H1 nor H4 showed any cleaved residue even after several cleavage cycles. To avoid possible loss of protein by extraction with organic solvents in the sequencing procedure, we then attached 100 nmol of each protein to isothiocyanato glass. Edman degradation was then carried out as described by Allen (1981), and no cleaved amino acids were detected. In order to show that the histones were, indeed, bound to the glass, it was necessary to release them by trypsin as follows: After staining gels or autoradiographs were scanned with a Cary 210 spectrophotometer equipped with a gel scanner and a digital interface port. Data were collected directly into a Hewlett-Packard 9845S computer through a 16-bit parallel interface. A digital filter was used to reduce graininess in the autoradiograph scans, and areas were determined by numerical integration with an interactive program written for the purpose, by either trapezoidal or cubic spline methods. The filter and integration programs, in BASIC, are available on request.

**Results**

**Nomenclature.** We have identified bands 1, 3, 4a, 4b, and 6 of the Mölberg & Rusch (1969) nomenclature as analogous to mammalian histones H1, H2A, H3, H2B, and H4, respectively (see Discussion). For clarity, we will refer to these histones as Physarum histone H1, etc., anticipating their identification later under Discussion.

**Histone Preparation.** Physarum nuclei were extracted with guanidine hydrochloride as described under Materials and Methods, and the extract was absorbed to Bio-Rex 70. Figure 1 shows the elution pattern obtained on a gradient from 5% to 40% guanidine hydrochloride in phosphate buffer (GP buffers) [cf. Bustin & Cole (1969) and Rasmussen et al. (1962)]. A partly resolved group of peaks in the absorbance
profile was observed, eluting at 9–15% guanidine hydrochloride. Gel electrophoresis of these fractions showed that they contained proteins previously identified as Physarum histones (Mohberg & Rusch, 1969; Corbett et al., 1977; Coté et al., 1982). Physarum histone H1 appears to have split into two subfractions, but it is not separated from the core histones, presumably due to histone aggregation (Figure 1).

In subsequent experiments, the Bio-Rex 70 column was eluted with three steps, 5%, 12%, and 40% GP buffer. Practically all the histones eluted with the 12% step with traces of the core histones found in the 40% step (Figure 2, tracks 2, 3, and 4). Histones were recovered from the GP buffer either by dialysis or by precipitation, as described under Materials and Methods. Figure 2 shows comparative gel electrophoresis of histones prepared by these methods (track 8) and by the method described by Mohberg & Rusch (1969) (track 1). Clearly, the guanidine hydrochloride method produces a pattern of histone bands similar to that produced by the Mohberg and Rusch method although the precipitation variation of the guanidine hydrochloride method leaves Physarum histone H4 and some of the other core histones behind in the first acetone supernatant (tracks 5 and 6). The material produced by the Mohberg and Rusch method in our laboratory is normally only 20–30% protein (by dry weight). The remaining material is assumed to be contaminating polysaccharide. In the guanidine hydrochloride method, this contamination is removed quantitatively by the Bio-Rex 70 chromatography. In our experience with many preparations, the guanidine hydrochloride method gives more reproducible yields of Physarum histone H3. We currently use the precipitation variant to prepare Physarum histone H1 and the dialysis variant to prepare core histones. Gel scans show that about 50% of the isolated protein is in the major histone bands, and the yield of total protein from 1 × 10¹⁰ nuclei (about 11 mg of DNA) is typically 20 mg.

**Analysis of Total Histone.** Total histones were analyzed by gel electrophoresis with the acid–urea–Triton system summarized in Table I (Bonner et al., 1980) and stained with Coomassie Brilliant Blue. This system gives a good separation of the five major histone components. Figure 3, unlike most other gel systems we tried (Table I), which do not separate...
Physarum histones H3 and H2B well (Figure 3B,C). The use of acid–urea–Triton gels has greatly simplified the study of Physarum histones. Physarum histones H1, H3, and H4 show multiple components on charge-sensitive gels, which are discussed further below. Figure 3C shows a 17.5% NaDodSO₄ gel of Physarum histones in which slight resolution of Physarum histones H3 and H2B is apparent. Bands from an acid–urea–Triton gel were cut out and rerun into a 17.5% NaDodSO₄ gel. However, Physarum histone H3 shows two minor components in the 17.5% NaDodSO₄ gel (Figure 3C, lane 4), and Physarum histone H1 shows a substantial band running at an apparent molecular weight of 23,000 (Figure 3C, lane 3). These components were not characterized further.

A two-dimensional gel pattern was obtained with the acid–urea–Triton system in the first dimension and the acid–urea–CTAB system (containing cetyltrimethylammonium bromide) in the second dimension (Bonner et al., 1980; Table I). Figure 4 shows that this system gives excellent resolution of the Physarum histones. Physarum histone H1 shows three spots; Physarum histone H2A migrates far from the diagonal, which includes Physarum histones H1, H2B, and H4. Physarum histone H2A shows only one major spot unlike mammalian histone H2A which has two major components. Physarum histone H2B also shows one major spot, close to the diagonal. Physarum histone H3 shows one major spot and a series of weak spots above and below the main component. Some gels show resolution of the major spot into two components as seen most clearly in the fluorograph (Figure 4C). The weak spots have not been identified. Three forms of Physarum histone H4, lying along the diagonal, are clearly visible. They represent 0, 1, or 2 mol of acetylysine/mol of protein, as described below.

These gels show a very large number of other proteins present in the histone preparation. Some of these may represent other histone or non-histone proteins such as HMG proteins (Goodwin et al., 1978; Matthews et al., 1979), H1⁶ (Smith & Johns, 1980), H2A₂ (West & Bonner, 1980), ubiquitin–H2A, or A24 (Goldknopf & Busch, 1977) and modification by sugars (Brightwell et al., 1975), and these possibilities are being investigated.

The electrophoretic pattern of Physarum histones was compared with that of mammalian (calf thymus) histones (Figure 3). Physarum histone H1 migrated consistently slower than mammalian histone H1. On acid–urea–Triton gels the Physarum histone H1 split into several components. This heterogeneity is destroyed by alkaline phosphatase pretreatment (K. Barnes and C. Crane-Robinson, personal communication) so it represents modification by phosphorylation, see below. Physarum histone H2A migrates more slowly than mammalian histone H2A in all gel systems. In acid–urea–Triton gels, both proteins migrate very slowly, behind histone H1. The low mobility in Triton-containing gels shows very clearly in the two-dimensional gel (Figure 4). Physarum histones H2B and H3 do not separate in acid–urea gels and are barely resolved in 17.5% NaDodSO₄ gels. Mammalian histone H2B migrates more rapidly than Physarum histone H2B in all the systems used. Mammalian histone H3 comigrates in acid–urea gels (Prior et al., 1980) and runs marginally faster on 17.5% NaDodSO₄ gels compared with Physarum histone H3. Physarum histone H4 has a mobility almost identical with the mobility of mammalian histone H4 in all systems, but on long gels a small difference is seen with Physarum histone H4 migrating more slowly. Similar results were obtained in comparisons with HeLa histones.

Molecular Weight Determination. Gels containing NaDodSO₄ were used for estimation of the molecular weights of Physarum histones. Figure 5 shows examples of the gel scans used. The gel electrophoresis systems described by Laemmli (1970), Panyim & Chalkley (1971), and Schreier et al. (1977)
FIGURE 4. *Physarum* histones separated in a two-dimensional gel system. (A) *Physarum* histones were prepared and electrophoresed as in Figure 3A. The resulting gel strip was polymerized onto an acid-urea-CTAB gel, electrophoresed, and stained with Coomassie Brilliant Blue (Bonner et al., 1980). The first dimension is from left to right; the second dimension is from top to bottom. (B) Map illustrating the position of histones in (A). (C) Fluorography of a two-dimensional gel. A *Physarum* macroplasmodium was pulse labeled with [3H]acetate for 3 min in S phase. Histones were extracted and separated on a two-dimensional gel as above that was then fluorographed (Laskey & Mills, 1975) for 49 days.

were used. The Panyim and Chalkley system uses pH 10, higher than the other systems that use pH 8.8. Calf thymus histones were used as molecular weight markers. A total of 10–15 measurements of molecular weight were made for each histone, and the mean and standard error are given in Table II. The standard error reflects the differences between the gel systems but does not include possible errors due to uncertainty in the calibration curve. This means that the estimate for *Physarum* histone H1 in particular may be less reliable than the standard error suggests. Better molecular weight values will come from sequence studies.

Fractionation of Total Histone. Figure 6 shows that *Physarum* histones can be separated into four components by chromatography on a column of Bio-Gel P-10 or P-60 as described under Materials and Methods. *Physarum* histones H1, H2A, H2B + H3, and H4 are separated as previously reported by Corbett et al. (1977). A partial separation of a component labeled “band 5” by Mohberg & Rusch (1969) is also observed. This may be a minor histone variant or a non-histone protein. It has not been characterized. When necessary, further purification was achieved with chromatography either on a Bio-Rex 70 column (H1), on a Sephadex G-75 column (H4) (Van der Westhuyzen & Von Holt, 1971), or on cellulose thin layers (H4) or by preparative gel electrophoresis (all histones). In order to separate *Physarum* histones H3 and H2B, we used a preparative acid-urea-Trition gel and eluted the bands electrophoretically as described under Materials and Methods. This method yields histones with at least 90% homogeneity when analyzed by rerunning on acid-urea-Trition gels (Figure 7) or by electrophoresis on 12.5%
of each of the peak fractions was analyzed by electrophoresis on a gel. The peak at fraction 20.5 cm was identified with HPLC, and the positions of the amino acid composition were stable during storage. The ultraviolet spectrum of each histone was measured, and there was no evidence for the presence of tryptophan (Allen, 1981).

Automatic Edman degradation of Physarum histone H2B gave the following preliminary N-terminal sequence: NH2-Pro-Asp-Lys-Gly-unknown-Thr-Lys-Glu-[or Pro]-Gly-Gly-(Glu)-(Ala)-(Ala)-(Ala)-(Glu)-(Glu).... The residues in parentheses were identified by HPLC, GC, and TLC. Manual Edman degradation of Physarum histone H2A gave the following pre-

Table III: Amino Acid Composition (mol %) of Physarum Plasmoidal Histones

<table>
<thead>
<tr>
<th></th>
<th>H1</th>
<th>H2A</th>
<th>H2B</th>
<th>H3</th>
<th>H4</th>
<th>N-T*</th>
<th>C-T*</th>
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</thead>
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<td>10.25</td>
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<td>2.15</td>
<td>4.10</td>
<td>1.35</td>
<td>0</td>
</tr>
</tbody>
</table>

* N-T is the amino-terminal chymotryptic fragment of H1. C-T is the carboxy-terminal chymotryptic fragment of H1. b Cys was determined as cysteic acid after performic acid oxidation. c nd, not determined.
was not due to total loss of the protein during the Edman degradation cycle by binding Physarum histones H1 and H4, separately, to isothiocyanato glass beads (Allen, 1981). Edman degradation of these immobilized histones also failed to release an N-terminal amino acid. We confirmed that protein was bound to the glass beads by digesting them with trypsin and observing the expected release of peptides. We concluded that the amino termini of these histones are blocked.

Physarum histone H1 was digested with chymotrypsin (Chambers et al., 1983; Rall & Cole, 1971), and the products were analyzed by gel electrophoresis. Figure 8 shows that the histone is initially cut to yield two large fragments, one of which is then degraded further. Chambers et al. (1983) identified the fragments as the N-terminal and C-terminal regions of Physarum histone H1. The two large fragments were separated by chromatography on Sephadex G-75 into the larger (C terminal) or the smaller (N terminal) essentially as described by Chambers et al. (1983), and then purified by thin-layer electrophoresis where the C-terminal fragment moved very rapidly toward the cathode while the N-terminal fragment moved slowly in the same direction. Figure 8B shows the fragments were pure as judged by gel electrophoresis, and Table III shows the amino acid composition of each fragment. The two fragments account for the complete molecule. Physarum histone H4 was also subjected to partial digestion with chymotrypsin, and the results are shown in Figure 9 together with similar digests of calf thymus H4. The patterns of digestion of the two histones are similar but not identical, and this is confirmed by amino acid analysis of the peptides (S. Fried, unpublished results).

Histone Modifications. Physarum has been extensively used for studies of histone acetylation and phosphorylation. The experiments described here confirm and extend those studies. Physarum macroplasmodia were pulse labeled with $^3H$-acetate for 3 min during S phase of the cell cycle. Figure 10 shows a scan of the fluorogram of an acid–urea–Triton gel of pulse-labeled histones. All the core histones show heavy labeling, indicative of postsynthetic modification by acetylation.
Physarum Plasmodial Histones Are Analogous to Mammalian Histones. (A) Histone H1. This Physarum histone is the most different from its analogous mammalian histone, as judged by its low electrophoretic mobility. Nevertheless, it was correctly identified as an H1 histone by Mohberg & Rusch (1969). Mohberg & Rusch later (1970) published an amino acid composition for Physarum band 1 that was essentially correct although marred by the presence of an "unidentified acidic amino acid" that can now be seen to be due to oxidation during the hydrolysis (Corbett, 1979). Similar amino acid analyses of Physarum histone H1 from plasmodia have been published by Tyrsin et al. (1977a), Fischer & Laemmli (1980), and Cruikshank & Walker (1981). A comparison of the Physarum histone H1 amino acid analysis published here (Table III) with an amino acid analysis of calf thymus H1 (Johns, 1976) clearly shows the similarity, but there are also substantial differences. For example, Physarum histone H1 contains histidine and methionine, which are absent in calf thymus H1. Physarum histone H1 is slightly less basic, overall, than calf thymus H1. The N terminus of Physarum histone H1 is blocked as is the N terminus of calf thymus H1. Initial studies with chymotrypsin have shown that Physarum histone H1, like calf thymus H1, has a sensitive phenylalanine residue about 100 residues from the amino terminus (Chambers et al., 1983). We have now shown that the two fragments produced by cleavage at this phenylalanine have polarized amino acid compositions. All the hydrophobic residues, except for a small number of valines, are found in the smaller, amino-terminal, fragment (Table III). This fragment comigrates with the analogous fragment from calf thymus H1 (Figure 8), suggesting that the amino-terminal region of Physarum histone H1 may be very similar to the same region of calf H1. The carboxy-terminal fragment from Physarum histone H1 splits into two components, like the analogous fragment of calf H1, probably due to sequence microheterogeneity. However, the Physarum fragment migrates much more slowly, suggesting that the larger molecular weight of Physarum histone H1 (Table IV) is due to an extended carboxy-terminal region. Physarum histone H1 undergoes a cell cycle dependent phosphorylation that has also been observed in mammalian cells [reviewed by Matthews (1980)]. The phosphorylation occurs on multiple sites in mammalian cells (Langan, 1978), and this has now been confirmed for Physarum (Figure 12) although the precise number of sites remains to be determined.
just like that of calf thymus H2A (Chahal et al., 1980).

**Histones** above. Determined from sequence (see Table IV for details). Some of these values are low because incorrect values for calf thymus histone molecular weights were used. Molecular weights determined from sequence (see Table II for details). Jockusch & Walker, 1974. Some of these values are low because incorrect values for calf thymus histone molecular weights were used. Tyrsin et al., 1977b. Some of these values are low for the same reason as in footnote e above. Allfrey et al., 1977. H1 was not determined. Fischer & Laemmli, 1980. Only H1 was determined. Côté et al., 1981. Histones isolated from *Physarum* amoebae (all the others are from plasmodia).

**(B) Histone H2A.** This component has received very little attention in the *Physarum* literature to date. It does not comigrate with any calf thymus histone, but Figure 3 shows that the mobility drops dramatically in Triton-containing gels, just like that of calf thymus H2A (Chahal et al., 1980). The two-dimensional gel (Figure 4) shows unusual such behavior is since the great majority of proteins run on or close to the diagonal in this system (Bonner et al., 1980). A comparison of the amino acid compositions of *Physarum* histone H2A (Table III) and calf thymus histone H2A (Von Holt et al., 1979) shows that these two proteins are much closer to each other than to any other histone and confirms the identification of *Physarum* histone H2A as an H2A-like histone. The amino-terminal region of histone H2A varies widely in sequence between species (Von Holt et al., 1979) so it is not surprising that there is no homology (except Lys-5) between *Physarum* and calf thymus H2As in the first 12 amino acids. The apparent molecular weight of *Physarum* band 3, 19 100 ± 200, is much higher than the molecular weight of calf thymus histone H2A, 13 960 (Table IV). This difference has led to difficulties in identifying the *Physarum* histone H2A (Jockusch & Walker, 1974; Tyrsin et al., 1977b; Lipinska & Klyszejko-Stefanowicz, 1981) in earlier studies from other laboratories.

*Physarum* histone H2A is rapidly acetylated during S phase of the cell cycle (Figure 10) but is not phosphorylated to a significant extent in randomly growing cultures (Figure 11). Phosphorylation of histone H2A has been correlated with transcriptional activity in cultured *Peromyscus* cells (Halleck & Gurley, 1980). This correlation does not appear to extend to *Physarum* since the microplasmodia are transcriptionally active but histone H2A is not extensively phosphorylated.

**(C) Histone H3.** Mohberg & Rusch (1969) showed that *Physarum* band 4 (histones H2B and H3) contained two components by differential precipitation. These components will separate, poorly, on NaDodSO4 gels or acid-urea gels (Jockusch & Walker, 1974; Johnson et al., 1978a; Tyrsin et al., 1977b) (Figure 3) and more clearly on acid-urea-Triton gels (Figure 4). A major difficulty with *Physarum* histone H3 has been poor recovery and instability. The guanidine hydrochloride extraction procedure has eliminated these problems and allowed a more detailed characterization. A comparison of the amino acid compositions of *Physarum* histone H3 (Table III) and calf thymus histone H3 (Von Holt et al., 1979) shows that the two compositions are almost identical except that *Physarum* histone H3 has one cysteine instead of two. W. Schiebel (unpublished results) and Prior et al. (1980) had previously shown the presence of cysteine in *Physarum* histone H3. They found no cysteine in the other *Physarum* histones, in agreement with the amino acid compositions reported here (Table III). The molecular weight of *Physarum* histone H3 may be slightly higher than that of calf thymus histone H3 but the difference is not significant at the present level of accuracy.

*Physarum* histone H3 is rapidly acetylated. The complex pattern of acetylated species shown in Figure 10 appears to result from eight overlapping components. This is consistent with two H3 subcomponents, each having 1-4 mol of acetyl/mol of histone. However, these data do not rule out the possibility of one H3 subcomponent with site-specific effects of acetylation on gel mobility.

**(D) Histone H2B.** A comparison of the amino acid compositions of *Physarum* histone H2B (Table III) and calf thymus histone H2B (Von Holt et al., 1979) shows that these are analogous proteins although they are not as close as the H3s. The amino-terminal region of histone H2B varies widely in sequence between species (Von Holt et al., 1979) so it is not surprising that there is virtually no homology between *Physarum* histone H2B and calf thymus H2B in the first few amino acids. The apparent molecular weight of *Physarum* histone H2B is 14 200 ± 100, again slightly higher than the molecular weight of the analogous calf thymus histone (Table IV). Tyrsin et al. (1977a) identified *Physarum* band 5 as an H2B-like histone. This minor band has not been investigated in the present study so its relationship to the major H2B-like histone in *Physarum* is unknown. *Physarum* histone H2B is rapidly acetylated during S phase.

**(E) Histone H4.** The comparison between the amino acid composition of *Physarum* histone H4 (Table III) and calf thymus H4 (Von Holt et al., 1979) shows very substantial similarity between the two proteins. The differences between these H4s are reproducible with the *Physarum* histone H4 isolated either by gel filtration or by preparative electrophoresis. *Physarum* histone H4 almost comigrates with calf thymus histone H4, but on high-resolution gels (acid-urea-Triton or 17.5% NaDodSO4 systems) the *Physarum* histone H4 is seen very slightly behind the calf thymus H4. From the NaDodSO4 gels this gives a molecular weight of 11 500 ± 100, again slightly higher than the molecular weight of the analogous calf thymus histone (Table IV). The N terminus of *Physarum* histone H4 is blocked as is the N terminus of calf thymus H4 (DeLange et al., 1969).

*Physarum* histone H4 is rapidly acetylated. Partial chymotryptic digestion of calf thymus histone H4 yields a stable intermediate, residues 1-37, that contains the acetylation sites (DeLange et al., 1969). *Physarum* histone H4 appears to be digested in an analogous way with respect to this N-terminal acetylated peptide (Figure 9), suggesting that, in *Physarum*, the acetylation sites may also be clustered at the N terminus. Further work on the sequence of *Physarum* histone H4 is in progress.

**Amoebal Histones.** As this paper was in the final stages...
of preparation, an analysis of histones from Physarum amoebae was published (Coté et al., 1982). The amino acid compositions of the amoebal core histones (Coté et al., 1982) are very similar to those of plasmodial core histones (Table III), and Coté et al. were able to show that amoebal and plasmodial histones comigrated in short acid–urea gels or in short NaDodSO₄ gels. It seems likely that any differences are minor. However, the H1 histones do show significant differences, which probably represent the presence of different subfractions of H1 or different proportions of the subfractions. There is evidence from high-resolution chromatography on Bio-Rex 70 columns that plasmodial H1 has at least three subfractions, not due to phosphorylation (Corbett et al., 1977, and data not shown). This difference may be due to the different strains of Physarum used in the two studies or to a change in H1 associated with differentiation from amoebae to plasmodia.

**Physarum Non-Histone Proteins.** Physarum plasmodia contain a small group of proteins that can be extracted from nuclei by 0.35 M NaCl and are soluble in 2% trichloroacetic acid, similar to the HMG proteins isolated from mammalian cells (Matthews et al., 1979; Goodwin et al., 1978). Coté et al. (1982) isolated an acid-soluble protein that they call "AS." AS is probably equivalent to Mohberg & Rusch's (1969) band 5 and to the major component of fraction 36 in Figure 6 of this paper. The amino acid composition was determined by Coté et al. (1982) and is very close to that of mammalian HMG 14 (Walker et al., 1979) except for the presence of a small proportion of hydrophobic residues. The apparent molecular weight (see Figure 6B) is also appropriate for HMG 14, but more detailed characterization is required before an assignment can be made.

Another potentially interesting protein is the 23 000 molecular weight band seen in lane 3 of Figure 3C where the band containing Physarum histone H1 was cut from an acid–urea–Triton gel and reelectrophoresed on a 17.5% NaDodSO₄ gel. This could be a degradation product of Physarum H1, but the absence of other possible degradation products makes this unlikely. The molecular weight of 23 000 is too high for it to be the protease-resistant hydrophobic core of Physarum histone H1, assuming this is reasonably conserved (Allan et al., 1980). The 23 000 molecular weight band is not labeled with 32P when Physarum histone H1 is labeled. The apparent change in mobility from a gel containing Triton X-100 to a gel containing NaDodSO₄ is a characteristic of histone H2A from both Physarum and mammalian cells, and the apparent molecular weight of the 23 000 molecular weight band is approximately equal to that of Physarum histone H2A + ubiquitin. These properties are consistent with the possibility that the 23 000 molecular weight band is the Physarum equivalent of A24. A24 is a conjugate protein with ubiquitin linked to lysine-119 of histone H2A by an isopeptide linkage to the C-terminal glycine of ubiquitin (Goldknopf & Busch, 1977). The mobility of mouse A24 (uH2A) in gels containing Triton X-100 is slightly less than the mobility of histone H2A (Pantazis & Bonner, 1981) whereas the 23 000 molecular weight band in Physarum runs ahead of Physarum histone H2A. As in the case of protein AS, the 23 000 molecular weight band needs further characterization.

**Physarum Nucleosomes.** The molecular weight of an octamer of Physarum core histones is 120 200 compared with 130 500 for that of calf thymus. The complete nucleosome has 170–190-bp DNA (Johnson et al., 1976), and the molecular weight of Physarum octamer + H1 is about 150 000 compared with 130 500 for that of calf thymus. Hence the protein:DNA ratio, on the average, in Physarum nucleosomes is higher than in calf thymus. Physarum nucleosomes, particularly those in active regions of the chromatin, have an unusual property of forming a stable intermediate form during micrococcal nuclease digestion. This stable intermediate was called "peak A" by Stiron et al. (1977), and Johnson et al. (1978a,b, 1979) showed that the peak A particles either were formed very rapidly from the active regions of the ribosomal genes or existed in those regions in vivo (Scheer et al., 1981). Most of Physarum chromatin could be converted to peak A particles (Johnson et al., 1978b).

The availability of pure, characterized Physarum histones makes mixed reconstitution experiments possible that may give information about the function of individual histones within the nucleosome. The present study also lays a sound basis for exploiting the naturally synchronous cell cycle in Physarum to study histone modification and structural transitions in chromatin.

**Acknowledgments**

Many people have contributed to the present state of knowledge of Physarum histones. We wish to acknowledge particularly the contributions of Dr. E. M. Bradbury, who introduced us to Physarum, and of our colleagues at Portsmouth Polytechnic, England, where this work began, namely, Drs. R. J. Inglis and S. Corbett, S. Miller, V. J. Robinson, and A. Skinner. HeLa histones were a generous gift from P. Yau. We thank T. J. Ward for excellent technical help. We are grateful to S. Fried for permission to use her data on partial chymotryptic digestion of Physarum histone H4 and to S. Van Patten and others in the Department of Biological Chemistry, University of California, Davis, for advice and help.

**References**


