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 minichromosome 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 minichromosome 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 $CaCl_2$ 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; Cotê et al., 1982). While we have found the $CaCl_2$ 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

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harvested during the exponential growth phase either for immediate analysis or as "seed" for macroplasmodial cultures. Mitosis was observed by phase-contrast microscopy in smears taken from the edges of macroplasmodia and fixed in ethanol (Guttes & Guttes, 1964). The time between the second and third mitoses after fusion was normally in the range 8–10 h.

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 (500*g*, 5 min) and then used for preparing nuclei. Macroplasmodia 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_2PO_4 had been omitted (Bradbury et al., 1973). A total of 5 mCi of $^{32}PO_4^{3-}$ (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 was added to labeled nuclei.

For labeling with [^{3}H]acetate, macroplasmodia 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 macroplasmodium 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}H]acetate (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 macroplasmodia 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[1]–0.1% (w/v) Triton X-100–1 mM phenylmethanesulfonyl fluoride, pH 7.1] by blending for 30 s at high speed on a Waring blender in a 1-L cup. Single macroplasmodia were homogenized in a Potter homogenizer (Waterborg & Matthews, 1982). The foam was allowed to settle for 10 min and the homogenate centrifuged (50*g*, 5 min) or collected by syphoning. The supernatant was filtered through two milk filters and the filtrate centrifuged (1500*g*, 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 phenylmethanesulfonyl 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 (50*g*, 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 (1000*g*, 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_3CCOOH 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^{10} nuclei was resuspended in 2–3 volumes of 40% GP buffer [40% (w/v) guanidine hydrochloride–50 mM KH_2PO_4–50 mM K_2HPO_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 (30000*g*, 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 (30000*g*, 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 230 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 lyophilization. For the best recovery of histone H1 compared to core histones and the non-histone proteins, the pooled peak fractions were made 25% Cl_3CCOOH by adding 100% (w/v) Cl_3CCOOH while stirring. The suspension stood for at least 1 h on ice, and the precipitate was collected by centrifugation (30000*g*, 45 min). The precipitate was washed thoroughly with acetone (Johns, 1976) and dried under vacuum. This latter procedure gives poor recovery of histone H4, which extracts 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.

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; NaDodSO$_4$, sodium dodecyl sulfate; Gdn·HCl, guanidine hydrochloride; HPLC, high-pressure liquid chromatography; GC, gas chromatography; TLC, thin-layer chromatography.

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 $NaDodSO_4$ system described by Laemmli (1970). For comparative studies, the $NaDodSO_4$ 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 I.

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/cm^2 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 I) 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 ahead of the protein, which comigrated with the methyl green. A 6 mm wide band containing the methyl green marker was cut out, and histones were eluted by diffusion into 1 mL of 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.

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 4 volumes of 20% (w/v) 2,5-diphenyloxazole (PPO) in glacial acetic acid. PPO was precipitated in the gel by shaking the gel for 2 h in water. The gel was dried and exposed to preflashed Kodak XR-5 film, at −70 °C (Laskey & Mills, 1975).

Two-dimensional gels were run with the acid–urea–Triton system in the first dimension. After being stained with Coomassie Brilliant Blue, the required lane was cut from the acid–urea–Triton gel and equilibrated for 30 min in 20 mL of 0.045 M NH_4OH–1% (w/v) cysteamine. A 1-mL aliquot of acetic acid was added, and the gel lane was fixed in this solution for 15 min and then embedded in 1% (w/v) agarose in 0.045 M NH_4OH–1 M acetic acid–1% cysteamine on top of the second-dimension gel. Electrophoresis was carried out until the methylene blue marker dye had run about 16 cm. The gel was stained with Coomassie Brilliant Blue or prepared for fluorography.

Silver Staining. (A) Method I. $NaDodSO_4$ (12.5%) gels were stained with silver nitrate according to Oakley et al. (1980). In most cases gels were first stained with Coomassie Blue and after being destained subsequently stained with silver stain. Proteins were fixed in the gel by soaking them for 30 min in 50% (v/v) methanol–7% (v/v) acetic acid. Then gels were transferred to 5% methanol–7% acetic acid and gently agitated overnight. For a permanent fixing of the proteins, the gel was soaked for 30 min in 40% glutaraldehyde. The solution was prepared fresh each time. The gel was then thoroughly rinsed at least 3 times with double-distilled H_2O and further washed overnight. A 31.5-mL aliquot of freshly prepared 0.36% NaOH was mixed with 2.1 mL of NH_4OH; then 1.2 g of silver nitrate dissolved in 6 mL of H_2O was slowly added, while stirring the solution vigorously. The mixture was immediately diluted to 150 mL and used at once. Water was drained off the gel, and the gel was incubated for 10 min in the silver nitrate solution by gentle shaking. The gel was removed from the ammoniacal silver solution and placed in 150 mL of distilled water and washed. Reducer solution was prepared fresh each time containing 0.005% citric acid–0.019% formaldehyde. The gel was then placed in 150 mL of reducer solution and was shaken for 5–10 min. The gel was removed from reducer solution as soon as the background started to darken; then it was washed several times in distilled water.

(B) Method II. Later, a faster method for silver staining polyacrylamide gels according to Wray et al. (1982) was used. Gels were silver stained after being destained of Coomassie stain. Gels were soaked for 1 hr in 50% methanol (reagent grade). The ammoniacal silver solution was prepared fresh before use as follows: 4 mL of a 20% $AgNO_3$ solution in double-distilled H_2O was slowly added to a solution of 21 mL of 0.36% NaOH–1.4 mL of 14.8 M NH_4OH. The solution was stirred vigorously while adding the silver nitrate and then immediately diluted with H_2O to 100 mL and used as soon as possible. The gel was stained for 15 min under constant gentle shaking. After being washed in double-distilled H_2O several times for 5 min, the gel was developed in a solution of 2.5 mL of 1% citric acid–0.25 mL of 38% formaldehyde in 500 mL of H_2O. The gel was removed from the developing solution as soon as the background started to darken. The gel was then rinsed in water. The gel was placed in a 50% methanol solution to stop the developing process.

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 21 000 and 22 000 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 *Physarum* 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 *Physarum* 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. Physarum histones, like mammalian histones, can be fractionated on gel filtration columns (Sommer & Chalkley, 1974; Von Holt & Brandt, 1977; Corbett et al., 1977; Chahal 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% NaN_3 at 10 mL/h. The sample (up to 5 mL) is loaded in 8 M deionized urea–0.02 M HCl–1% (v/v) β-mercaptoethanol. This column gives *Physarum* histone H1, H2A, and H4 completely separated from other histones, but *Physarum* 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

		separating gel						
mneumonic	ref	acrylamide:bis-(acrylamide) [% (w/v)]	detergent	urea (M)	buffer and other constituents	stacking gel	upper reservoir buffer; lower reservoir buffer	marker dye
acid–urea–Triton	Bonner et al., 1980	15:0.1	Triton X-100 (8 mM)	8	1 M acetic acid, 50 mM NH_4OH	yes	0.1 M glycine–1 M acetic acid; 0.1 M glycine–1 M acetic acid	methylene blue
acid–urea–CTAB[a]	Bonner et al., 1980	15:0.1	none	6	1 M acetic acid, 50 mM NH_4OH	yes	0.1 M glycine–1 M acetic acid–0.15% CTAB; 0.1 M glycine–1 M acetic acid	methylene blue
acid–urea (Bonner)	Bonner et al., 1980	15:0.1	none	8	1 M acetic acid, 50 mM NH_4OH	yes	0.1 M glycine–1 M acetic acid; 0.1 M glycine–1 M acetic acid	methylene blue
acid–urea (Panyim & Chalkley)	Panyim & Chalkley, 1969	15:0.5	none	2.5	0.9 M acetic acid	no	0.9 M acetic acid; 0.9 M acetic acid	methyl green
elution	West & Bonner, 1980	(0.5% agarose)	none	none	1 M acetic acid, 50 mM NaOH	yes	1 M acetic acid–0.1 M betaine–0.15% CTAB; 1 M acetic acid–50 mM NaOH	methyl green
12.5% $NaDodSO_4$	Laemmli, 1970	12.5:0.34	$NaDodSO_4$ (0.1%)	none	0.375 M Tris, pH 8.8	yes	25 mM Tris–0.19 M glycine–0.1% $NaDodSO_4$; 25 mM Tris–0.19 M glycine–0.1% $NaDodSO_4$	bromophenol blue
15% $NaDodSO_4$ (pH 8.8)	Laemmli, 1970	15:0.26	$NaDodSO_4$ (0.1%)	none	0.375 M Tris, pH 8.8	yes	25 mM Tris–0.19 M glycine–0.1% $NaDodSO_4$; 25 mM Tris–0.19 M glycine–0.1% $NaDodSO_4$	bromophenol blue
15% $NaDodSO_4$ (pH 10)	Panyim & Chalkley, 1971	15:0.1	$NaDodSO_4$ (0.0625%)	none	25 mM glycine, pH 10, 10% glycerol	no	25 mM glycine, pH 10–0.05% $NaDodSO_4$; 25 mM glycine, pH 10–0.05% $NaDodSO_4$	bromophenol blue
17.5% $NaDodSO_4$	Schreier et al., 1977	17.5:0.07	$NaDodSO_4$ (0.1%)	none	0.37 M Tris, pH 8.8	yes	0.025 M Tris–0.19 M glycine–0.1% $NaDodSO_4$, pH 8.8; 0.025 M Tris–0.19 M glycine–0.1% $NaDodSO_4$, pH 8.8	bromophenol blue

[a] CTAB, cetyltrimethylammonium bromide.

Table II: Molecular Weights of *Physarum* Histones[a]

	$NaDodSO_4$ gel system[b]				
Physarum histone	1	2	3	4	average ± standard error (*n*)
H1	28 300	27 200	33 900	30 700	30 692 ± 1280 (10)
H2A	nd	18 400	19 500	19 300	19 067 ± 203 (11)
H3	nd	15 600	15 800	14 500	15 512 ± 134 (15)
H2B	nd	14 100	14 300	14 100	14 188 ± 87 (13)
H4	nd	11 440	11 600	11 500	11 542 ± 73 (11)

[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% $NaDodSO_4$ (Laemmli, 1970); (2) 15% $NaDodSO_4$, (3) 15% $NaDodSO_4$, pH 10 (Panyim & Chalkley, 1971); (4) 17.5% $NaDodSO_4$ (Schreier et al., 1977).

× 1.5-cm diameter) of Bio-Gel P-10 was used with the same sample and eluant buffers, with a flow rate of 4 mL/h. This column gives some overlap, especially of *Physarum* histones H1 and H2A (Figure 7) but is valuable for rapid separations and initial partial purification. The differences between Bio-Gel P-10 and P-60, if the same size columns are used, are minor. Bio-Gel P-60 separates *Physarum* histones H1 and H2A better, but the performance deteriorates with use more rapidly than with Bio-Gel P-10. For some experiments, histone H4 was further purified on a column (130 cm long × 1.0-cm diameter) of Sephadex G-75 superfine eluted with 5% acetic acid at 0.03 mL/min.

Thin-layer chromatography was used for analysis or purification of peptides. Peptide mixtures were applied to cellulose thin layers (Polygram Cel 300, 20 × 20 cm; Macherey-Nagel and Co., Duren, West Germany). The sample was applied close to the anode. Thin-layer plates were wetted by dipping them into electrophoresis buffer [pyridine–acetic acid–acetone–water (2:4:15:79), pH 4.4]. Electrophoresis was carried out for 105 min at 480 V (15–20 mA) in electrophoresis buffer. After electrophoresis, the plates were dried and subjected to ascending chromatography perpendicular to the direction of electrophoresis in pyridine–butanol–acetic acid–water (50:75:15:60) for 4 h. Peptides were detected either by spraying the plates with a ninhydrin solution (3 g of ninhydrin–30 mL of collidine–100 mL of acetic acid–870 mL of ethanol) or by wetting them with 5% (v/v) pyridine in acetone and spraying them with 0.005% (w/v) fluorescamine (Hoffman-La Roche, Basel, Switzerland) in acetone. The fluorescamine-treated plates were examined in ultraviolet light (360 nm).

Amino Acid Analysis. A total of 2.5 nmol of purified protein or peptide was dissolved in 200–500 μL of 5.7 N HCl (3× distilled)–0.02% β-mercaptoethanol. Hydrolysis took place for 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 methyllysine, but these were not quantitated. Cysteine was determined as cysteic acid as follows: 5–10 nmol of protein or peptide was dissolved in 500 μL of formic acid, and 20 μL of H_2O_2 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)-4′-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 being dried over NaOH, a tryptic digest was done at 37 °C for 4 h in 0.2 M *N*-methylmorpholine acetate, pH 8.0, at an enzyme to protein ratio of 1:50. The glass beads were centrifuged, and the supernatant was lyophilized and applied to a cellulose thin layer and chromatographed as described above. After spraying with ninhydrin in both cases, we observed several stained bands, indicating that both proteins were bound to the isothiocyanato glass.

Chymotryptic Digest. After column chromatography on Bio-Gel P-10, the fractions containing H1 were combined and lyophilized. The protein was then taken up in 50 mM Tris-HCl, pH 8.0, at a concentration of 1 mg/mL. Chymotryptic digestion was performed for 10 min at 26 °C at an enzyme to protein ratio of 1:500. The digest was stopped with glacial acetic acid added to give a final concentration of 5% and immediately applied either on a column of Sephadex G-75 superfine (130 cm × 1.2 cm) for separation of fragments or on a column of Sephadex G-25 fine (30 × 0.7 cm) for desalting the sample. The desalted fractions were lyophilized and applied to thin-layer plates. Electrophoresis was carried out as above. The peptides were detected with fluorescamine, scraped off, and then eluted from the cellulose with 50% (v/v) acetic acid and dried down under vacuum over NaOH. The purity of the fragments was checked on 12.5% NaDodSO$_4$ gels. The amino acid composition of both fragments was determined.

Histone H4 was prepared by Bio-Gel P-10 chromatography from total *Physarum* histones or from commercially available calf thymus histones (Sigma). At a concentration of 1.3 mg of histone H4/mL of 20 mM Tris-HCl, pH 7.4, it was digested with α-chymotrypsin, treated with TLCK [(N^{α}-tosyl-L-lysyl)chloromethane hydrochloride], at room temperature (22 °C) for 20 min at substrate to enzyme ratios of 50:1 to 4000:1. The digestion was stopped by the addition of 0.5 volume of 0.75 M acetic acid–7.5 M urea–15% (w/v) sucrose–1% (w/v) methyl green, and the peptides were separated on a preelectrophoresed acid–urea gel (Panyim & Chalkley, 1969) until the green marker dye reached the end of the gel. The gel was stained overnight on 0.1% (w/v) Naphthalene Black 12B (BDH) in 7% (v/v) acetic acid–20% (v/v) methanol and destained in 7% acetic acid–20% methanol in the presence of AG 1-X8 anion-exchange resin (Bio-Rad).

Gel Scanning. Stained 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 Mohberg & 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

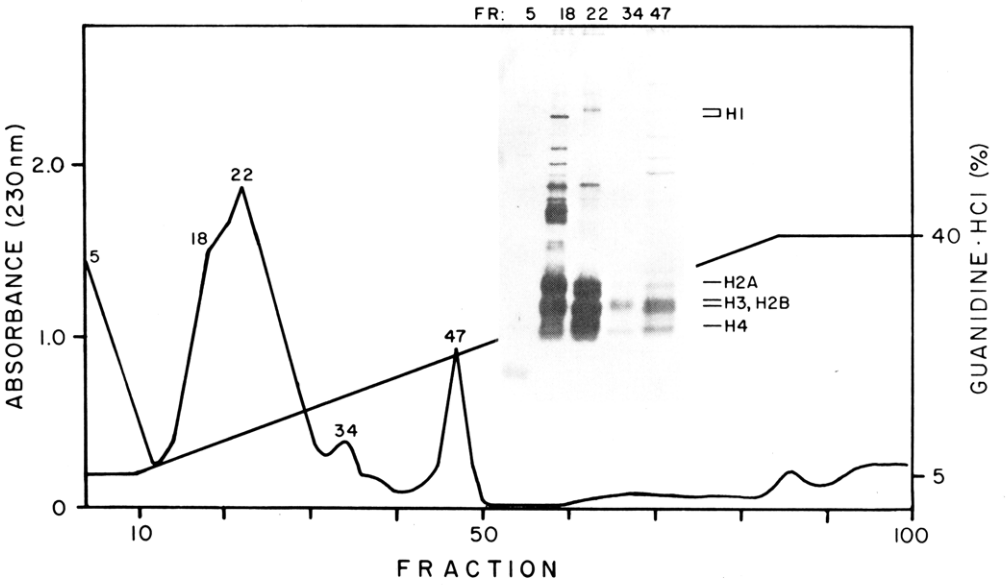

FIGURE 1: Elution of *Physarum* histones from a Bio-Rex 70 column. *Physarum* nuclear proteins were absorbed to Bio-Rex 70; the resin was washed with 5% GP buffer and packed into a column (20 × 0.9 cm). The column was eluted with a linear gradient from 5% GP buffer to 40% GP buffer at a flow rate of 2 mL/h; fractions were collected at 15-min intervals. For the first hour, the resin was still settling (fractions 1–4, not shown). The absorbance of subsequent fractions was measured at 230 nm, corrected for the absorbance of the guanidine hydrochloride in the elution buffer and plotted against fraction number. The peak fractions (numbers 5, 18, 22, 34, and 47) are indicated on the elution profile. The guanidine hydrochloride gradient was monitored by its refractive index and is also shown in the figure. (Inset) 12.5% $NaDodSO_4$ gel electrophoresis of peak fractions from the column. Peak fractions (FR) were analyzed directly by gel electrophoresis with silver staining (method I under Materials and Methods).

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; Cotê 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 1×10^{10} 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

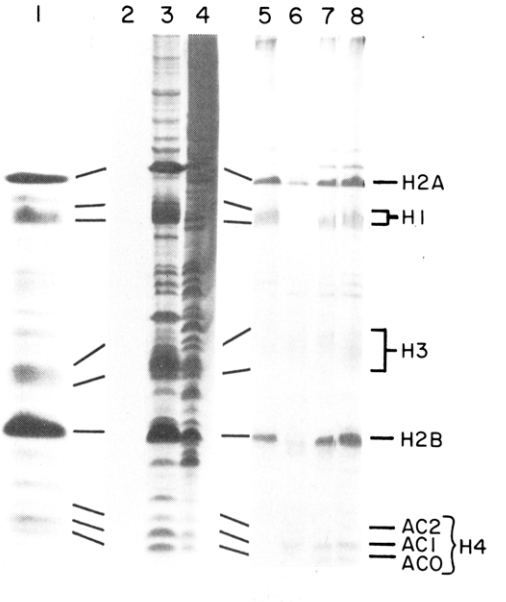

FIGURE 2: Comparison of *Physarum* histones prepared with different methods. The histones were extracted as indicated below, electrophoresed in an acid–urea–Triton gel, and stained with Coomassie Brilliant Blue. (Lane 1) Extraction with 1 M $CaCl_2$; (lanes 2–8) extraction with 40% GP buffer. Lane 2 shows the eluate of a Bio-Rex 70 column at 5% GP buffer, lane 3 is the eluate at 12% GP buffer, and lane 4 is the eluate at 40% GP buffer; the three samples were dialyzed against 5% acetic acid and lyophilized prior to electrophoresis. Lanes 5–8 demonstrate the effect of recovering the histones after ion-exchange chromatography: in lane 5 the histones were precipitated with 25% trichloroacetic acid; lane 6 is the first acetone supernatant. Note the lack of *Physarum* histone H1 in lane 6. Lane 7 shows the histones after short dialysis followed by trichloroacetic acid precipitation, and lane 8 shows histones dialyzed against H_2O followed by lyophilization.

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

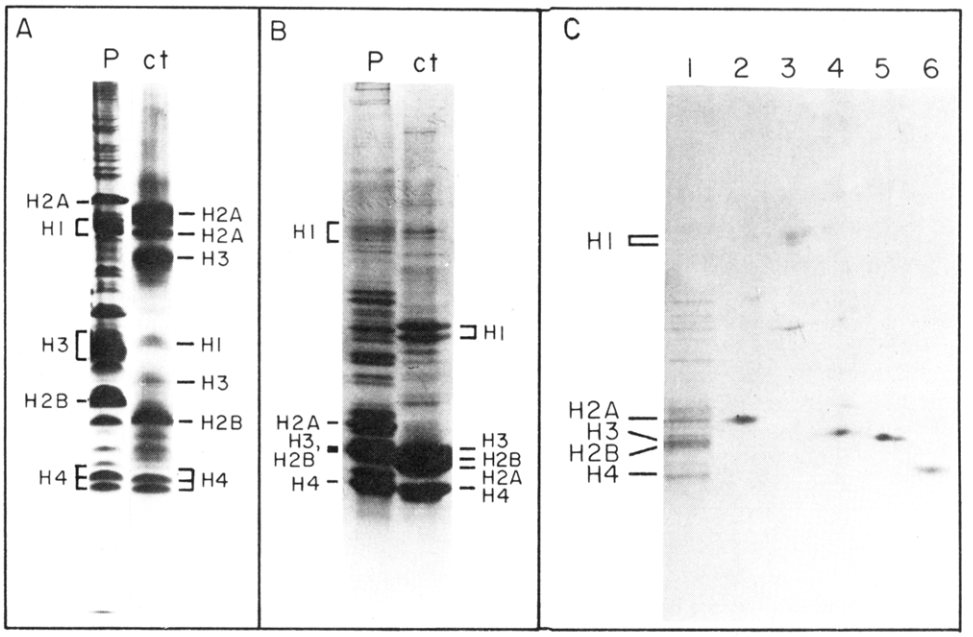

FIGURE 3: *Physarum* and calf thymus histones in acid–urea–Triton and $NaDodSO_4$ gel electrophoresis. The proteins were stained with Coomassie Brilliant Blue. (A) *Physarum* (P) and calf thymus (ct) histones were separated on an acid–urea–Triton gel. The histone bands are labeled. The bands not labeled in the *Physarum* pattern are a reproducible set of non-histone proteins not yet characterized. (B) The same histones were separated on 17.5% $NaDodSO_4$ gels. *Physarum* H1 is faint on these gels because it stains much more slowly and is not fully stained in this experiment (Bates et al., 1981). (C) 17.5% $NaDodSO_4$ gel electrophoresis (Schreier et al., 1977). Lane 1 shows total *Physarum* histone. Lanes 2–6 show reelectrophoresis of bands cut from the acid–urea–Triton gel shown in (A): (lane 2) H2A; (lane 3) H1; (lane 4) H3; (lane 5) H2B; (lane 6) H4.

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% $NaDodSO_4$ 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% $NaDodSO_4$ gel, tracks 2–6 of Figure 3C, to provide an exact correlation between the bands on the two gel systems. The major band of *Physarum* histones H2A, H2B, and H4 from the acid–urea–Triton gel reelectrophoresed as a single major band in the 17.5% $NaDodSO_4$ gel. However, *Physarum* histone H3 shows two minor components in the 17.5% $NaDodSO_4$ 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 acetyllysine/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), $H1^0$ (Smith & Johns, 1980), H2A.2 (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% $NaDodSO_4$ 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% $NaDodSO_4$ 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 $NaDodSO_4$ 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)

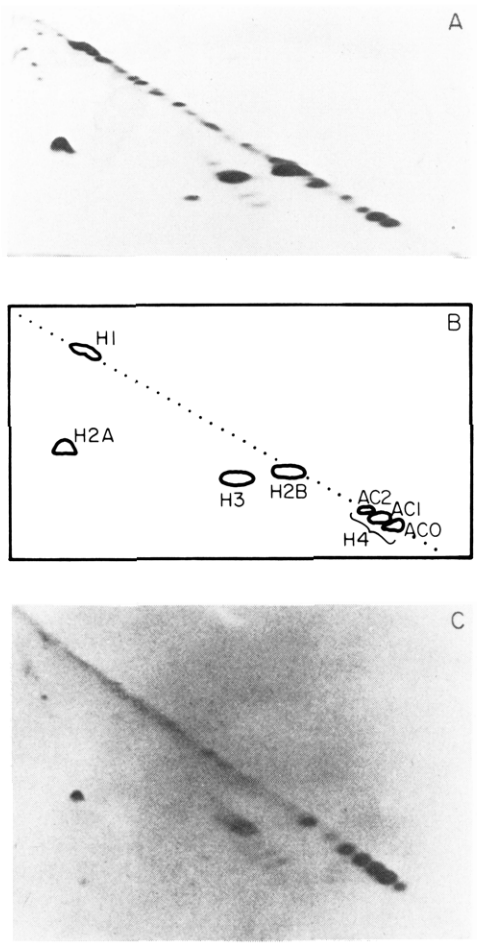

FIGURE 4: *Physarum* histones separated in a two-dimensional gel system. (A) *Physarum* histones were prepared and electrophoresed as in Figure 3A. The resulting gel strip was polymerized onto an acid–urea–CTAB gel, electrophoresed, and stained with Coomassie Brilliant Blue (Bonner et al., 1980). The 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 [^{3}H]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.

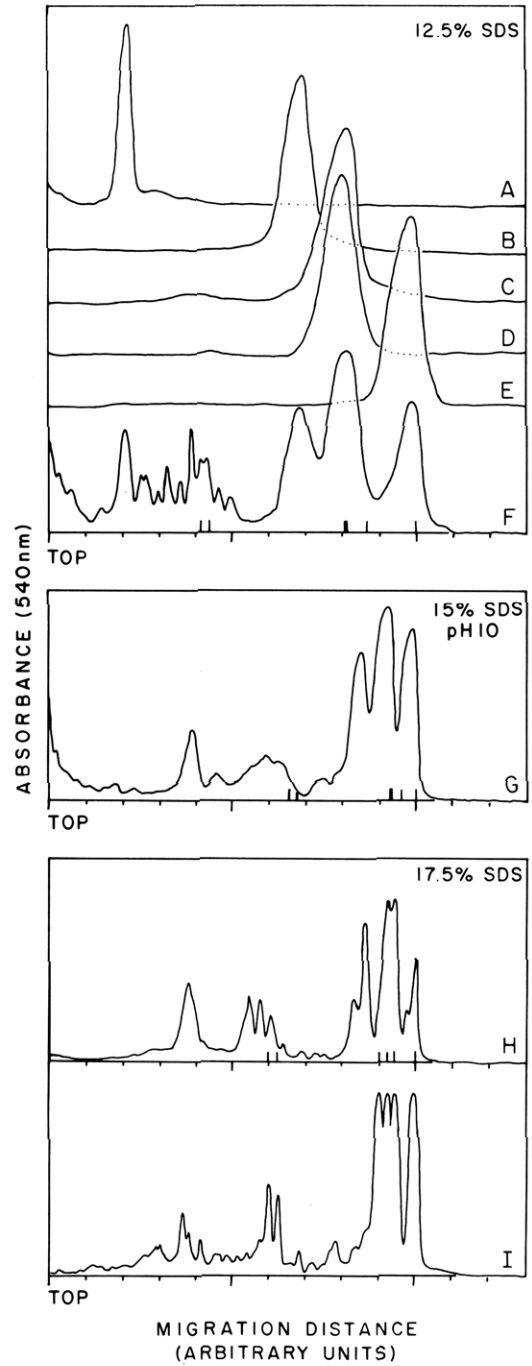

FIGURE 5: Molecular weight gels for *Physarum* histones. (A–F) 12.5% $NaDodSO_4$ (Laemmli, 1970); (G) 15% $NaDodSO_4$ (pH 10) gel (Panyim & Chalkley, 1971); (H, I) 17.5% $NaDodSO_4$ gels (Schreier et al., 1977). The gels were stained with Coomassie Brilliant Blue and scanned. (A–E) Histone purified by electroelution from acid–urea–Triton gels. (Note the absence of minor bands, unlike Figure 3. These cleaner preparations were obtained by taking smaller cuts from a less loaded preparative gel.) (A) through (E) are *Physarum* histones H1, H2A, H3, H2B, and H4, respectively. (F–H) *Physarum* total histone. The positions of calf thymus histone molecular weight markers are shown by vertical tics rising from the abscissa: (from left to right) calf H1 (two species), H3, H2B, H2A, and H4. (I) HeLa total histone.

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–Triton 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–Triton gels (Figure 7) or by electrophoresis on 12.5%

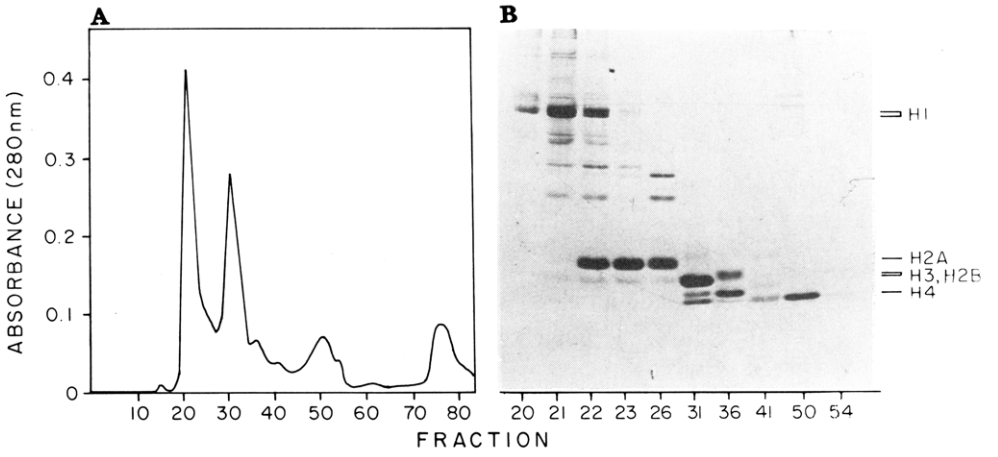

FIGURE 6: Elution profile of *Physarum* histones chromatographed on a column of Bio-Gel P-10. Approximately 10 mg of protein was dissolved in 0.5 mL of sample buffer (8 M deionized urea–20 mM HCl–1% β-mercaptoethanol) and applied to the column. The column (65 cm long × 1.5-cm diameter) was eluted with 20 mM HCl–50 mM NaCl–0.02% NaN_3 at a flow rate of 4 mL/h, and fractions were collected at 15-min intervals. (A) Absorbance at 280 nm of each fraction. (B) An aliquot (10 μL, fractions 20–23, 31, 50 or 20 μL, fractions 26, 36, 41, 54) of each of the peak fractions was analyzed by electrophoresis on a 12.5% $NaDodSO_4$ gel, which was stained with Coomassie Brilliant Blue. Each lane of the gel is labeled with the fraction number corresponding to the fractions in (A), and the positions of *Physarum* histones on the gel are shown on the right of the gel. The peak at fraction 76 did not contain protein but did include the urea and mercaptoethanol from the sample buffer.

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

	H1	H2A	H2B	H3	H4	N-T[a]	C-T[a]
Asx	3.98	6.96	4.67	5.09	6.46	6.85	1.90
Thr	6.70	4.26	6.89	7.02	8.14	6.86	6.38
Ser	8.71	7.81	9.31	4.66	2.45	7.19	11.57
Glx	6.78	10.48	9.28	10.63	6.27	11.84	2.57
Pro	10.23	4.47	3.90	4.94	1.47	7.07	18.20
Gly	4.62	11.12	7.36	6.14	14.65	6.47	2.66
Ala	18.07	10.26	10.40	12.53	6.95	11.77	27.74
Val	3.74	5.44	7.04	4.10	6.68	4.51	2.16
Met	0.80	0.34	1.46	0.78	0.71	1.00	0
Ile	2.78	5.26	3.61	5.88	5.27	3.40	0.32
Leu	3.70	8.47	4.97	8.53	6.99	4.97	0.97
Tyr	1.25	3.03	4.35	2.15	4.10	1.35	0
Phe	1.63	1.38	1.96	3.87	2.38	2.34	0
His	3.78	2.41	3.99	1.80	3.02	2.56	0
Lys	17.56	10.67	16.15	9.37	9.86	15.39	23.96
Arg	5.19	7.83	5.25	12.46	14.61	6.42	1.30
Cys[b]	0.20	0.20	0.28	0.71	0.23	nd[c]	nd

[a] 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.

$NaDodSO_4$ gels (Figure 5). The electrophoretic elution procedure gave recoveries of up to 90%, and dried histone fractions were stable during storage.

Initial Studies of Primary Structure. Table III shows the amino acid composition of each *Physarum* histone. Cysteine was determined separately after formic acid oxidation and found only in *Physarum* histone H3, which had 1 mol of cysteine/mol of histone. The ultraviolet spectrum of each histone was measured, and there was no evidence for the presence of tryptophan in any *Physarum* histone. None of the histones could be stained with *p*-(dimethylamino)benzaldehyde, which is also consistent with the absence of tryptophan (Allen, 1981).

Automatic Edman degradation of *Physarum* histone H2B gave the following preliminary N-terminal sequence: NH_2-Pro-Asp-Lys-Gly-unknown-Thr-Lys-Glu[or Pro]-(Gly)-Gly-(Glu)-(Ala)-(Ala)-(Ala)-(Gly)-(Glu)-.... The residues in parentheses were identified by HPLC only; the others were identified with HPLC, GC, and TLC. Manual Edman degradation of *Physarum* histone H2A gave the following preliminary N-terminal sequence: Ala-Gly-Pro-Glu-(X)-Thr-Ser-(X)-Ala-Thr-(X)-Glu-.... The (X) residues are not positively identified, but they may be Lys since the protein was bound to glass beads (see Materials and Methods) through its lysine residues. Manual Edman degradation of *Physarum* histones H1 and H4 gave no result although a glucagon sample run in parallel gave the expected amino acid released. We showed that the absence of a released N-terminal amino acid

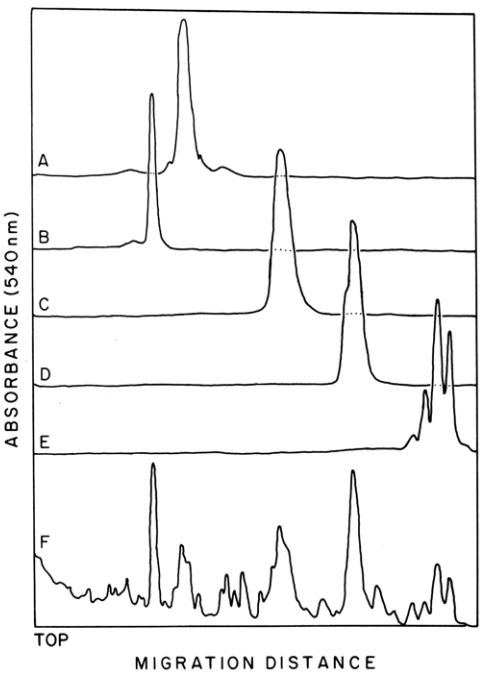

FIGURE 7: Analysis of isolated histones on acid–urea–Triton gels. (A–E) Gel scans of *Physarum* histones isolated by electroelution from acid–urea–Triton gels. The homogeneity of each fraction was estimated from the area under the major peak compared with the total area under the scan. Areas were determined numerically by trapezoidal or cubic spline methods: (A) *Physarum* histone H1 (90% homogeneous); (B) *Physarum* histone H2A (91% homogeneous); (C) *Physarum* histone H3 (93% homogeneous); (D) *Physarum* histone H2B (98% homogeneous); (E) *Physarum* histone H4 (91% homogeneous). (F) Gel scan of total *Physarum* histones. H1 and H3 are resolved into several components. This resolution is lost in (A) and (C) above because the gels were overloaded to reveal any minor impurities.

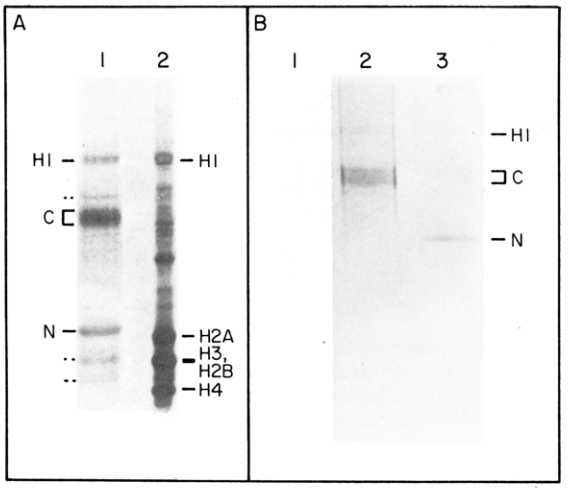

FIGURE 8: Chymotryptic digestion of *Physarum* histone H1. Gel electrophoresis on 12.5% $NaDodSO_4$ gels stained with silver nitrate (Oakley et al., 1980). (A) (Lane 1) Partial chymotryptic digest of *Physarum* histone H1. The positions of undigested *Physarum* histone H1, the major C-terminal fragments (C), and the major N-terminal fragment (N) are marked on the left. The three dotted lines indicate bands that have not been characterized further. (Lane 2) Total *Physarum* histone run as a marker. (B) A partial chymotryptic digest of *Physarum* histone H1 was fractionated by chromatography on Sephadex G-75 superfine, and the peak fractions were analyzed: (lane 1) undigested *Physarum* histone H1; (lane 2) major C-terminal fragments; (lane 3) major N-terminal fragment.

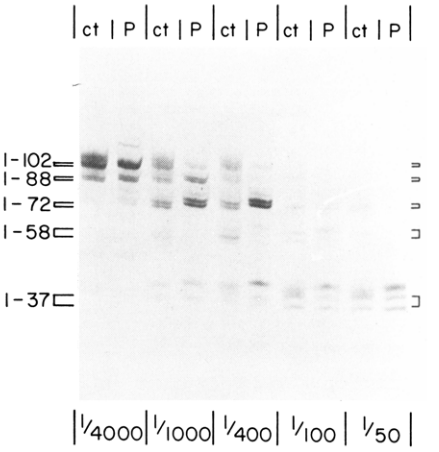

FIGURE 9: Partial chymotryptic digestion of *Physarum* and calf histones H4. *Physarum* (P) and calf thymus (ct) histones H4 were digested with chymotrypsin for 20 min at room temperature (22 °C). The enzyme:substrate (w/w) ratios were 1:4000, 1:1000, 1:400, 1:100, and 1:50 as indicated at the bottom of the figure. The digests were analyzed on acid–urea gels that were stained with amido black. The fragments of calf thymus histone H4 that include the N terminus are indicated at the left of the gel. 1–102 is the intact molecule. Each fragment is a doublet because of the presence of acetyllysine in the N-terminal region of approximately 50% of the calf thymus histone H4. These fragments were identified by comparison with gels of calf thymus H4 characterized by S. Van Patten and D. A. Walsh (unpublished results).

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 [^{3}H]-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

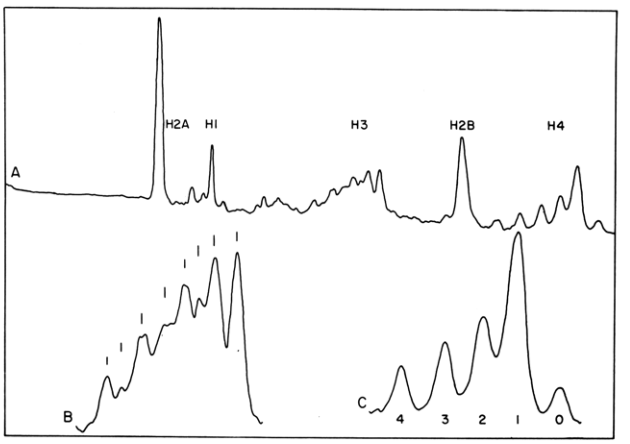

FIGURE 10: Acid–urea–Triton gel electrophoresis of *Physarum* histone pulse labeled with [^{3}H]acetate. A *Physarum* plasmodium, in S phase of the cell cycle, was labeled for 3 min with [^{3}H]acetate as described under Materials and Methods. Total histone was isolated and analyzed on an acid–urea–Triton gel that was then fluorographed (Laskey & Mills, 1975). The fluorography was scanned in the gel scanner. (A) Total histone. (B) Enlarged view of the H3 region of A. The vertical lines indicate the positions of eight peaks that are tentatively interpreted as four states of acetylation of two subspecies of *Physarum* histone H3. (C) Enlarged view of the H4 region of A. The five bands represent 0–4 acetyllysines/molecule of *Physarum* histone H4 as indicated on the figure. Label in the component with zero acetyllysines arises from *N*-acetylserine, which is producing during histone synthesis.

(see also the two-dimensional fluorograph, Figure 4). The clearest pattern is given by *Physarum* histone H4, which shows five bands corresponding to 0–4 mol of acetyllysine/mol of histone. Analogy with mammalian H4 (DeLange et al., 1969) and the presence of an acetylserine resonance in the nuclear magnetic resonance spectrum (S. S. Chahal and P. D. Cary, unpublished results) suggest that labeling of the unmodified form of *Physarum* histone H4 is probably due to acetylserine at the blocked amino terminus since the label is not recovered in amino acids after acid hydrolysis, if the labeling time is less than 15 min (V. Jane Robinson, unpublished results). Note that *Physarum* histone H1 shows a similar degree of labeling,

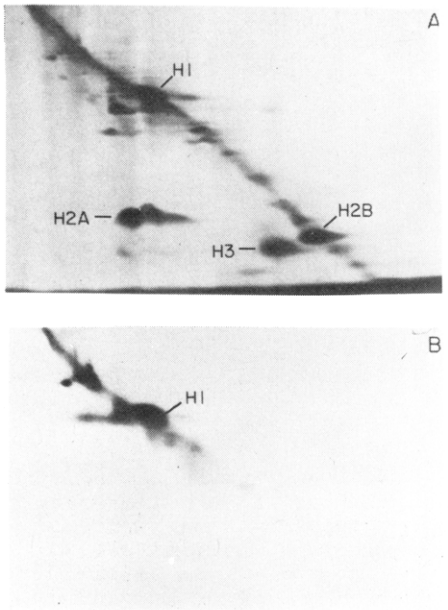

FIGURE 11: ^{32}P labeling of *Physarum* histones. *Physarum* microplasmodia were labeled with [^{32}P]phosphate for 48 h, and total histone was isolated. In this experiment *Physarum* histone H4 was not recovered (see section on histone preparation). The total histone was analyzed by two-dimensional gel electrophoresis (Bonner et al., 1980), and the gel was stained with Coomassie Brilliant Blue. The gel was photographed and then subjected to autoradiography for 36 h with an intensifying screen at −70 °C. (A) Coomassie Brilliant Blue stained gel. (B) Autoradiograph. The first dimension was from left to right, and the second dimension was from top to bottom.

probably also due to an amino-terminal acetylserine. Neither *Physarum* histone H1 nor the unmodified form of *Physarum* histone H4 is labeled if the pulse of [^{3}H]acetate is given to plasmodia in G2 phase. *Physarum* histone H3 shows a complex band pattern that can sometimes be resolved into eight major bands (Figure 10B). *Physarum* histones H2A and H2B are highly acetylated in S phase but no clear resolution of acetylated species was detected in Coomassie-stained acid–urea–Triton gels.

Histone phosphorylation was assessed by labeling microplasmodia with [^{32}P]phosphate for several generations (Bradbury et al., 1973). Figure 11 shows the two-dimensional gel electrophoresis pattern of the histones. *Physarum* histone H1 is the only major labeled histone although other, nonhistone, proteins are labeled. The same histones were separated in an acid–urea gel, and the labeled band of *Physarum* histone H1 was cut out, eluted with acetic acid (Bernabeau et al., 1980), and digested with trypsin. The tryptic peptides were separated by thin-layer chromatography and electrophoresis. Figure 12 shows the ninhydrin-stained peptides and the ^{32}P-labeled peptides as revealed by autoradiography. In each of three separate preparations of ^{32}P-labeled *Physarum* histone H1, there were 14 labeled spots plus the origin. The origin was eluted, redigested, and chromatographed and electrophoresed again. The radioactivity remained at the origin, showing that the label at the origin was not due to partial digestion. The phosphopeptides comigrated with faint ninhydrin spots located in positions consistent with the phosphopeptides having a lower overall positive charge than the nonphosphorylated peptides. One phosphopeptide had an overall negative charge. The presence of 15 phosphopeptides (14 spots plus origin) does not imply 15 phosphorylation sites per molecule because there may be multiple spots representing a single site due to sequence microheterogeneity and to partial cleavage of Lys–Lys or Lys–Arg bonds (Langan, 1978).

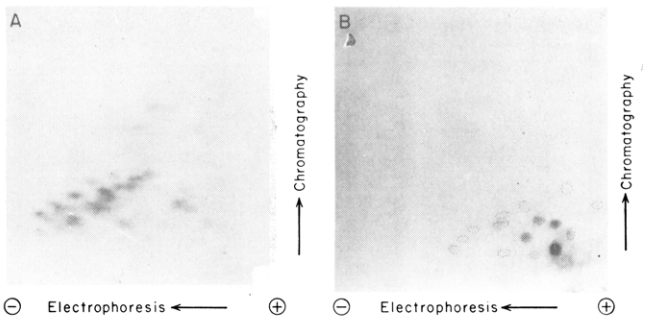

FIGURE 12: Tryptic peptide maps of ^{32}P-labeled *Physarum* histone H1. *Physarum* histone H1, labeled as described under Materials and Methods, was digested with trypsin and analyzed by two-dimensional thin-layer electrophoresis and chromatography as described under Materials and Methods. The thin-layer plate was sprayed with ninhydrin and photographed. It was then subjected to autoradiography for 3 days with an intensifying screen at −70 °C. (A) Ninhydrin stained map. (B) Autoradiograph. The first dimension (electrophoresis) was from right to left, and the second dimension (chromatography) was from bottom to top.

Discussion

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.

Table IV: Molecular Weights of *Physarum* Histones

histone	band[a]	calf thymus[b]	*Physarum*					
			this paper	I[c]	II[d]	III[e]	IV[f]	V[g]
H1	1	21 500	30 700	24 500	22 700		27 500	29 700
H2A	3	13 960	19 100	14 500	15 700	17 500		15 700
H2B	4b	13 774	14 190	14 000	14 300	15 100		14 700
H3	4a	15 273	15 500	14 000	15 000	15 600		14 700
H4	6	11 236	11 540	10 500	10 500	11 400		11 300

[a] Nomenclature of Mohberg & Rusch (1969). Where another author's assignment of *Physarum* histones to mammalian equivalents differed from that reported here, the band nomenclature has been used to make sure that equivalent histones were compared. [b] Molecular weights determined from sequence (see Table II for details). [c] Jockusch & Walker, 1974. Some of these values are low because incorrect values for calf thymus histone molecular weights were used. [d] Tyrsin et al., 1977b. Some of these values are low for the same reason as in footnote *c* above. [e] Allfrey et al., 1977. H1 was not determined. [f] Fischer & Laemmli, 1980. Only H1 was determined. [g] Coté 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 how 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 $NaDodSO_4$ gels or acid–urea gels (Jockusch & Walker, 1974; Johnson et al., 1978a,b; 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 acetate/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% $NaDodSO_4$ systems) the *Physarum* histone H4 is seen very slightly behind the calf thymus H4. From the $NaDodSO_4$ 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 (Cotê et al., 1982). The amino acid compositions of the amoebal core histones (Cotê et al., 1982) are very similar to those of plasmodial core histones (Table III), and Cotê et al. were able to show that amoebal and plasmodial histones comigrated in short acid–urea gels or in short $NaDodSO_4$ 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). Cotê 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 Cotê 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% $NaDodSO_4$ 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 ^{32}P when *Physarum* histone H1 is labeled. The apparent change in mobility from a gel containing Triton X-100 to a gel containing $NaDodSO_4$ 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 108 500 for that of calf thymus although the DNA length in the core particle is preserved (Johnson et al., 1976). 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 Staron 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.

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