ISOLATION AND FRACTIONATION OF NUCLEAR COMPONENTS FROM *PHYSARUM FLAVICOMUM*

A Thesis

Presented to

The Faculty of the Department of Biology

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

---

by

Mabel Chung Yee

August, 1973

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ABSTRACT

This research is designed to isolate histone from purified *Physarum flavicomum* plasmodial nuclei. Attempts are made to fractionate and characterize this histone in order to compare this histone with other eucaryotic systems.

An isolation procedure is improved for obtaining large quantities of *P. flavicomum* plasmodial nuclei with a high degree of purity. While most of the mechanical and enzymatic treatments have failed to yield complete breakage of nuclei, pretreatment with dithiothreitol (DTT) has been found to be an effective method for increasing nuclei breakage.

Using a high concentration of salt (2 M NaCl) at neutral pH an acidic protein is co-extracted from purified plasmodial nuclei along with the basic histone proteins. This acidic material is characterized as actin-myosin-like protein by its amino acid composition and molecular weight. SDS-gel-electrophoresis gives an estimated molecular weight of $41,000 \pm 4,000$ for this actin-myosin-like protein.

Different reagents are used for the extraction of the basic histone material from purified plasmodial nuclei. Acid extraction (0.4 N H_2SO_4) is impractical since extremely low yields are obtained. Among the different salts used, $CaCl_2$, coupled with TCA precipitation and dilute acid re-extraction, yields the most histone material with the least amount of acidic protein contamination.

Gel filtration and carboxymethyl cellulose chromatography are unsatisfactory for the fractionation of the plasmodial histone. Fractionation based on solubility differences gives fractions with very low purity.

The amino acid composition of whole plasmodial histone indicates that it is somewhat similar to calf thymus and *Tetrahymena* and most similar to *P. polycephalum* histones.

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INTRODUCTION

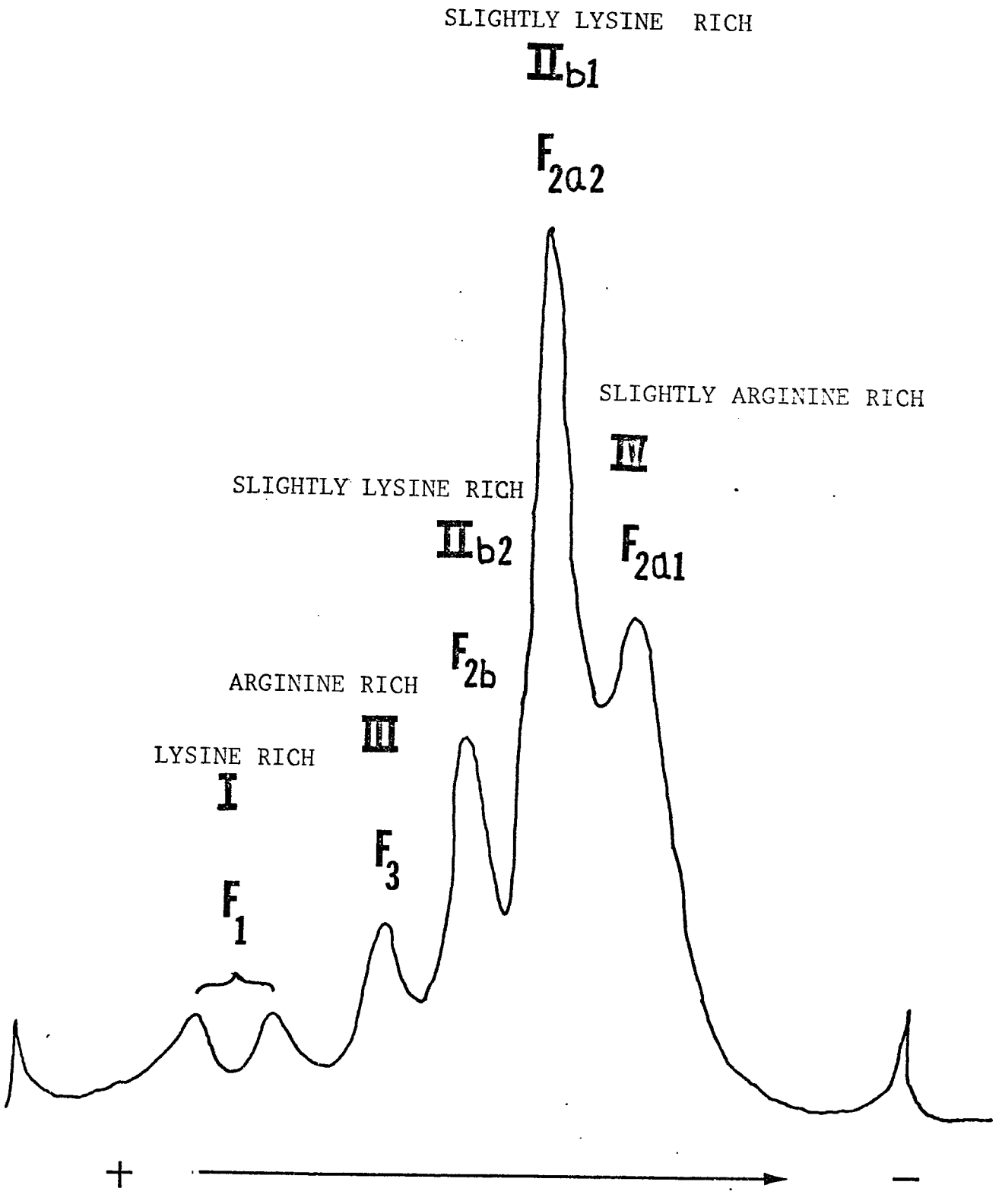
Histones are basic proteins of the nucleus that at some time are associated with DNA. Basic nuclear material, nuclein, was first isolated by Friedrich Miescher (1). Since this first isolation of the histones, they have been shown to exist, associated with DNA, in all the animal somatic cell nuclei examined. It is now generally accepted that they are present in the somatic cell nuclei of all eucaryotic organisims.

Stedman and Stedman (2) were the first to demonstrate that histone is not a homogenous protein. They suggested that histone is in fact a mixture of proteins with similar properties. The different fractions are classified on the basis of their lysine and arginine content. Fraction I (very lysine rich), fraction IIb1 (slightly lysine rich), fraction IIb2 (slightly lysine rich), fraction III (very arginine rich), and fraction IV (slightly arginine rich). A typical gel scan of calf thymus whole histone is presented in Fig. 1.

All the histones so far studied from different organisms, are found to have the five basic fractions. Differences detected are either in the relative quantities of each fraction or the additional presence of some unusual fraction.

The Myxomycetes (true slime molds) are free living, eucaryotic organisms which have characteristics in common with both plants and animals but are less complex. The life cycle of a Myxomycete exhibits a diploid, motile, multi-nucleate plasmodium or vegetative

Fig. 1. Disc electrophoresis of whole calf thymus histone. Standard whole histone from Worthington is subjected to pH 4.4 electrophoresis. Gels are stained with 0.1% Amido black in 7% acetic acid, and destained with 7% acetic acid-10% ethanol. Densitometric tracings are prepared in a Gilford linear transport set at 575 nm. The direction of electrophoresis is from left to right as indicated by the arrow. The sharp spikes at the beginning and end of the scan are the top and bottom of the gel, respectively.


SLIGHTLY LYSINE RICH
IIb1
F2a2
SLIGHTLY ARGININE RICH
IV
SLIGHTLY LYSINE RICH
IIb2
F2a1
F2b
ARGININE RICH
III
LYSINE RICH
I
F3
F1
+
−

stage which is capable of differentiating. At this plasmodial stage, rhythmical protoplasmic streaming is observed. A naturally occurring synchronous mitosis also takes place within a single plasmodium.

The plasmodium of Physarum polycephalum has been shown to have no G1 phase in its mitotic cycle (3). The cells after mitosis go into the S phase with no lag in between. Rusch (4) has shown that DNA and histones are synthesized exclusively in the S phase of the cell cycle. Mohberg and Rusch (5) isolated histones from P. polycephalum plasmodial nuclei. They have shown that the histone pattern is highly conserved during the mitotic cycle (6).

This research is therefore designed to isolate histone from purified Physarum flavicomum plasmodial nuclei. Attempts are made to fractionate and characterize this histone in order to compare this histone with other eucaryotic systems.

MATERIALS AND METHODS

Organism and growth conditions. *Physarum flavicomum* variety 1 was obtained from Henney and Henney (7) and was maintained in pure shake culture in a soluble, semi-defined medium developed in our laboratory (8). The identity of the culture was confirmed after inducing the plasmodium to sporulate. Cultural conditions and minimal growth requirements for this strain have been described by Henney and Lynch (9).

The cultures are maintained by sterile inoculation of 1 ml, presettled, 4 day old microplasmodia into a 500 ml, cotton plugged, glass Erlenmeyer flask containing 200 ml semi-defined, sterile media and 0.2 ml of sterile 0.25% (w/v) hematin (Sigma Chemical Co., St. Louis, Mo.) in 1% NaOH. The cultures are grown at 25 C in the dark on a Gyrotory shaker (New Brunswick Scientific Co., New Brunswick, N. J.) at 170 rpm. Batch cultures are grown in 7 liters of semi-defined media containing 7 ml of 0.25% (w/v) hematin in 1% NaOH and 3.5 ml of antiform 60 silicone emulsion (General Electric, Waterford, N.Y.). The apparatus is a 9 liter pyrex bottle which is prepared and sterilized as described by Teague (10). The media is inoculated with 10 ml of presettled microplasmodia. Cultures take about 5 days to develop maximum growth yield without entering stationary phase or "slime phase", which is characterized by copious extra-cellular slime production and a reduction in average cell size.

Cell harvest. The microplasmodia will settle to the flask bottom after aeration is stopped. The carboys are placed in a 4 C cold room for 10 to 15 min, followed by decantation of excess media. The remaining cells and media are centrifuged in a Sorval model RC-2 refrigerated centrifuge (Ivan Sorval, Inc., Norwalk, Conn.) at 2000 x g for 5 min at 2 C. The media is decanted. The cells are resuspended in 2 volumes of 0.25 M sucrose solution containing 0.01 M EDTA (Fisher Scientific Co., Houston, Texas) and 0.01 M Tris-HCl pH 7.2 (Sigma Chemical Co.) and the 2000 x g centrifugation is repeated. Approximately 25 g (wet weight) or 27 ml packed cell volume per liter of medium can be expected. EDTA aids in better breakage when washed plasmodia is subsequently blended. Higher concentration of EDTA used will cause leaching of the plasmodia as examined under light microscope.

Cells for nuclei isolation must be used fresh. Frozen cells give very poor yields and the isolated nuclei are damaged badly.

Nuclei isolation. The method of Mohberg and Rusch (11) is modified to accomodate large samples yielding nuclei of very high purity. Washed packed cells are suspended in 10 volumes of homogenizing medium containing 0.25 M sucrose (Imperial granulated sugar), 0.01% Triton X-100 (Rohm and Haas Co., Philadelphia, Pa.), 0.01 M Tris-HCl pH 7.2 and 0.005M $CaCl_2$ (J. T. Baker Chemical Co.). Sucrose is present to maintain the integrity of nuclei. A pH lower than 7.0 is deleterious to nuclei. Higher percentage of Triton X-100 will cause damage to nuclei and also decrease the yield.

Clumping of nuclei was prevented with 5mM $CaCl_2$, but higher concentrations may cause irreversible binding of calcium ions to nuclear membrane. Cells are then homogenized in a two-speed Waring Laboratory Blender (model 1042) at high speed at 120 volt setting for 2 min. All procedures are performed at 4 C unless stated otherwise.

The cell suspension is filtered by gravity through two thicknesses of milk filters (Rapid Flo, single gauge-faced, Johnson and Johnson) clamped in a two piece 130 mm diameter polyethylene Buchler funnel. Filters were saturated with the homogenizing medium before use. If centrifugation, even at low speed, is used in place of filtration at this stage, unbroken microplasmodia and trapped slime will sediment simultaneously with nuclei thereby increasing the percentage of contamination. The filtrate is suspended in 25 times the original packed cell volume. This suspension is then refiltered by gravity through the two layers of milk filter. Samples of the filtrate at this stage when stained with nuclear stain (0.1% Azure C in 0.25 M sucrose) and examined under light microscope shows individual nuclei with no debris attached.

Total filtrate in 100 ml quantities is underlaid with 50 ml of 1 M sucrose containing 0.01% Triton X-100, 0.01 M Tris-HCl pH 7.2 and 5 mM $CaCl_2$. Underlying is achieved by siphoning the heavy sucrose through small plastic tubing. One can handle 6 centrifuge bottles at one time giving a very distinct interface. Round bottom polycarbonate centrifuge bottles (250 ml) are used to give better pelleting of nuclei. The filled bottles are centrifuged in a Portable refrigerated International centrifuge Model PR-2 with a 259swinging bucket

head at 1000 x g for 10 min.

The yellowish crude nuclei pellet is resuspended in 15 times the original packed cell volume of homogenizing medium and subjected to Waring high speed blending at 120 volt setting for 1 min. The nuclear suspension is underlaid once more with 1/2 volume of 1 M sucrose medium and centrifuge at 1000x g for 10 min. The slightly yellow nuclei pellet when stained and examined with the light microscope shows very little debris but the nuclei still have some strands of material attached to the membrane.

The nuclear pellet is suspended in twice the original packed cell volume of wash medium containing 0.25 M sucrose, 0.02 M EDTA, 0.5 M KCl (Fisher Scientific Co.), and 0.01 M Tris-HCl with the final pH of the solution adjusted to 6.3. 20 mM EDTA is the maximum concentration tolerable before nuclear membrane damage will occur. KCl in the medium helps to solubilize the viscous material present. The nuclear suspension is blended at low speed 75 volt setting for 1 min. After centrifugation at 2000 x g for 5 min, the nuclear pellet is exposed to repeated washing until 95% purity is obtained (usually a total of three washes is sufficient). Nuclear purity is routinely checked by staining nuclei with 0.1% Azure C in 0.25 M sucrose and examining them with the light microscope at 1000 x magnification. Concentration of nuclei is determined by counting an aliquot in a hemacytometer.

Nuclei breakage. Purified nuclei can be frozen in pellet form without detectable change either morphologically or chemically over a period of up to one month. When purified nuclei are transferred from

the isotonic sucrose solution to an hypotonic environment of either 0.20 mM EDTA pH 7.2 or distilled water, no breakage of the nuclei is observed. The change in osmotic pressure causes a very slight swelling of the nuclei. Repeated freezing and thawing also gives the same result without breakage of the nuclei.

Mechanical treatment with either the Dounce homogenizer or Waring blending, in hypotonic solution, gives no breakage. Grinding with 5 volumes of acid cleaned glass powder, or 3 volumes of alumina, at 4 C for 30 min, produced distorted nuclear shape but no visible breakage. Sonication for 20 min gives about 70% breakage. Repeated grinding with dry ice also gives 70% breakage. Exposure of nuclei to French press at 19,000 psi would only give about 70% breakage. Nuclei suspended in 0.20 mM EDTA pH 7.2 when exposed to N_2 at 2,000 psi in a stainless steel Parr 4635 Cell Disruption Bomb (Parr Instrument Co., Moline, Ill.) for 30 min at 4 C would only give 50% breakage. Unbroken nuclei can be centrifuged down at 50,000 x g and reexposed to N_2 pressure until all nuclei are broken.

Incubation of nuclear suspension with Pancreatic Lipase (Worthington Biochemicals) at a constant readjusted pH of 8.0 for 30 min only gives 50% breakage. When the nuclear suspension is incubated at 5 mM DTT at pH 9.2 at room temperature for 30 min, a very viscous suspension is obtained and 100% breakage is detected. DTT has been shown to aid in membrane breakage in other organisms (12).

Chromatin preparation. The viscous suspension of nuclear material after DTT incubation is dialyzed against 50 volumes of 0.2 mM EDTA pH 7.2 with 2 additional changes over a period of 24 h at 4 C.

At the end of dialysis, no trace of DTT should remain in the sample. The nuclear material (crude chromatin) is centrifuged at 4 C in a Beckman Model L3-50 ultracentrifuge with a #40 or 30 fixed angle rotor at 54,000 x g for 15 min. The clear viscous material on top is designated as true chromatin and the gelatinous opaque material at bottom is referred to as chromatin pellet.

Extraction of basic protein from chromatin. Three different methods of extracting basic proteins are used. The first method is the extraction of true chromatin with NaCl in the presence and absence of urea. An equal volume of either 4 M NaCl or 4 M NaCl and 10 M urea is added. to true chromatin. The solution is allowed to stir in the cold for 30 min. The sample is then centrifuged at 30,000 x g for 18 h at 4 C in a Beckman L3-50 ultracentrifuge. The supernatant which contains the dissociated protein material is dialyzed against distilled water for 24 h and lyophilized

The second method is the extraction with $CaCl_2$ from the chromatin pellet (5). The chromatin pellet is homogenized in 10 volumes of 1 M $CaCl_2$ and the viscous suspension is stirred at 4 C for 24 h. After centrifugation at 50,000 x g for 15 min, enough 100% TCA (w/v) (Fisher Scientific Co.) is added to the supernatant, giving a final TCA concentration of 25%. Precipitation is complete after standing at 4 C for 2 h. The TCA solution is centrifuged at 50,000 x g for 15 min. Centrifuge bottles are drained well and cold 0.04 N H_2SO_4 is added. The precipitate is scraped off the sides of the centrifuge bottles by use of a rubber policeman. The suspension is homogenized by use of 2 or 3

passes with a glass homogenizer. After dialysis against 100 volumes of 0.04 N H_2SO_4 at 4 C for 24 h, the suspension is centrifuged at 50,000 x g for 15 min. The supernatant is dialyzed in the cold against 100 volumes of distilled water and lyophilized to dryness.

The third method is the extraction of chromatin pellet with 0.4 N H_2SO_4. Chromatin pellet is homogenized with 10 volumes of 0.4 N H_2SO_4 and stirred in the cold for 30 min. The sample is centrifuged at 35,000 x g for 15 min. Supernatant is saved and the pellet re-extracted with 5 volumes of 0.4 N H_2SO_4. The combined extract is dialyzed against distilled water and lyophilized to dryness.

Chemical fractionation of whole histone. The method of Johns (13) produces little fractionation due to the small amount of sample available. A modified procedure is used, which is designed to handle small samples with high purity fractionation for all animal histones (14).

Fraction F1 (I, very lysine rich). Whole histone (50-100 mg) is dissolved in 1 mM HCl (1 ml/5 mg protein), the solution is centrifuged at 12,000 x g for 10 min and the supernatant containing the histone collected. Sufficient concentrated PCA (perchloric acid) is added dropwise with shaking to make the final solution 0.5 M in PCA. After standing for 15 min, the solution is centrifuged at 12,000 x g for 5 min and the pellet is kept. The supernatant, containing histone F1, is treated with 3 M H_2SO_4 to a final concentration of 0.2 M, followed by precipitation with a 6 volume excess of acetone. The solution is stored overnight at -14 C before collection of the F1 pellet.

Fraction F2B (IIb2, slightly lysine rich). The histone insoluble in 0.5 M PCA is collected and dissolved in 1 mM HCl (1 ml/5 mg protein). Concentrated PCA is added to a final concentration of 0.5 M and after 15 min the solution is centrifuged at 12,000 x g for 5 min. The supernatant is discarded. The pellet containing histones F2b, F3 and F2a is washed once with 0.5 M PCA and then extracted into an equal volume of 80% ethanol-5% HCl with a glass homogenizer and left for 10 min. After centrifugation, the supernatant containing histones F3 + F2a is kept and the pellet again dissolved in 1 mM HCl and treated with 0.5 M PCA as described above. The pellet is extracted into the 80% ethanol-5% HCL. The histone F2b pellet after six such cycles is washed with acetone and dried *in vacuo*.

F3 (III, very arginine rich) + F2a (IIb1, slightly lysine rich and IV, slightly arginine rich). The supernatants from the first three histone F2b washings with ethanol-HCl is precipitated by addition of 3 M H_2SO_4 to a final concentration of 0.2 M followed by addition of a 4 volume excess acetone. The pellet is washed with acetone and dried *in vacuo* completely. The pellet is dissolved in ethanol-HCl and A_{230} is recorded and adjusted to $A_{230} = 6$. The solution is dialyzed against 7 volumes of anhydrous ethanol for 5 h with vigorous stirring. The contents of the dialysis bag are removed, centrifuged at 12,000 x g for 5 min and the pellet (F3) is washed four times with acetone and dried. Supernatant which contains the F2a is preciptated in acetone-H_2SO_4, washed once with acetone and then dried.

Column chromatography. Ten g of Sephadex G-100, fine or G-75, medium (Pharmacia Fine Chemicals, Piscataway, N. J.) is allowed to swell in the eluting buffer of 0.01 N HCl for 75 h at room temperature. The gel suspension is degassed by aspiration for 30 min. Two sections of 9 mm x 120 cm glass columns (Glenco, Houston, Texas) are connected to give a total column length of 240 cm. The column is mounted and checked with a level to make sure that the column is vertical. Ten ml of 0.01 N HCl is poured into the column to cover the bed support. No air should remain in or under the bed support and the column outlet is closed. A slurry of resin is poured down the side of the chromatographic tube by use of a small funnel thus avoiding bubble formation. The gel is allowed to settle by gravity. After 15 min intervals the excess buffer on the top is removed by use of a syringe fitted with a capillary tubing. Gel suspension is added continuously until the desired height of 220 cm is obtained. The resin is allowed to settle overnight with a few cm of buffer on top to ensure that packing is complete. The column is then filled with buffer and connected to a buffer reservoir. The flow rate is adjusted by controlling the outlet valve. Equilibration is complete after running the column under constant pressure for 24 h. All buffers used have 0.02% sodium azide (Mallinckrodt Chemical Works, St. Louis, Mo.) to prevent bacterial and fungal growth.

Void volume determination is achieved by use of blue dextran (Pharmacia Fine Chemicals). Five ml of 2% blue dextran in 10% sucrose is applied to the top of the column with the outlet valve closed. The column is then filled by carefully overlayering buffer on top of the

sample through a thin capillary tubing attached to a syringe. The column is connected for elution at constant pressure. For a properly packed column the blue dextran elutes as a relatively narrow single band and not a diffused smear. The void volume, volume required before blue dextran is eluted, is recorded. The column is now ready for sample application. All Sephadex columns used are run at room temperature.

Electrophoresis. Analytical polyacrylamide gel electrophoresis (15) is used to follow separation and purification of basic nuclear protein. The polyacrylamide is made from stock solutions prepared and stored in amber bottles at 4 C. The 7.5% acrylamide, 6.25 M urea separating gels are made by mixing two parts acrylamide solution containing 30% acrylamide (Eastman Kodak Co., Rochester, N.Y.) and 0.08% bis-acrylamide (Eastman Kodak); one part buffer containing 48% 1 N KOH, 17.2% glacial acetic acid and 4% TEMED (Eastman Kodak); and five parts freshly prepared 10 M urea in 0.2% ammonium persulfate (E. Merck Ag., Darmstadt, Germany). Acid cleaned glass tubing coated with column coat (Canalco), free-standing in rubber stoppers, are 70% filled with the above solution and carefully overlayered using a 20-gage needle and a glass syringe with a solution of 3 M urea. Gels are allowed to polymerize at room temperature for 30 min until a flat line demarking the gel-urea surface appears.

The stoppers and urea are gently removed from the tubes and the upper portion of the tubes are inserted through rubber grommets in the bottom of a 1 liter electrically inert reservoir. The empty

portion of the glass tubes and the reservoir are filled with a 3.2% β-alanine (Eastman Kodak Co.) and a 0.08% glacial acetic acid buffer pH 4.4. The lower ends of the electrophoresis tubes are inserted into another reservoir of equal capacity and construction filled with the same buffer. Platinum electrodes inserted in the top and bottom buffer chamber are aligned so the distances from each tube to the electrodes are the same. Samples dissolved in 8 M urea and 0.25 M H_2SO_4 are carefully layered on top of each gel. A charge of 4 ma/gel is directed across the gels for 1 1/2 h with the top as anode.

Gels are stained for protein in 0.1% Amido Black (Curtin) in 7% acetic acid for 30 min. The gels, reamed free from the glass tube with a water jet delivered by a 25-gage needle and syringe, are dropped directly into this stain. They are then diffused free of excess stain in a solution of 7% acetic acid-10% ethanol.

SDS-polyacrylamide gel electrophoresis (16) is used for determination of molecular weight. For the 10% acrylamide solution, 22 g of acrylamide and 0.06 g of bis-acrylamide are dissolved in water to give 100 ml of solution. Insoluble material is removed by filtration through Whatman No. 1 filter paper (Curtin). The solution is kept at 4 C in a dark bottle. For a typical fun of 12 gels, 15 ml of gel buffer containing 0.2% SDS (Nutritional Biochemicals Corp., Cleveland, Ohio) and 0.2 M sodium phosphate buffer pH 7.2 is deaerated and mixed with 13.5 ml of acrylamide solution. After further deaeration, 1.5 ml of freshly made ammonium persulfate solution (15 mg/ml) and 0.045 ml of TEMED are added. Before the gel hardens, a few drops of water are layered on

top of the gel solution. After 30 min an interface can be seen indicating that the gel has solidified.

All protein samples are dissolved in 0.1 M sodium phosphate buffer pH 7.2 with 0.1% mercaptoethanol (Eastman Kodak) and 0.1% SDS. Recrystallized urea is added to give a final concentration of 8 M The protein solution is then incubated at 70 C for 1 h. The tracking dye (0.05% bromphenol blue, E. Merck Ag.) is added to each sample before application to the gels. Electrophoresis buffer of 0.1 M sodium phosphate buffer pH 7.2 and 0.1% SDS is carefully layered on top of each sample to fill the tubes. The two compartments of the electrophoresis apparatus are filled with the same buffer. Electrophoresis is performed at a constant current of 4 ma/gel for 18 h. Under these conditions the marker dye would have moved three-quarters through the gel. After electrophoresis, the gels are removed from the tubes by squirting water from a syringe between gel and glass wall. The gels are stained in 0.1% Amido black in 7% acetic acid for 1 h. Destaining is achieved by placing gels in large volumes of 7% acetic acid-10% ethanol overnight. The positions of the blue protein zones and the distance traveled by the tracking dye are recorded and a standard curve of relative mobility is established. The relative mobility of the unknown sample is compared to the standard curve and its molecular weight extrapolated.

Preparative polyacrylamide gel electrophoresis is used to separate the basic protein from the acidic material that is co-extracted by 1 M $CaCl_2$. 7.5% acrylamide, 6.25 M urea gels are polymerized in

a 2.2 cm diameter column-coated glass tubing with one end sealed with 2 layers of parafilm and a tight fitting O ring. Tubes are filled to 3 cm height and overlayered carefully with 3 M urea. Polymerization is complete in 30 min at room temperature. The glass tubing is filled with the 3.2% β-alanine, 0.08% glacial acetic acid pH 4.4 buffer. The bottom of the tube is immersed in a 1 liter plastic beaker containing the same buffer. The gel is pre-electrophoresed at 25 ma with anode at the top for 1 h. A dialysis bag filled with 2 to 3 ml of the pH 4.4 buffer is placed over the end of the gel with a tight fitting O ring and carefully checked for air bubbles and leakage. Samples of a 3-5 mg protein in 8 M urea and 0.25 M H_2SO_4 is applied to the top of the gel and carefully overlayered with the buffer. The gel with the sample is exposed to 25 ma for 5 h with the anode on top. The contents of the dialysis bag is carefully and quantatively removed. The gel is reamed free from the glass tube and immersed in 0.1% Amido black in 7% acetic acid for staining over-night. Destaining is achieved by placing the gel in large volumes of the 7% acetic acid-10% ethanol solution with constant changes.

Hydrolysis of protein. Lyophilized samples of known protein concentration in acid cleaned hydrolysis flasks are dissolved in 2 ml of 5.7 N redistilled, constant boiling HCl (Matheson, Coleman & Bell). Each sample is shell frozen in acetone and dry ice and twice evacuated by vacuum pump to ensure a complete removal of air from the flask. The flask is clamped and sealed with a Propane torch (Turner Corp., Sycamore, Ill.).

The sealed sample is placed in an constant environment of 110 $\pm$ 1 C for 22 h. HCl is removed by flash drying on a rotary evaporator (Buchler Instruments, Fort Lee, N. J.). The dried sample is dissolved in the appropriate buffer and analyzed by the amino acid analyzer (model K8000, Phoenix Precision Instrument Co.).

Performic acid oxidation of cystine (17). Upon normal acid hydrolysis, the cysteine content of a protein is partially destroyed. In order to get an accurate determination of the cysteine content, the cystine and cysteine have to be oxidized to cysteic acid before exposure to acid hydrolysis. One ml of 30% H_2O_2 (Fisher Scientific) is added to 9 ml of 88% formic acid (Fisher Scientific). The mixture is allowed to stand for 1 h at room temperature and subsequently cooled to 4 C before use. To 0.5 ml of lyophilized protein, 2 ml of the performic acid solution is added. The reaction mixture is kept at 4 C for 4 h and then 0.3 ml of 48% hydrobromic acid (Fisher Scientific) is added with swirling of the reaction flask in an ice bath. The reaction flask is placed in a vacuum dessicator which contains a plate of NaOH pellets. The dessicator is evacuated first by use of a water aspirator for 20 min, then carefully switched to a vacuum pump till completely dry. The sample is then ready for acid hydrolysis.

Determination of tyrosine and tryptophan (18). Spectrophotometric determination of tyrosine and tryptophan in proteins offers an advantage over chemical methods because it does not require hydrolysis, which often leads to partial decomposition of these two amino acids. Standard solutions of D,L-tyrosine (Sigma Chemical Co.) and D,L-tryptophan

(Sigma Chemical Co.) are made up in 0.1 N sodium hydroxide and diluted to give a final amino acid concentration of 0.05 mM to 0.1 mM.

All measurements are carried out in a Beckman DK-2A Ratio recording spectrophotometer with 1 cm cells. Scans of individual tryptophan or tyrosine are made from 220 nm to 320 nm. Known amounts of tyrosine and tryptophan are mixed to give a tyrosine/tryptophan molar ratio of 0.5 to 1.0 and 220 nm to 320 nm scans are made. Depending on the ratio of these two amino acids, there is a bathochromic shift of 1 to 3 nm. A line is drawn tangent to the two maxima of the characteristic absorbance peaks. A reference value is set up on known molar ratio of tyrosine/tryptophan by the following equation:

$$S = \frac{(a/b) \quad 10^3}{A_{max}}$$

where (a) is the change in absorbance at intercept; (b) is the change in wavelength at intercept and A_{max} is the maximum absorbance.

The unknown samples of 0.5 mg protein is dissolved in 1 ml of 0.1 N sodium hydroxide and scanned immediately from 220 nm to 320 nm. A line tangent to the maxima absorbance is drawn and the S value calculated. By comparison to the standard ratio already established, the ratio and concentration of tyrosine/tryptophan in the unknown sample is extrapolated.

Protein Analysis. Protein concentrations are determined by the method of Lowry, et al (19). Determination employs a

standard curve prepared by subjecting known amounts of crystalline BSA (Sigma Chemical Co.) to the test. Absolute determination of BSA standard solution concentration utilizing spectrophometric assay is performed as described by Teague (20). An additional blank with the same amount of urea as in the unknown is prepared when samples are in urea solution in order to account for any interference by urea.

For total protein determination on microplasmodia, a small aliquot of cells from the 2000 x g centrigugation is weighed to determine the wet weight. 1.0 ml of 10% TCA (w/v) and 1 ml of acetone is added to the sample, and the pellet resuspended by vibrating on a Super-mixer. The yellow supernatant is poured off and the pellet subjected to the TCA-acetone once more to ensure the complete removal of the pigment which otherwise would have interfered with the Lowry protein test. The colorless pellet is dissolved in 0.4 N NaOH, 5 ml/g wet weight. An appropriate aliquot is used for the Lowry test.

D (-) Ribose determination. The orcinol test for D (-) ribose (21, 22) is modified and used as explained below. The standard used is a solution of D (-) ribose (Sigma Chemical Co.). This is kept at 4 C and diluted with 0.5 N PCA to give a series of concentrations from 1.25 to 50 μg per ml.

The orcinol acid reagent is made by adding 2 ml of a 10% $FeCl_3.6H_2O$ solution to 400 ml of concentrated HCl. The orcinol reagent is a 6% solution of purified orcinol (Sigma Chemical Co.)

in 95% ethanol.

The test is run by adding 3 ml of standard, sample, or 0.5 N PCA blank to a test tube. 6 ml of the orcinol acid reagent is added and mixed. 0.4 ml of the 6% alcoholic orcinol is added next, and the tubes are heated in a boiling water bath for 20 min. The liquid level in the tubes has to submerge below the level of the boiling water for consistent results. After the tubes are cooled to room temperature, O. D. readings are made at 660 nm. The standard curve is established and the unknown samples are compared to it.

RNA determination. To determine the amount of ribose that is released from the purines of the RNA, the base ratio figures of *P. rigidum* (23) is used to estimate the total amount of ribose attached to the purines and pyrimidines in *P. flavicomum*. Approximately 57.8% of the ribose in RNA is attached to the purines. Since 43.8% of the RNA is made up of D (-) ribose, the total RNA can easily be calculated by the following equation:

$$\frac{R}{P \times C} = \text{mg RNA}$$

R = mg D (-) ribose as determined from orcinol curve.

P = Percentage of purines in total RNA as established by the *P. rigidum* base ratio studies (57.8%)

C = Percent D (-) ribose in the total RNA established from the base ratio studies of *P. rigidum* (43.8%)

DNA determination with diphenylamine (24). Analytical grade fresh acetaldehyde (Fisher Scientific Co.) tightly capped in small portions of 10 ml is stored at 4 C. 1 ml of the cooled acetaldehyde

is transferred in a cooled pipette into 50 ml of water. This aqueous acetaldehyde (1.6%) is kept at 4 C in a well-stoppered bottle and the solution is discarded after one month. 1.5 g of diphenylamine (Eastman Kodak) that has been recrystallised twice with petroleum ether (J. T. Baker Chemical Co.) is dissolved in 100 ml of glacial acetic acid (Fisher Scientific Co., Reagent A. C. S.) which has been kept in the dark. After 1.5 ml of concentrated sulfuric acid is added, the solution is mixed well. Just before use 0.1 ml of 1.6% aqueous acetaldehyde is added per 20 ml of reagent. DNA (Salmon sperm, highly polymerized, Mann Research Lab., N. Y., N. Y.) is dissolved at 0.5 mg/ml in 5 mM NaOH and kept as stock solution at 4 C. The absolute concentration in the stock solution is determined by the extinction coefficient, E_{260} = 210 at pH 12. For working standards a measured volume of stock standard solution is mixed with an equal volume of 1 N PCA and heated at 70 C for 15 min. A series of standard DNA concentrations from 10 to 100 μg is set up, using 0.5 N PCA to make all volume up to 1 ml. Unknown samples in 0.5 N PCA are made up in the same manner. The blank is composed of 1 ml 0.5 N PCA. Two volumes (2 ml) of the diphenylamine reagent mixture is added to each tube and mixed well. Tubes are then covered with marbles to prevent any loss of liquid due to evaporation. After incubation in the dark at room temperature for 18 h, the tubes are read both at 610 and 650 nm. $A_{610} - A_{650}$ readings are plotted to establish a DNA standard curve. Unknown sample concentrations are interpolated from the standard curve.

Treatment of microplasmodia for nucleic acid analysis. 1 g (wet weight) of plasmodia pellet is homogenized in 10 ml of 0.5 N PCA and incubated at 4 C for 20 min. After centrifugation at 15,000 x g for 10 min, the supernatant is discarded and the pellet is extracted 3 more times in the cold with 0.5 N PCA. The slightly yellow pellet is suspended in 2 ml of 0.5 N PCA and extracted at 70 C for 50 min. The supernatant is saved after centrifugation and the pellet is reexposed to the hot PCA extraction once more. The combined supernatant is then used for analysis of both DNA and RNA content.

Dialysis. All dialysis tubing (Curtin) used is cleaned by boiling in 2% sodium carbonate for 10 min, rinsing in water and boiling in 0.2% EDTA for 10 min. It is then rinsed in water exhaustively and stored in 0.2% sodium azide at 4 C to prevent fungal and bacterial growth. When needed, a suitable length of tubing, rinsed free of azide, is filled with the ends triple tied, and immersed in appropriate dialyzing medium at 4 C. Additional media changes over a 24 h period are made accordingly. Stirring with a Corning magnetic mixer or Lab-Stir prevented protein-membrane association and facilitated dialysis.

Lyophilization. Samples in distilled water are shell frozen in acetone and dry ice. Total volume in each lyophilizing flask should never exceed half the capacity of the flask for effective lyophilization. The lyophilizing flask is connected to a Virtis Bench top lyophilizer equipped with a Virtis vacuum trap

(Virtis Co., Gardiner, N. Y.). The whole apparatus is connected to a Sensaur Hitachi vacuum pump (Aloe Scientific, St. Louis, Mo.). When samples are down to dryness individual port holes are released carefully of the vacuum and the samples taken off.

Urea recrystallization. Urea crystals (Curtin) are dissolved in boiling 95% ethanol in a concentration of 1 gm/ml. The hot ethanolic solution is filtered by suction through a Buchler funnel lined with Whatman No. 1 filter paper. The filtrate is allowed to stand at 4 C overnight to assure complete recrystallization. Crystals are drained well and allowed to dry until no trace of ethanol is detected before use. Urea crystals once dissolved are used fresh. If allowed to stand, even at 4 C, the urea solution is acidified to pH 2 by addition of sulfuric acid in order to prevent cyanate formation.

Glassware. All glassware used have been immersed overnight in an acid cleaning solution containing 18% concentrated nitric acid and 82% concentrated HCl. The glassware is rinsed 5 times with tap water, 5 times with deionized water and finally twice with glass distilled water. The glassware is then dried in an oven.

Water. Double distilled water from a Corning AG-3 glass still is used exclusively.

RESULTS

Nuclei isolation. Most nuclei isolation methods that are applicable to other systems cannot be applied to plasmodial nuclei isolation due to two factors. The first is the presence of slime. During isolation, besides using very large volumes of homogenizing medium, a non-ionic detergent, Triton X-100, has to be present at a critical concentration so that slime will stay in solution and can be easily disposed of.

The second factor is the nature of the plasmodial nuclei. At the crude isolation stage, the nuclei are very fragile. Even in the presence of sucrose, any harsh mechanical treatment like prolonged blending or douncing will damage the nuclear membrane. Once the nuclei are free of contaminants, the nuclear membranes become very resistant to mechanical treatment and change in osmotic pressure. The membrane seems to be contractile in nature, in that it will swell and become slightly distorted but will not break easily. The findings from experiments employing mechanical methods of grinding, blending, douncing, exposure to high pressure and use of enzymatic treatment by lipase, agree with the findings of O'Farrell and Shall (25). Complete breakage is not achieved by any of the above treatments. DTT is the only reagent we found that will give a 100% breakage of nuclei.

Purified plasmodial nuclei. Plasmodial nuclei are approximately half the size of mammalian nuclei. The suspension of plasmodia used for isolation is composed of microplasmodia at

various stages of the cell cycle since no attempt was made to obtain synchronous cultures. In the crude homogenate, the size of the nuclei ranges from 2 to 6 μ in diameter, while purified nuclei are of the size from 4 to 6 μ in diameter with the average diameter of 5 μ. About 1% of the nuclei have two nucleoli. The nucleolus occupies approximately 50% of the nuclear volume. Purity of the nuclei preparation is routinely checked with the light microscope at 1000 x magnification. Samples have also been examined in the electron microscope. Even under these high magnifications, one can see that there are little or no contaminating slime filaments attached to the nuclear membrane. The nuclear membrane can be seen as an intact continuous structure and there appears to be no leakage of the nuclear material. The chemical composition of the purified nuclei is given in Table I. The amount of RNA present in the nucleus is relatively high. The high value most probably is not due to the presence of contaminating ribosomal RNA as indicated by the microscopic examination of the isolated nuclei. This may be an indication of the large amount of RNA that is present in the nucleolus.

Fig. 2 presents photographs of purified nuclei stained with Azure C taken using the light microscope. Fig. 3 presents electron microscope photographs of fixed purified nuclei.

TABLE I

Chemical composition of microplasmodia and purified nuclei.

Nuclear yield is 4×10^8 nuclei/gm wet weight of microplasmodia.

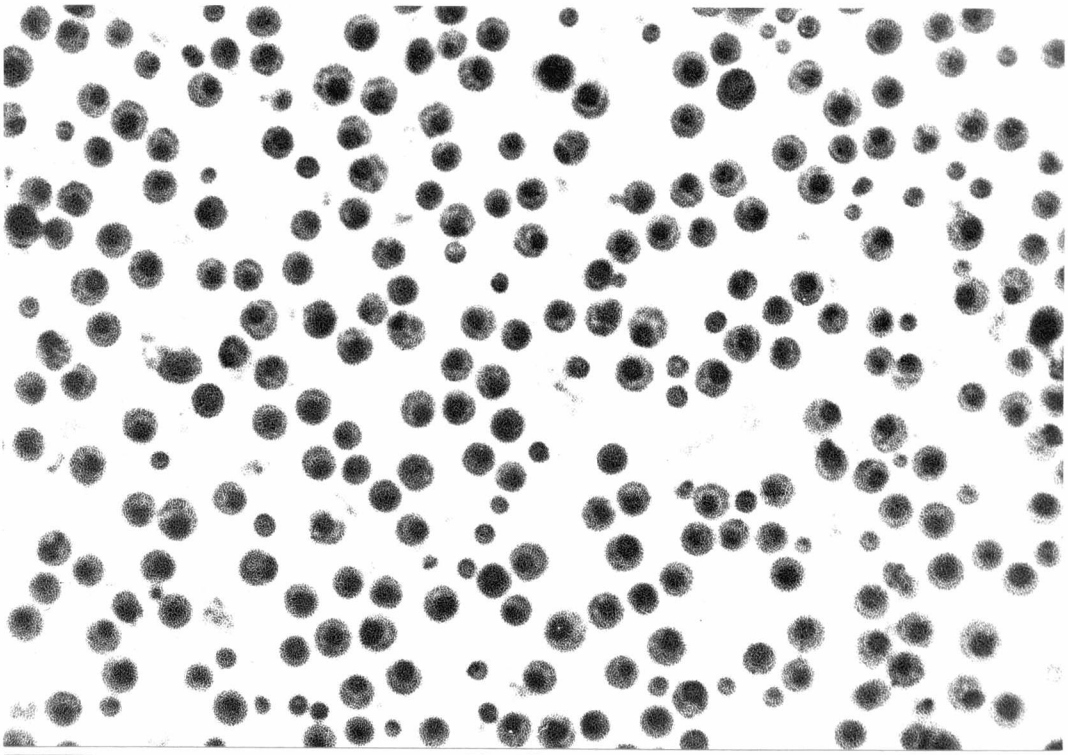
	mg/gm wet wt microplasmodia	pg/nucleus
Protein	28.00 ± 3.00	10.50 ± 0.50
RNA	2.91 ± 0.53	1.59 ± 0.21
DNA	0.30 ± 0.05	0.84 ± 0.05

[a] Average ± standard deviation.

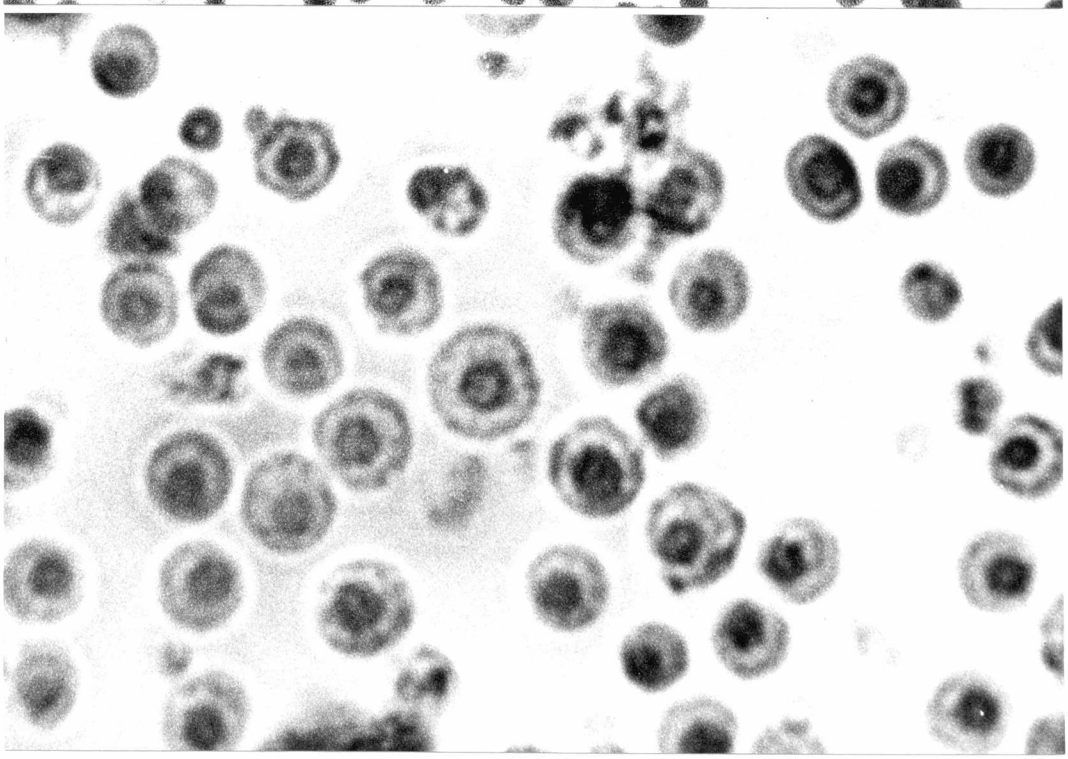
Fig. 2 Light microscope photographs of purified nuclei stained with 0.1% Azure C in 0.25 M sucrose. (A) shows a typical nuclei preparation with nuclei of different sizes. (B) shows the easily visible darkly stained nucleolus.

(A) 1,000 X

(B) 3,024 X



A

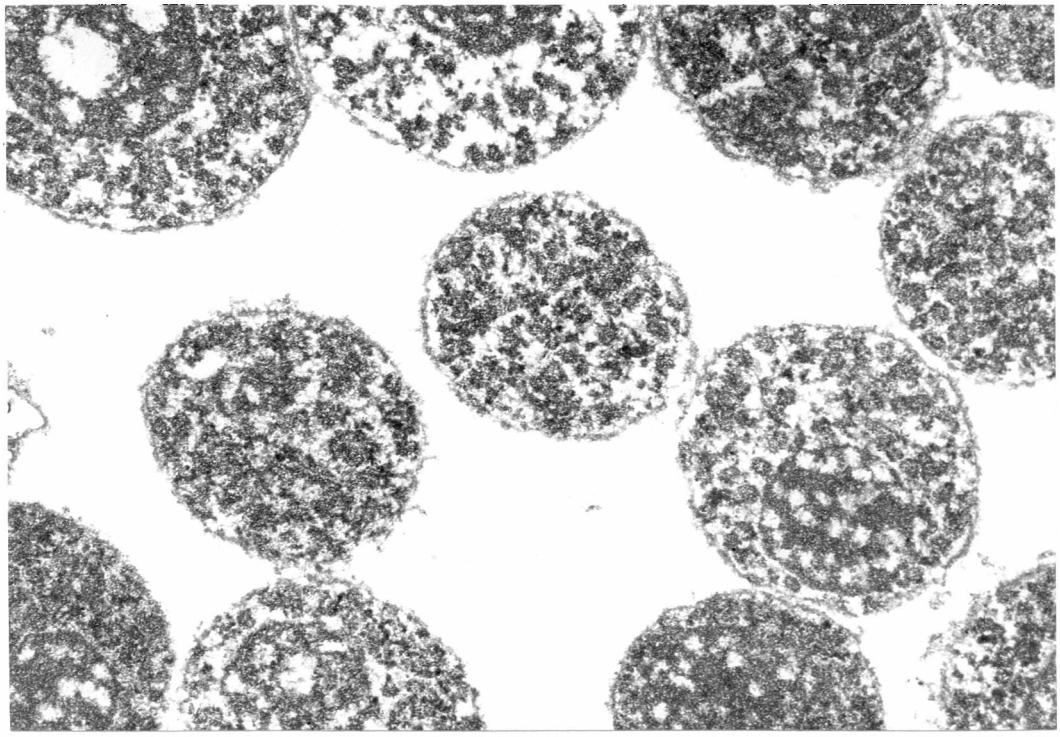


B

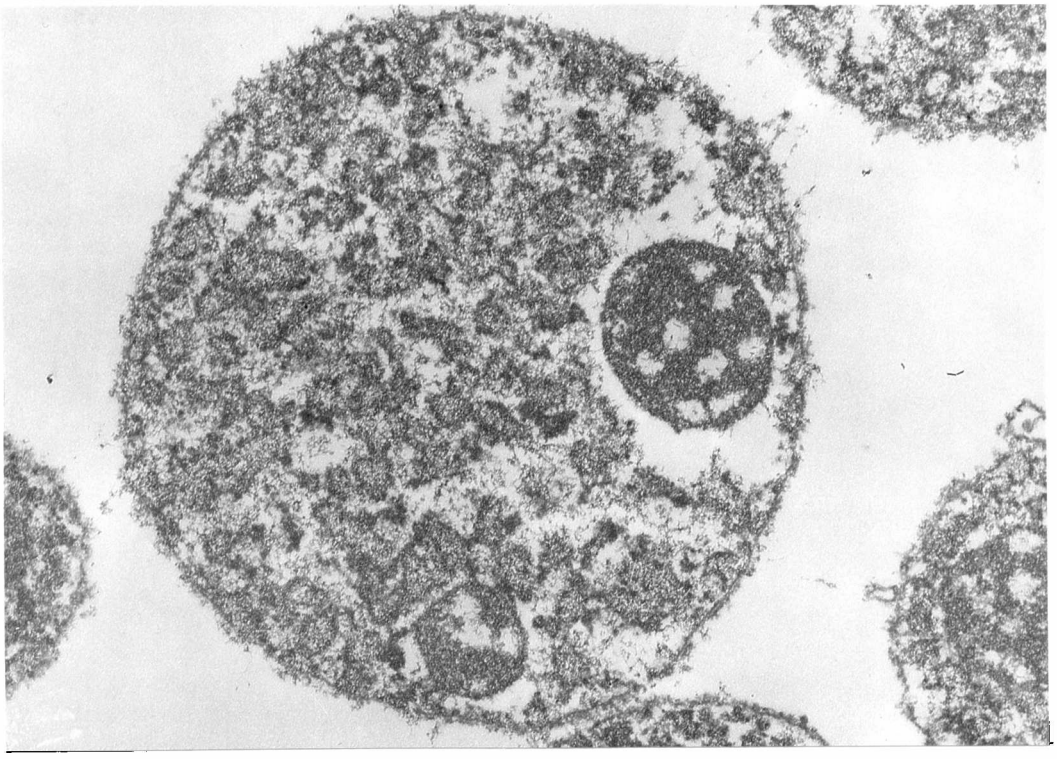
Fig. 3 Electron microscopic analysis of purified nuclei. The nuclei are first fixed in 2.5% glutaraldehyde and 0.1 M sodium phosphate buffer pH 7.4. The nuclei are then fixed in 1% osmium sulfate with 0.1 M sodium phosphate, pH 7.4. For full details of the preparation of these nuclei for electron microscopic analysis, refer to Lena W. Cheung's Thesis (Ultrastructural studies of the life cycle of Physarum flavicomum grown in pure culture, 1972, University of Houston). All electron microscopic analyses were performed by Miss Cheung.

(A) 9,000 X

(B) 15,000 X



A



B

Chromatin preparation. The broken nuclei suspension is referred to as the crude chromatin. The clear gelatinous supernatant after the 54,000 x g centrifugation is referred to as true chromatin, and the pellet is designated as chromatin pellet. The true chromatin, regardless of the method of nuclear breakage, typically shows an ultraviolet absorbance pattern similar to that presented in Table II. A_{260} is the absorbance for nuclei acid. A_{280} is the absorbance maximum for protein. A typical true chromatin preparation has an A_{260}/A_{280} of 1.5 to 2.0. The chromatin preparation should also exhibit little turbidty, that is, little absorption at wavelengths in which nucleic acids and proteins do not absorb, i. e. A_{320}.

Nuclear protein preparation. A nuclear protein preparation should have little or no nucleic acid present. An A_{260}/A_{280} of more than 1 suggests the presence of nucleic acid. 1 M $CaCl_2$ extraction followed by TCA precipitation and re-extraction with 0.04 N H_2SO_4 gives a nuclear protein preparation with an A_{260}/A_{280} of less than one. The ultraviolet absorbance pattern of different nuclear protein preparations utilizing different extraction reagents is given in Table III.

Acidic material when present in a nuclear protein preparation will not migrate in the pH 4.4 gel electrophoresis. The acidic material either stays on top of the gel or will migrate towards the anode. All nuclear protein preparations are subjected to the pH 4.4 disc gel electrophoresis to determine the nature of the extracted material. 0.04 N H_2SO_4 acid extraction gives pure

TABLE II

True chromatin preparation.

The clear gelatinous supernatant after the 54,000 x g centrifugation is referred to as true chromatin.

Method of nuclei breakage	% of total nuclear protein	A_{260}	A_{280}	A_{320}	A_{260}/A_{280}
Dry ice grinding	22.0	0.640	0.370	0.047	1.72
N_2 pressure	26.5	0.810	0.468	0.034	1.73
Sonication	23.8	1.850	1.100	0.040	1.68
DTT at pH 9.2	28.7	1.600	0.900	0.047	1.77
Average	25.2 ± 3.5				1.72 ± 0.05

TABLE III

Nuclear protein preparation.

Details given in text.

Method of nuclei breakage	Source	Extraction Reagent	A_{230}	A_{260}	A_{280}	A_{320}	A_{260}/A_{280}
DTT	True chromatin	2 M NaCl	1.350	0.532	0.458	0.020	1.16
DTT	True chromatin	2 M NaCl + 5 M urea	0.560	0.168	0.155	0.010	1.08
N_2	Chromatin pellet	1 M $CaCl_2$	0.570	0.500	0.420	0.025	1.19
N_2	True chromatin	1 M $CaCl_2$	0.820	0.400	0.340	0.010	1.18
N_2	True chromatin	0.4 N H_2SO_4	0.400	0.175	0.150	0.032	1.17
N_2	Chromatin Pellet	1 M $CaCl_2$; TCA precipitation; 0.04 N H_2SO_4	0.880	0.115	0.133	0.020	0.86
DTT	True chromatin	1 M $CaCl_2$; TCA precipitation; 0.04 N H_2SO_4	0.550	0.101	0.120	0.010	0.84
DTT	Chromatin pellet	1 M $CaCl_2$; TCA precipitation; 0.04 N H_2SO_4	0.580	0.103	0.118	0.012	0.87

basic nuclear protein with no detectable trace of acidic material as shown by the densitometric tracing in Fig. 4. The only disadvantage is the very low yield of less than 1%. It is therefore not feasible to use this method of extraction. NaCl extraction gives a 16% yield but more than 50% of the protein extracted is acidic in nature. With the NaCl + urea combination extraction, 75% of extracted material is acidic in nature. 1 M $CaCl_2$ extraction followed by TCA precipitation and 0.04 N H_2SO_4 re-extraction yields samples with less than 10% acidic material contamination. Nuclear protein yields are presented in Table IV.

Column chromatography. 2 M NaCl extractable material from true chromatin gives the 5 main histone bands but also a very heavy band of acidic material. The lysine rich histone bands are very light consisting of about 10% of the total histone material. Fractionation of the 2 M NaCl extractable material from true chromatin on Sephadex G-100 gives 3 protein peaks and one urea peak. Due to the low percentage of aromatic amino acids present the absorbance of histone is higher at 230 nm than at 280 nm (the usual wavelength for other proteins). The A_{230} profile is presented in Fig. 5. The urea peak after dialysis against distilled water gives no A_{230} reading. Each combined protein fraction is subjected to pH 4.4 disc electrophoresis. The densitometric tracings of the different fractions are presented in Fig. 6.

The first combined fraction is totally acidic in nature. The second combined fraction is composed of mostly slightly lysine rich

Fig. 4 Disc electrophoresis of 0.4 N H_2SO_4 extractable protein from chromatin pellet. Sample is subjected to disc electrophoresis at pH 4.4, stained with 0.1% Amido black in 7% acetic acid, and destained with 7% acetic acid-10% ethanol. Densitometric tracings are prepared in a Gilford linear transport set at 575 nm. The direction of electrophoresis is from left to right as indicated by the arrow.

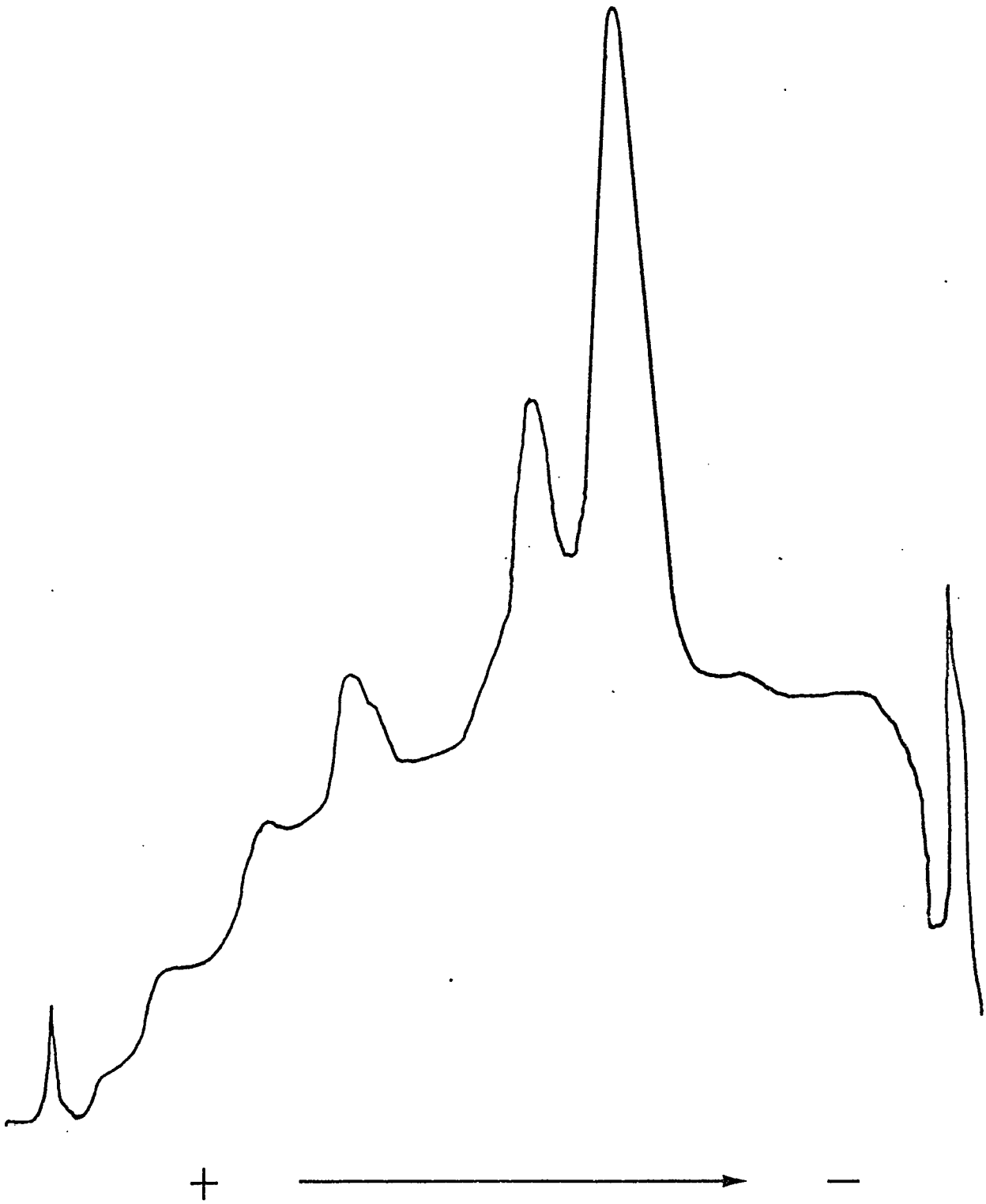


TABLE IV

Nuclear protein yields

Details given in text for each preparation.

Extraction reagent	Source	Method of nuclei breakage	Protein extracted % of total nuclear protein	% of acidic material present
0.4 N H_2SO_4	True chromatin	CO_2	0.58	0
0.4 N H_2SO_4	Chromatin pellet	CO_2	0.50	0
0.4 N H_2SO_4	True chromatin	DTT	0.25	0
0.4 N H_2SO_4	Chromatin pellet	DTT	0.50	0
0.4 N H_2SO_4	True chromatin	Sonication	0.30	0
0.4 N H_2SO_4	Chromatin pellet	Sonication	0.78	0
2 M NaCl	True chromatin	DTT	16.00	50
2 M NaCl + 5 M urea	True chromatin	DTT	30.00	75
1 M $CaCl_2$; TCA precipitation 0.04 N H_2SO_4	True chromatin	DTT	5.00	10
1 M $CaCl_2$; TCA precipitation 0.04 N H_2SO_4	Chromatin pellet	DTT	5.00	10
1 M $CaCl_2$; TCA precipitation 0.04 N H_2SO_4	Chromatin pellet	N_2	5.00	10

Fig. 5 Fractionation of 2 M NaCl extractable protein from true chromatin on Sephadex G-100. Column of 0.9 x 200 cm is eluted with 0.02 N HCl at a constant flow rate of 6 ml/h. Fractions of 2 ml each are collected and monitored at 230 nm. Tubes are combined to fractions as indicated by the dotted lines. Fraction 73-90 is the urea peak.

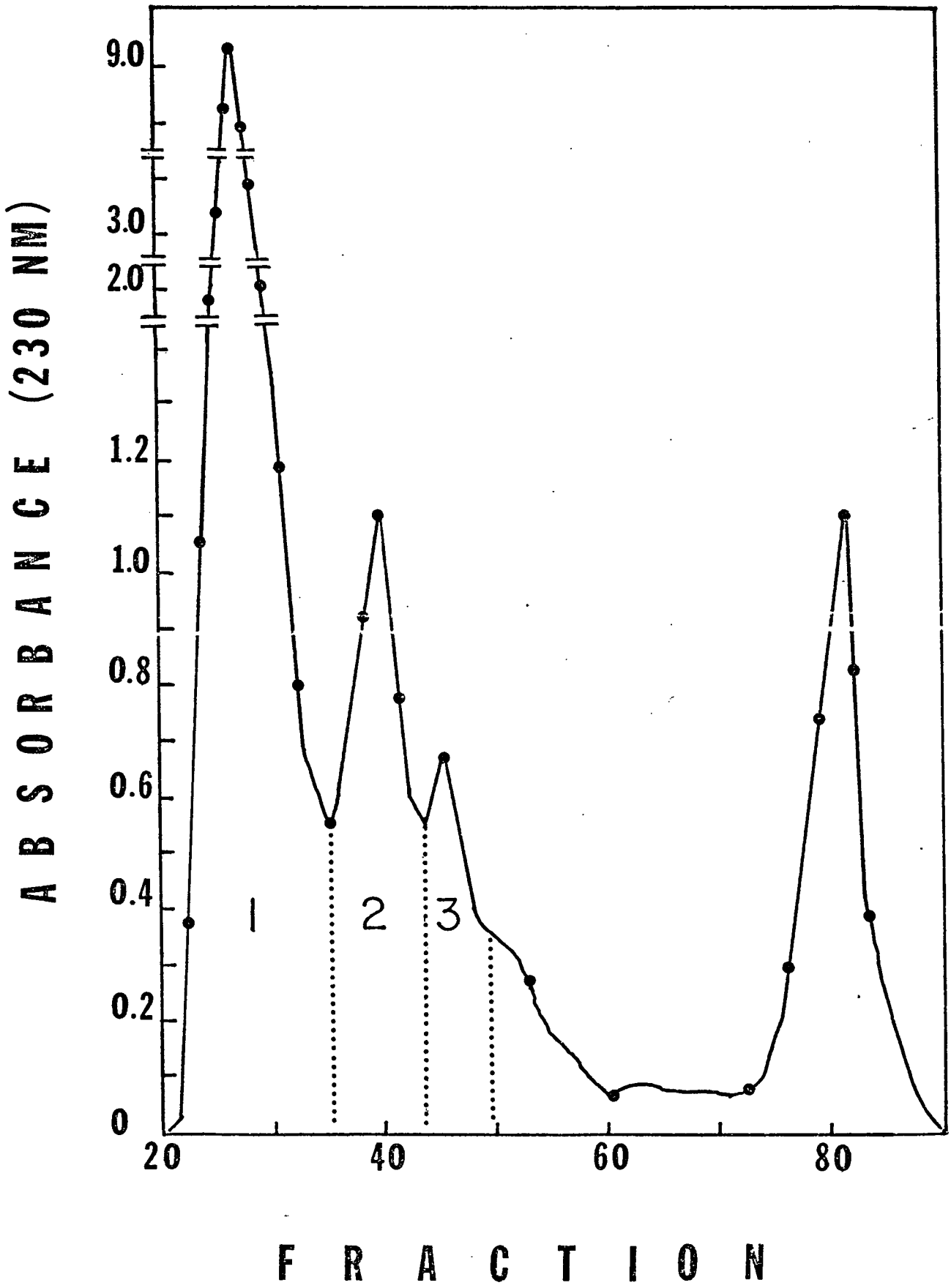
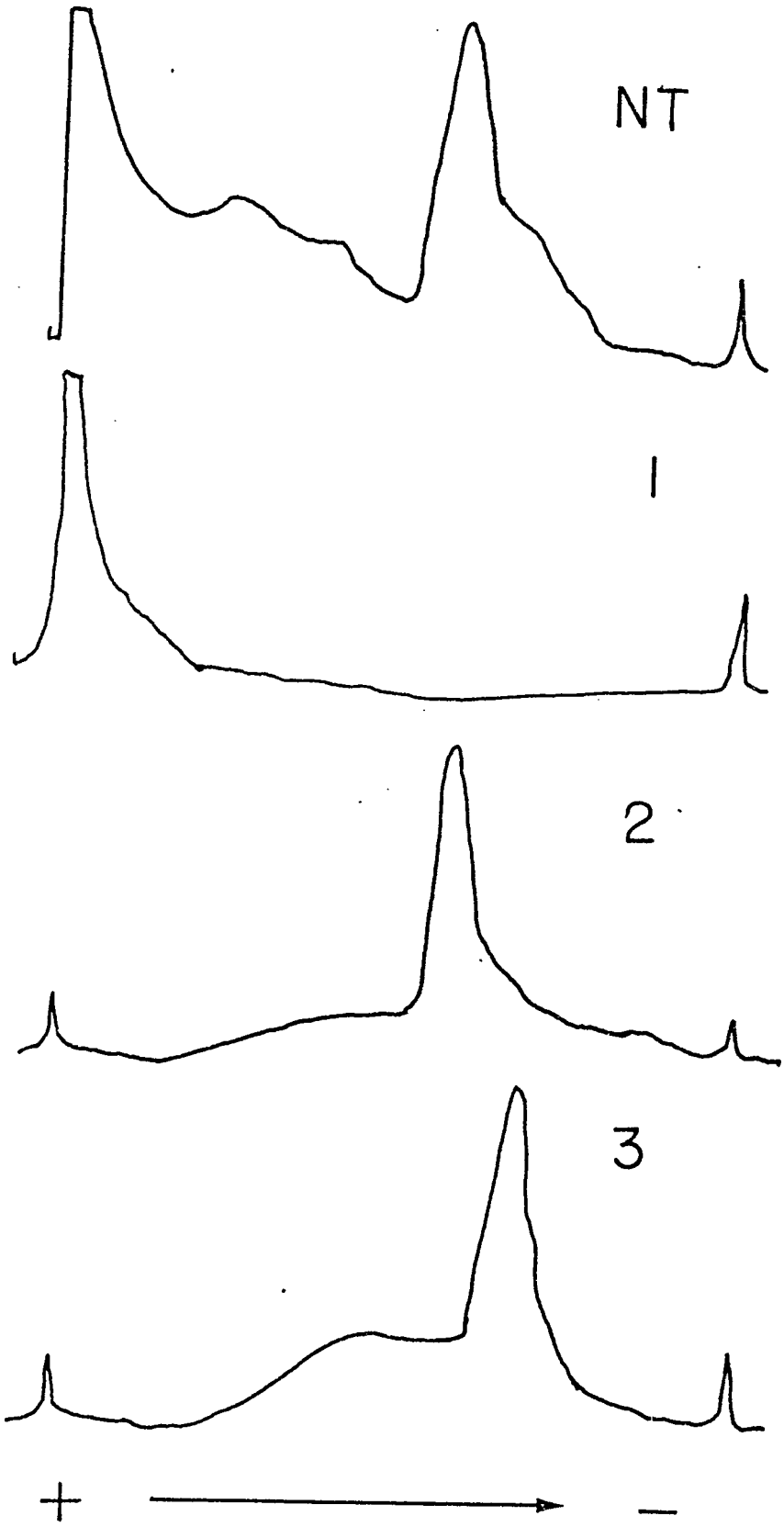

ABSORBANCE (230 NM)
9.0
3.0
2.0
1.2
1.0
0.8
0.6
0.4
0.2
0
1
2
3
20
40
60
80
FRACTION

Fig. 6 Disc electrophoresis of 2 M NaCl extractable protein from true chromatin. Whole (NT) and combined protein fractions (1, 2 & 3) from Sephadex G-100 are subjected to the pH 4.4 disc electrophoresis. Gel scans are made as described in Fig. 3. Direction of electrophoresis is indicated by the arrow.


NT
1
2
3
+
−

material but other minor bands are present. The third fraction is composed of mostly arginine rich histones with quite a bit of contamination from the lysine rich histones. There is no clear cut separation except the first peak, indicating that the different histone fractions are most probably very close in their molecular weight. This may also be due to problems of aggregation.

2 M NaCl + 5 M urea extraction from true chromatin gives a protein preparation with more of the lysine fraction extracted. Fractionation of the total extractable material on Sephadex G-100 gives 4 protein peaks and one urea peak. The A_{230} profile is presented in Fig. 7. Fig. 8 shows the densitometric tracings of the different combined fractions.

Fraction 1 is composed of 90% acidic material and 10% of histone material, most probably the lysine rich fraction. Fraction 2 is composed of the slightly lysine rich material. Fraction 3 gives both the lysine and arginine rich histone fractions. Fraction 4 gives no separation at all. It has all the histone fractions and therefore no gel tracing of this fraction is shown.

Regardless of the nature of the extractable material, Sephadex G-100 gives no clear cut separation. Each fractionated peak is cross contaminated with other fractions. This cross contamination is not due to overloading for when only half the amount of sample is applied the sample kind of contamination is present.

Fig. 7 Fractionation of 2 M NaCl + 5 M urea extractable protein from true chromatin on Sephadex G-100. Column of 0.9 x 200 cm is eluted with 0.02 N HCl at a constant flow rate of 6 ml/h. Fractions of 2 ml each are collected and monitored at 230 nm for protein. Tubes are collected into fractions as indicated by the dotted lines. Fraction 73-80 is the urea peak.

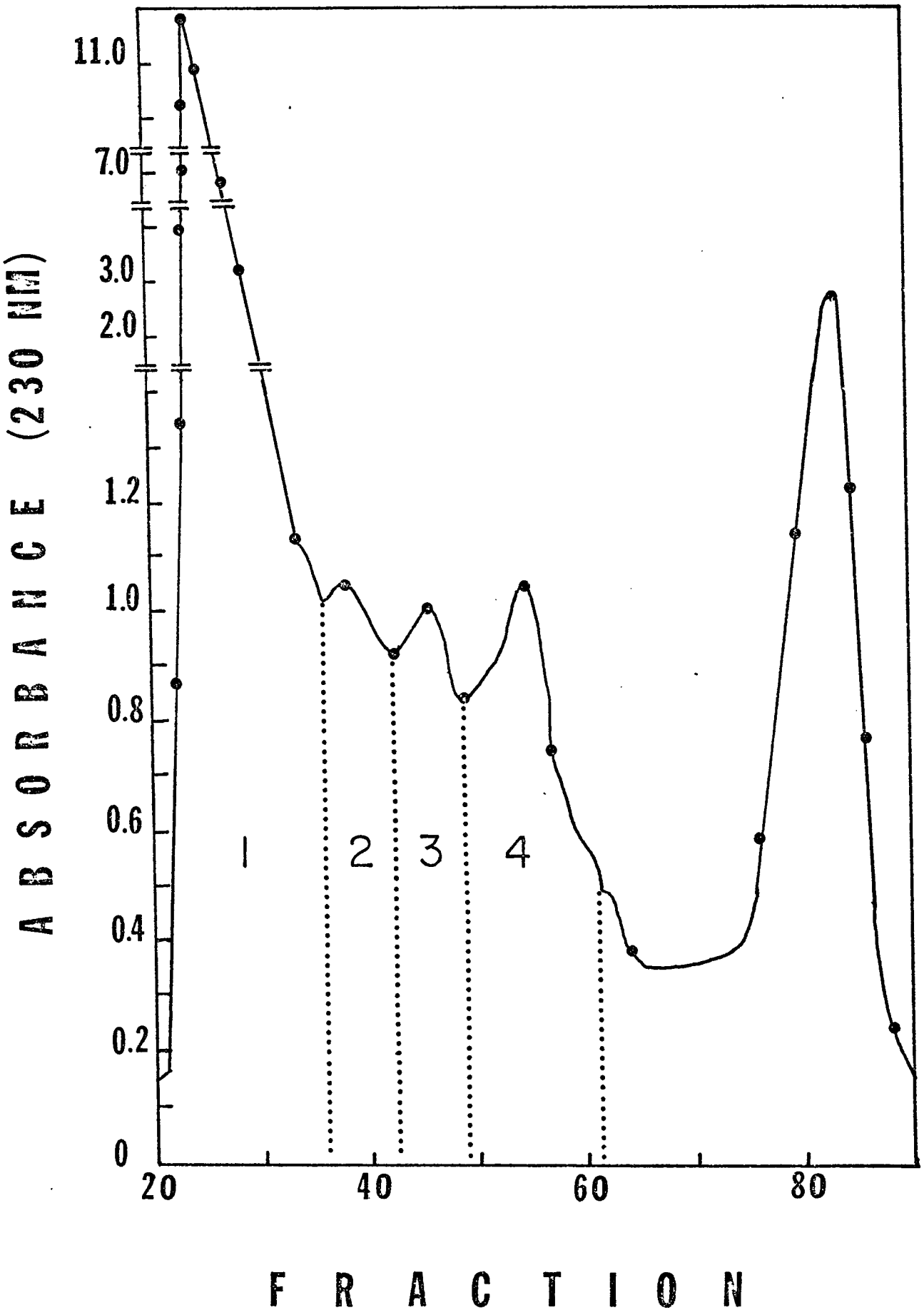
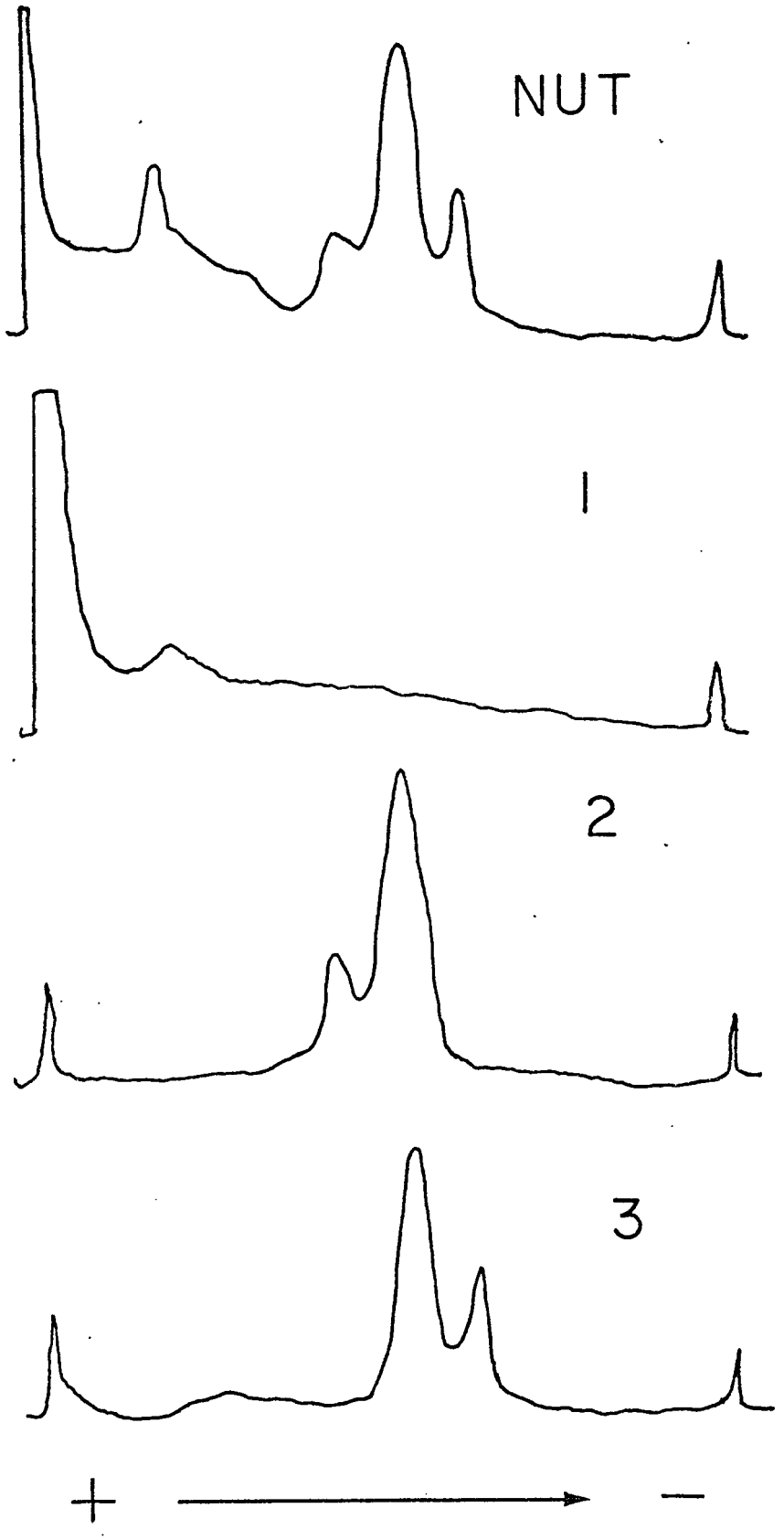

ABSORBANCE (230 NM)
11.0
7.0
3.0
2.0
1.2
1.0
0.8
0.6
0.4
0.2
0
1
2
3
4
20
40
60
80
FRACTION

Fig. 8. Disc electrophoresis of 2 M NaCl + 5 M urea extractable protein from true chromatin. Samples of whole (NUT) and combined fractions (1, 2 & 3) from Sephadex G-100 are subjected to pH 4.4 disc electrophoresis. Gels scans are prepared as described in Fig. 3. Direction of electrophoresis is indicated by the arrow.


NUT
1
2
3
+
−

1 M $CaCl_2$ extracts from chromatin pellet followed by TCA precipitation and 0.04 N H_2SO_4 re-extraction gives a protein sample with all the 5 main histone bands with only about 10% acidic material present. The fastest moving electrophoretic band (slightly arginine rich fraction) is present in a relatively small amount as compared to Calf thymus histones. Fractionation of the $CaCl_2$ extractable material on Sepahdex G-75 gives 3 protein peaks and one urea peak. The A_{230} profile is given in Fig. 9. Fig. 10 gives the densitometric tracings of the different fractions.

Fraction 1 is composed of mostly lysine rich histone with some of the acidic material present. Fraction 2 has most of the slightly lysine rich histone with some contamination from the arginine histones. Fraction 3 has most of the arginine histone but a detectable amount of slightly lysine rich histone is present.

Sephadex G-100 or G-75 as demonstrated is incapable of giving a clear cut separation. A summary of the Sephadex fractionation is presented in Table V. From the results obtained, there is an implication that the different histone fractions involved are most probably very close in their molecular weight and therefore cannot be separated solely on this basis.

An anionic Sephadex, SPC 50, which separates proteins on the basis of both their size and charge is used. Column of 0.9 x 200 cm equilibrated with 0.01 N HCl is loaded with one of the combined fractions from Sephadex G-75. Elution is achieved by use of a linear gradient of 0 to 1 M NaCl in 0.01 N HCl. Two A_{230} peaks

Fig. 9. Fractionation of 1 M $CaCl_2$, TCA precipitated and 0.04 N H_2SO_4 re-extracted protein from chromatin pellet on Sephadex G-75. Column of 0.9 x 200 cm is eluted with 0.02 N HCl at a constant flow rate of 6 ml/h. Fractions of 2 ml each are collected and monitored at 230 nm for protein. Tubes are combined to fractions as indicated by the dotted lines. Fraction 165-180 is the urea peak.

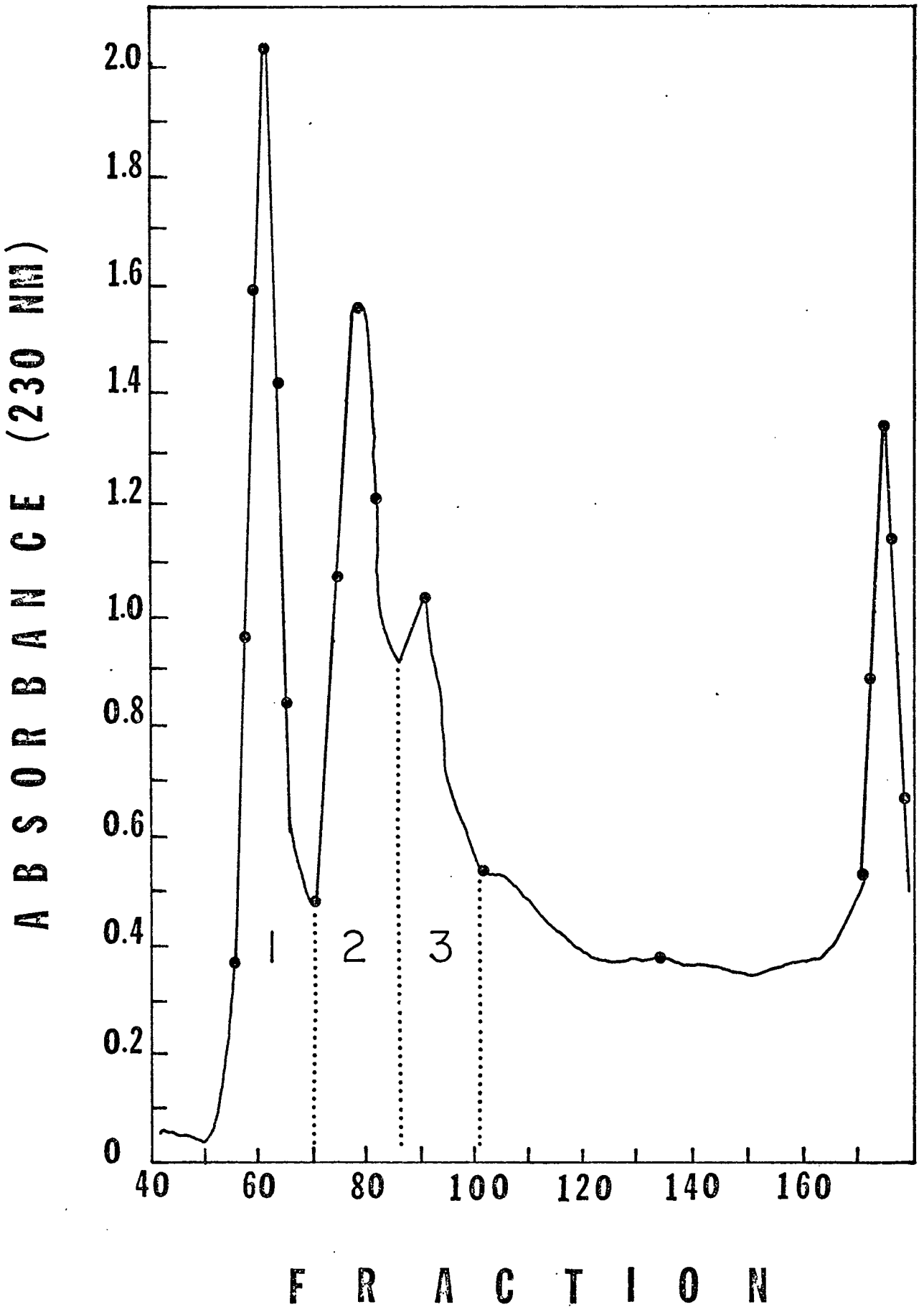

ABSORBANCE (230 NM)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
1
2
3
40
60
80
100
120
140
160
FRACTION

Fig. 10. Disc eletrophoresis of 1 M $CaCl_2$, TCA precipitated and 0.04 N H_2SO_4 re-extracted protein from chromatin pellet. Samples of whole (CCP) and combined fractions (1, 2 & 3) from Sephadex G-75 are subjected to pH 4.4 electrophoresis. Gels scans are prepared as described in Fig. 3. The direction of electrophoresis is indicated by the arrow.

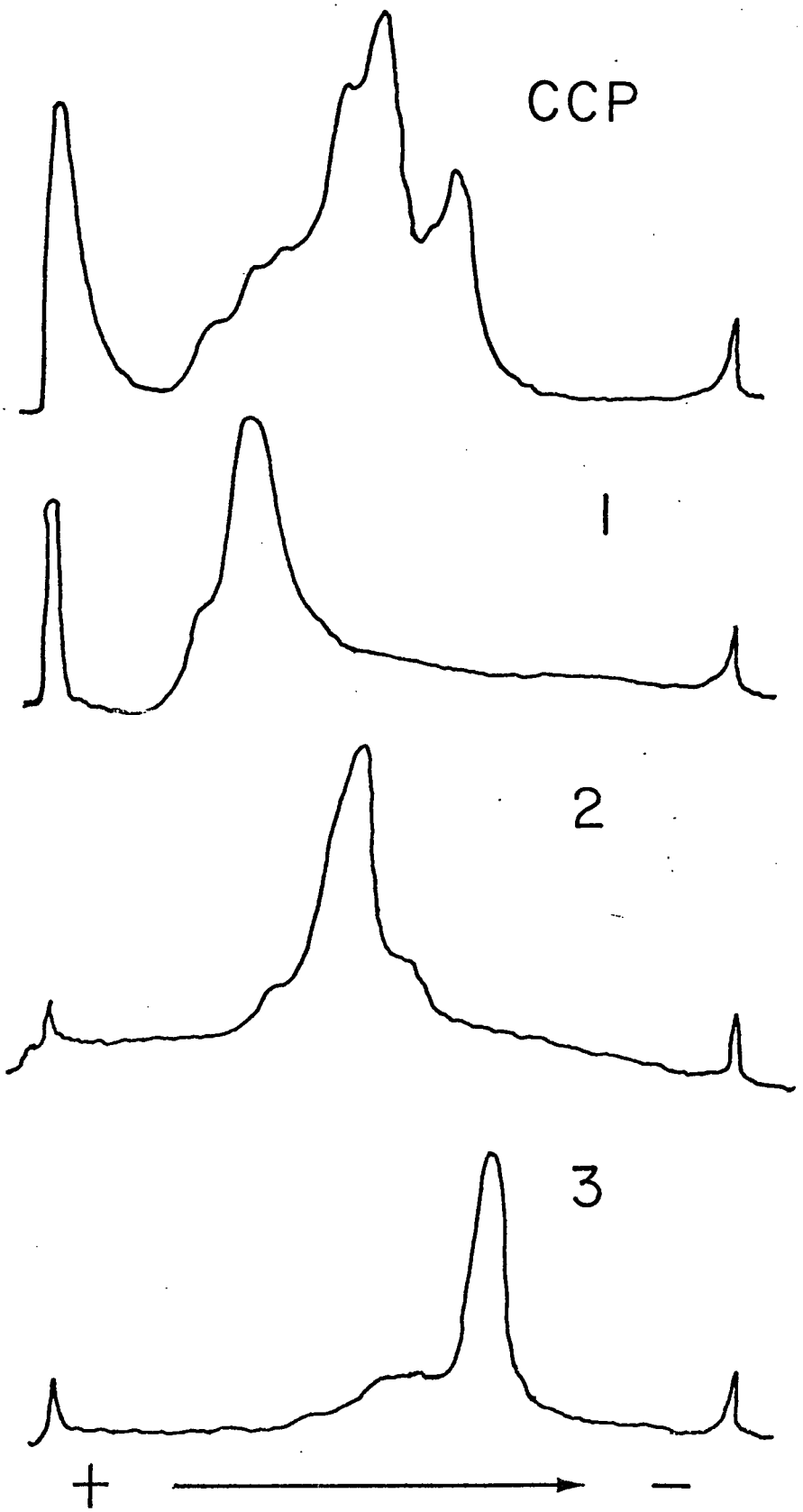

CCP
1
2
3
+
−

TABLE V

Sephadex column chromatography.

Details given in text.

Extraction reagent	Sephadex	Peak I Total A_{230}	%	Peak II Total A_{230}	%	Peak III Total A_{230}	%	Peak IV Total A_{230}	%
2 M NaCl	G-100	24.90	81.00	6.87	12.97	31.67	5.98	-	-
2 M NaCl + 5 M urea	G-100	48.00	71.00	6.00	8.8	5.6	8.25	8.25	12.00
1 M $CaCl_2$; TCA precipitation; 0.04 N H_2SO_4	G-75	14.64	35.00	15.24	37.00	11.91	28.00	-	-

are observed, but when each fraction is subjected to disc electrophoresis, no separation is detected

CM cellulose chromatography (26) is tried next. One of the combined fractions from Sephadex G-75 is loaded on a column of 1 x 12 cm. The column is eluted first with a linear gradient of 0 to 1 M NaCl in sodium acetate buffer pH 4.4(0.1 M in acetate). Second elution is achieved by use of 0.01 N HCl at pH 2.5 and finally with 0.02 N HCl at pH 2.0. Each elution step gives a clear cut A_{230} peak but when subjected to disc electrophoresis, no separation is detected.

The results further imply that the different histone fraction besides being very similar in their molecular weight, also have very little difference in their overall charge. There is also the strong possibility that aggregation of the different fractions occurs, thereby changing the property of each fraction and preventing effective separation.

Fractionation based on solubility. The method of Johns (13) is first applied to the unfractionated protein samples. Due to the small sample size (mg instead of gm quantities) results obtained are not satisfactory. The modified method of Oliver et al (14) is also used since it was developed for mg instead of gm of starting materials. This method is capable of fractionating histones obtained from any animal source into five major groups. However, result-

ing fractions shown in Fig. 11 to Fig. 14 are still not of high purity. There is the presence of minor bands in each of the fractionated samples. Also the yield is very low. With a starting sample of 60 mg, each of the four fractions is not more than 3 mg.

Fig. 11 shows that fraction I (very lysine rich) has 3 bands instead of the two bands as found in the Calf thymus standard from Worthington. Fig. 12 shows that fraction IIb2 (slightly lysine rich) has one major band plus one minor band, instead of the two major bands found in the standard. Fig. 13 of fraction III (arginine rich) shows that the fractionated sample is highly heterogenous, composing of at least 3 bands, while the Calf thymus standard has only one major band. Fig. 14 of fraction IIb1 + IV (slightly lysine and arginine rich) shows one major band with 2 minor bands, while the standard shows only 2 major bands.

Fig. 15 with all of the four fractionated samples present, clearly demonstrated the presence of minor bands in each of the fractions. The Physarum histones must have different solubility properties than the mammalian histones.

On disc electrophoresis the histone pattern of Physarum is very similar to that of Calf thymus histones. But results obtained so far from column chromatography and solubility fractionation show that the Physarum histones behave very differently from the Calf thymus histones. In order to see

Fig. 11 Disc electrophoresis of very lysine rich (I) histone fraction. (a) is the calf thymus standard purchased from Worthington with its two major bands. (b) is the isolated fraction from *P. flavicomum* nuclear protein with its three major bands. The slow moving first band is absent in the standard obtained from Calf thymus.

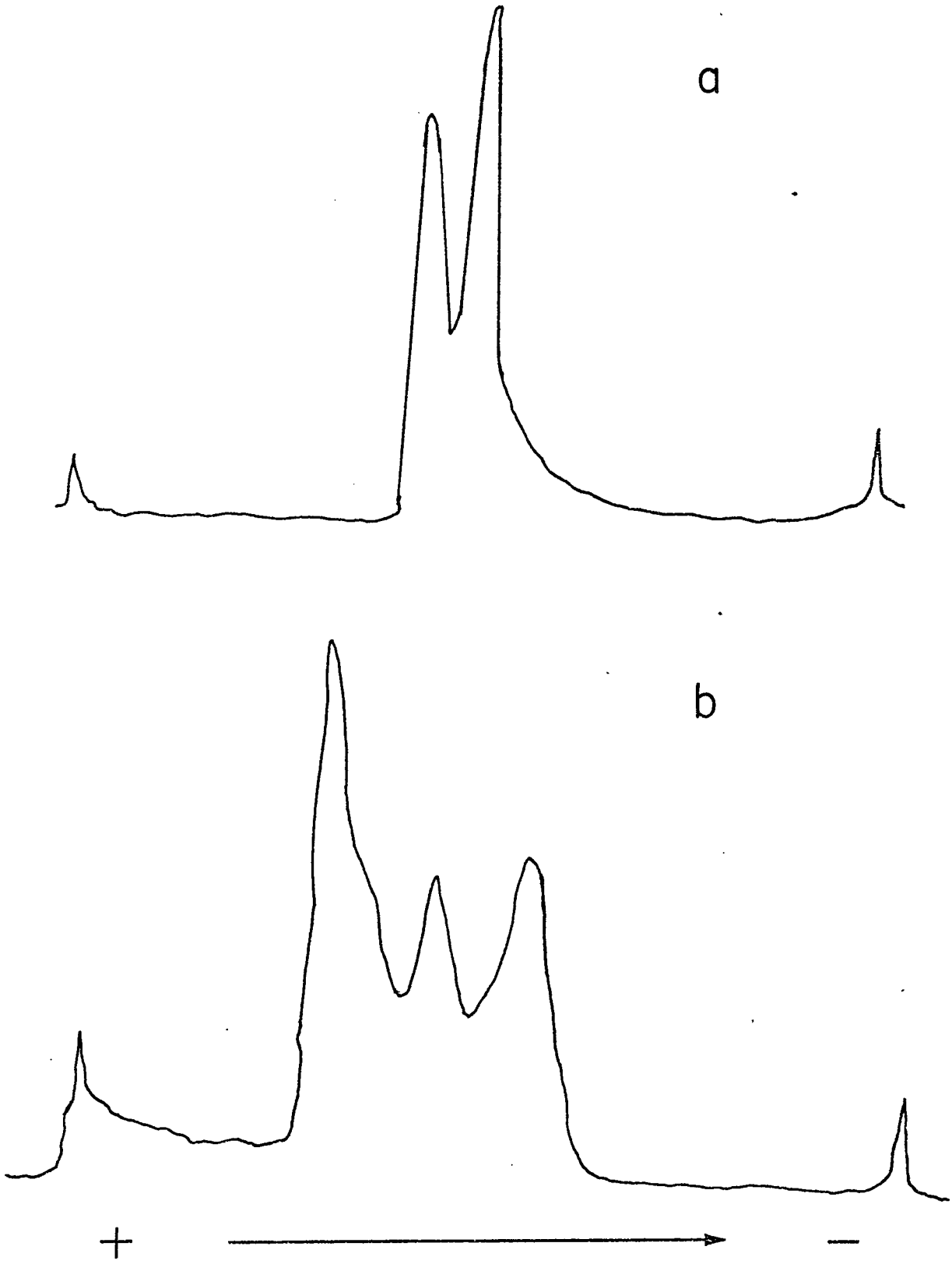

a
b
+
−

Fig. 12. Disc electrophoresis of the slightly lysine rich (IIb2) fraction. (a) is the Worthington standard from calf Thymus with its two major bands. (b) is the isolated fraction from *P. flavicomum* nuclear protein with only one major band and a very slight minor band.

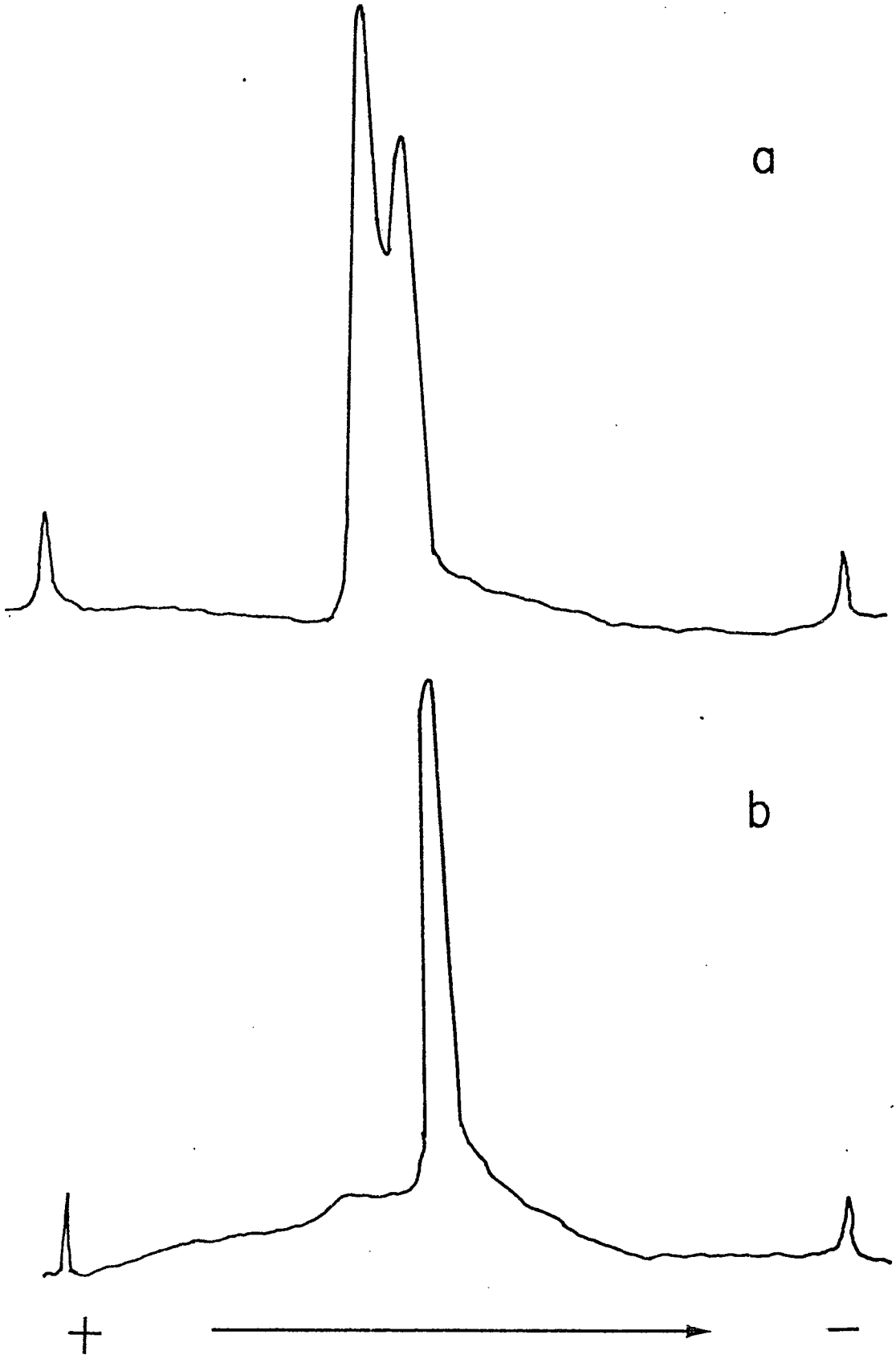

a
b
+
−

Fig. 13. Disc electrophoresis of very arginine rich (III) histone fraction. (a) is the Worthington standard from calf thymus with its one major band. (b) is the isolated fraction from *P. flavicomum* nuclear protein with its three major bands.

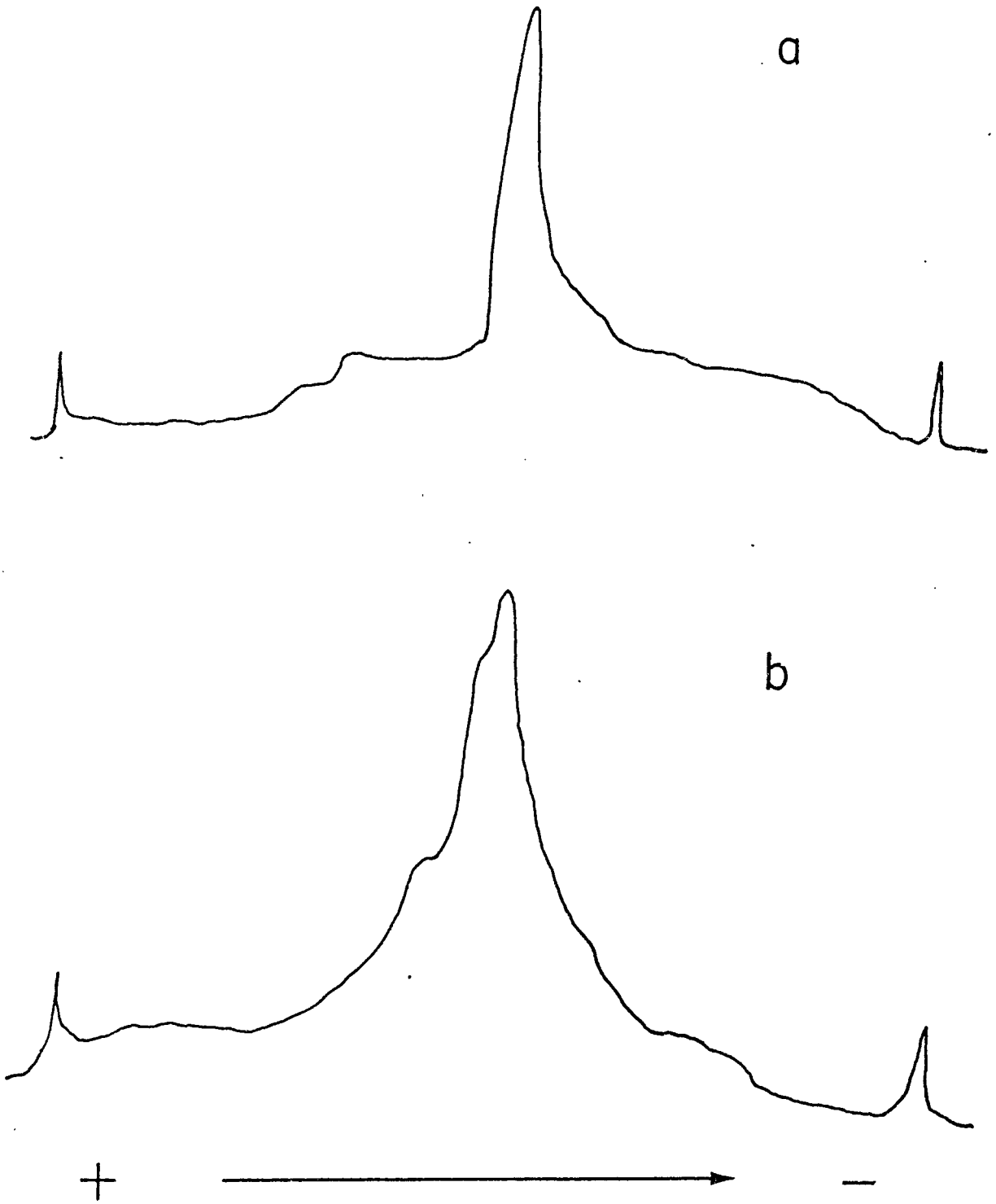

a
b
+
−

Fig. 14 Disc electrophoresis of slightly lysine and arginine rich (IIb1 + IV) histone fraction. (a) is the Worthington standard from calf thymus with its two major bands. (b) is the isolated fraction from *P. flavicomum* nuclear protein with one broad major band and two minor bands.

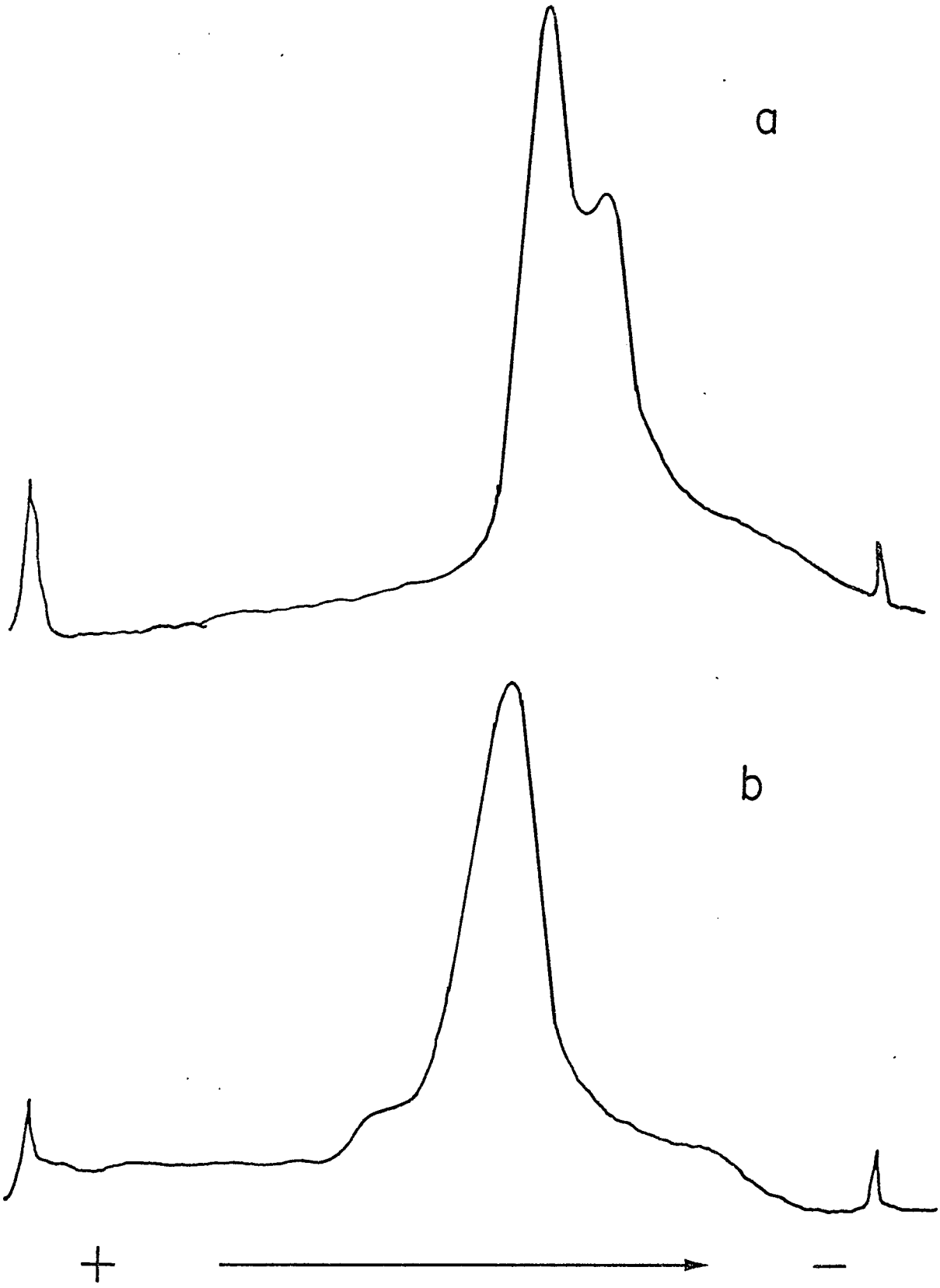
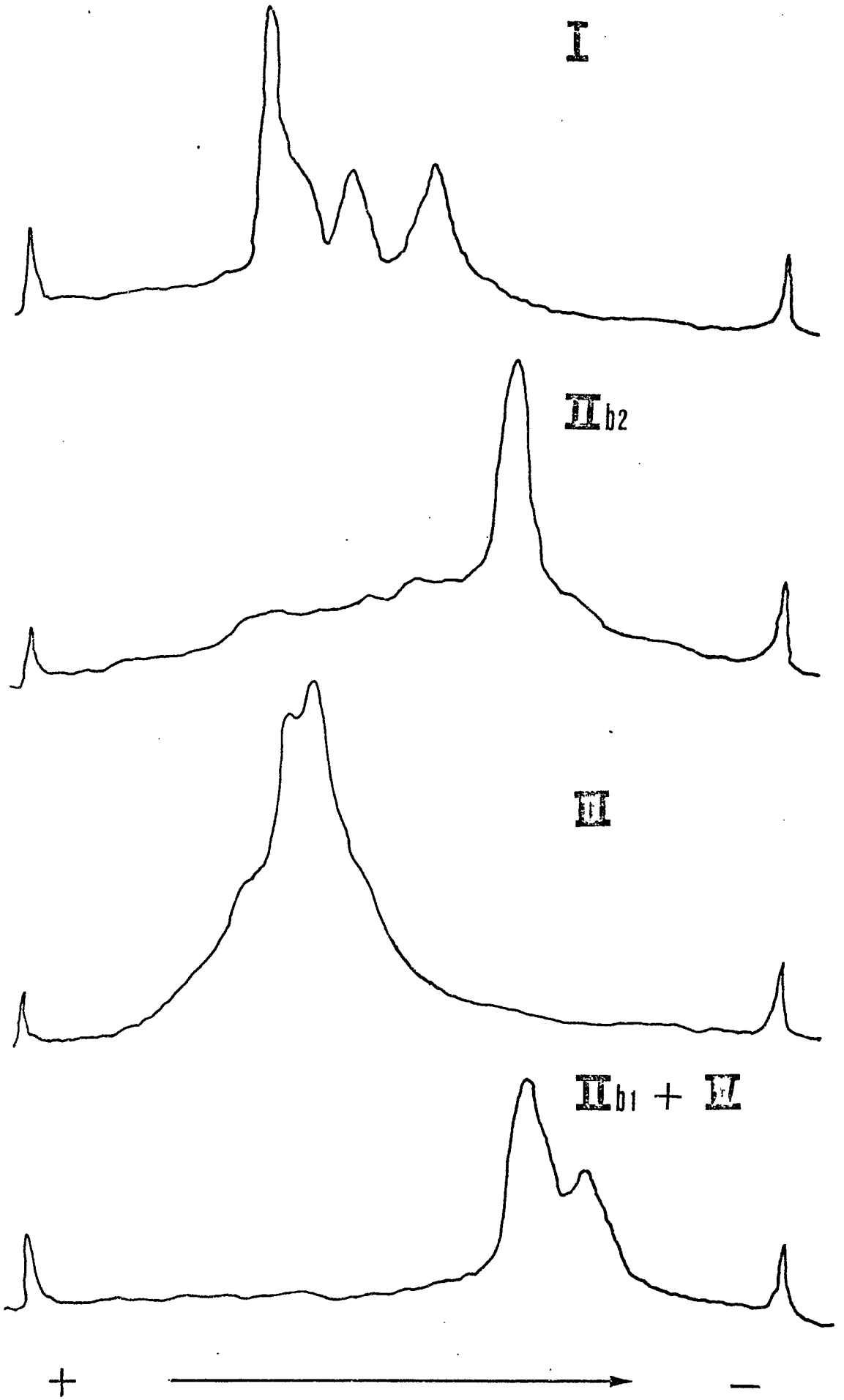

a
b
+
−

Fig. 15 Disc electrophoresis of all four isolated histone fractions from *P. flavicomum* nuclear protein.

I	(very lysine rich)	3 major bands
IIb2	(slightly lysine rich)	1 major band + minor bands
III	(very arginine rich)	3 major bands
IIb1	(slightly lysine rich) +	1 major band +
IV	(slightly arginine rich)	minor bands


I
IIb2
III
IIb1 + IV
+
−

if the Physarum histone is indeed the same or different from Calf thymus, an amino acid analysis is therefore performed on the whole histone sample.

Amino acid analysis of whole nuclear protein. Unfractionated 1 M $CaCl_2$, TCA precipitated and 0.04 N H_2SO_4 re-extracted material is first subjected to preparative electrophoresis to remove the acidic material present. Fig. 16 shows the densitometric tracings of the sample before and after preparative electrophoresis. The sample after electrophoresis, that is free of the acidic material, is then used for amino acid analysis.

Aliquots of the protein material are treated three different ways. One is hydrolysed directly and loaded on the Amino acid analyser for total amino acid analysis. A second sample is subjected to performic acid oxidation, then hydrolysed for the determination of cysteic acid. The results of the amino acid analysis are given in Table VI.

A third sample is dissolved in 0.1 N NaOH to be analyzed spectrophotometrically to determine the absence or presence of tryptophan. The scans are shown in Fig. 17. The maximum absorbance of the Physarum nuclear protein sample is at 293 nm, with no peak at the 282 nm tryptophan region. The absence of tryptophan confirms that the sample is composed of histone with no significant acidic protein contamination.

Fig. 16. Disc electrophoresis of whole unfractionated nuclear protein from *P. flavicomum* before and after preparative electrophoresis. (a) is the nuclear protein before preparative electrophoresis with the acidic material present on top of gel. (b) is the nuclear protein after preparative electrophoresis which contains only a trace amount of the acidic material.

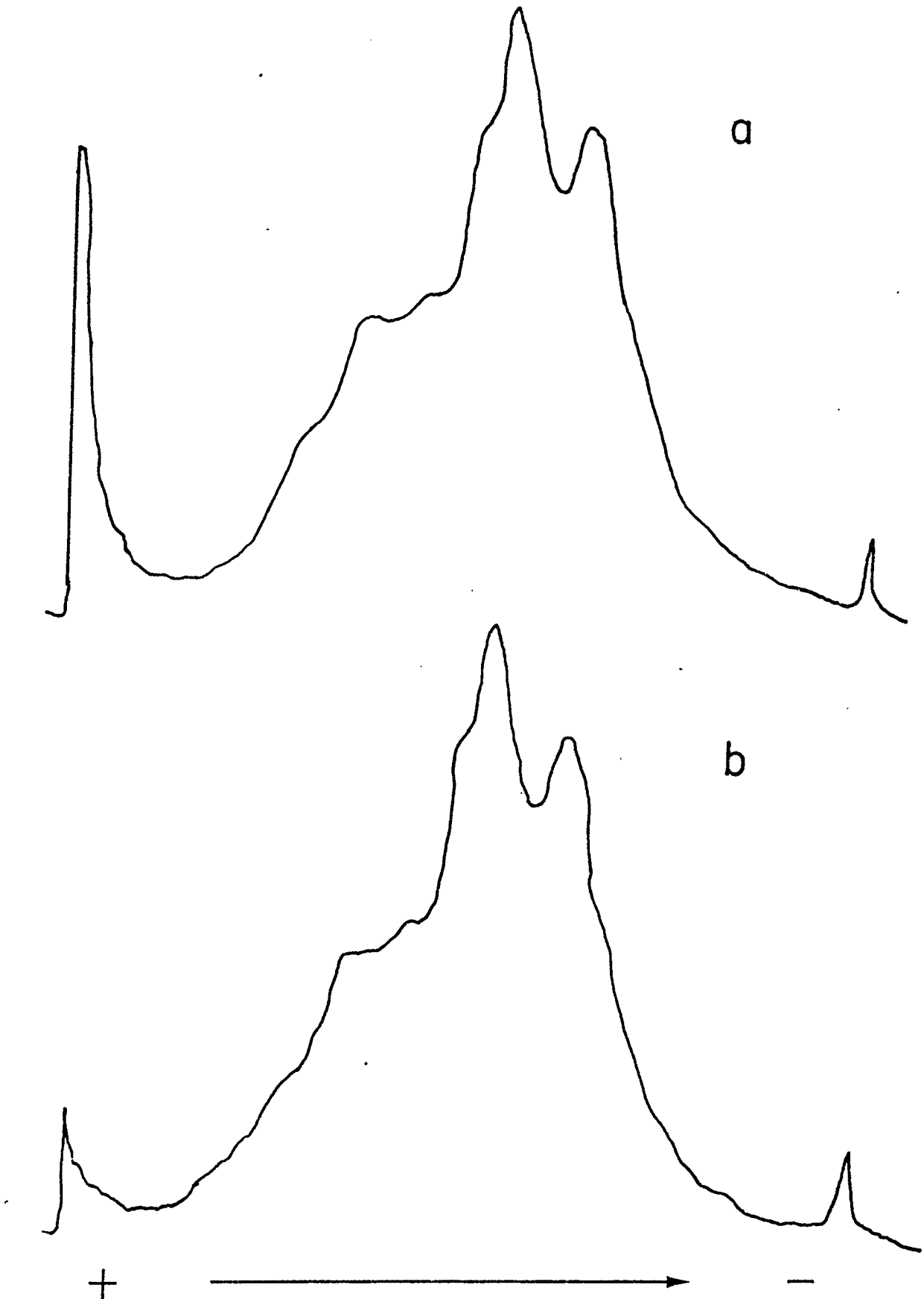

a
b
+
−

TABLE VI

Amino acid composition of whole histone from *Physarum flavicomum*, *P. polycephalum*, Calf thymus and *Tetrahymena pyriformis*

Amino acid	*P. flavicomum* nm/mg protein	*P. flavicomum* moles %	*P. polycephalum* moles % (27)	Calf thymus moles % (28)	*T. pyriformis* moles % (29)
Aspartic Acid (A)	240	6.1	7.5	4.9	7.5
Threonine	215	5.4	6.1	5.3	6.3
Serine	261	6.9	6.1	5.0	7.8
Glutamic Acid (A)	385	10.1	11.2	8.4	9.1
Proline	151	3.8	6.6	4.8	4.0
Glycine	507	13.3	7.7	8.7	7.4
Alanine	281	7.5	9.7	13.6	10.0
Valine	256	6.6	5.7	6.2	6.0
Cystine/2 *	trace	trace	-	trace	0.0
Methionine	28	0.7	1.0	0.9	1.2
Isoleucine	198	5.0	3.9	4.4	4.1
Leucine	286	7.3	6.6	7.7	6.2
Tyrosine	131	3.3	2.0	2.3	1.6
Phenylalanine	76	1.9	2.3	1.7	2.9
Lysine }	496	12.8	14.5	14.9	16.3
Histidine } (B)	146	3.7	2.1	2.3	2.6
Arginine }	215	5.6	7.1	8.9	7.0
Acidics (A)	625	16.2	18.7	13.3	16.6
Basics (B)	857	22.1	23.7	26.1	25.9
B/A		1.4	1.3	2.0	1.6
Lys/Arg		2.3	2.0	1.7	2.3

* Determined as cysteic acid.

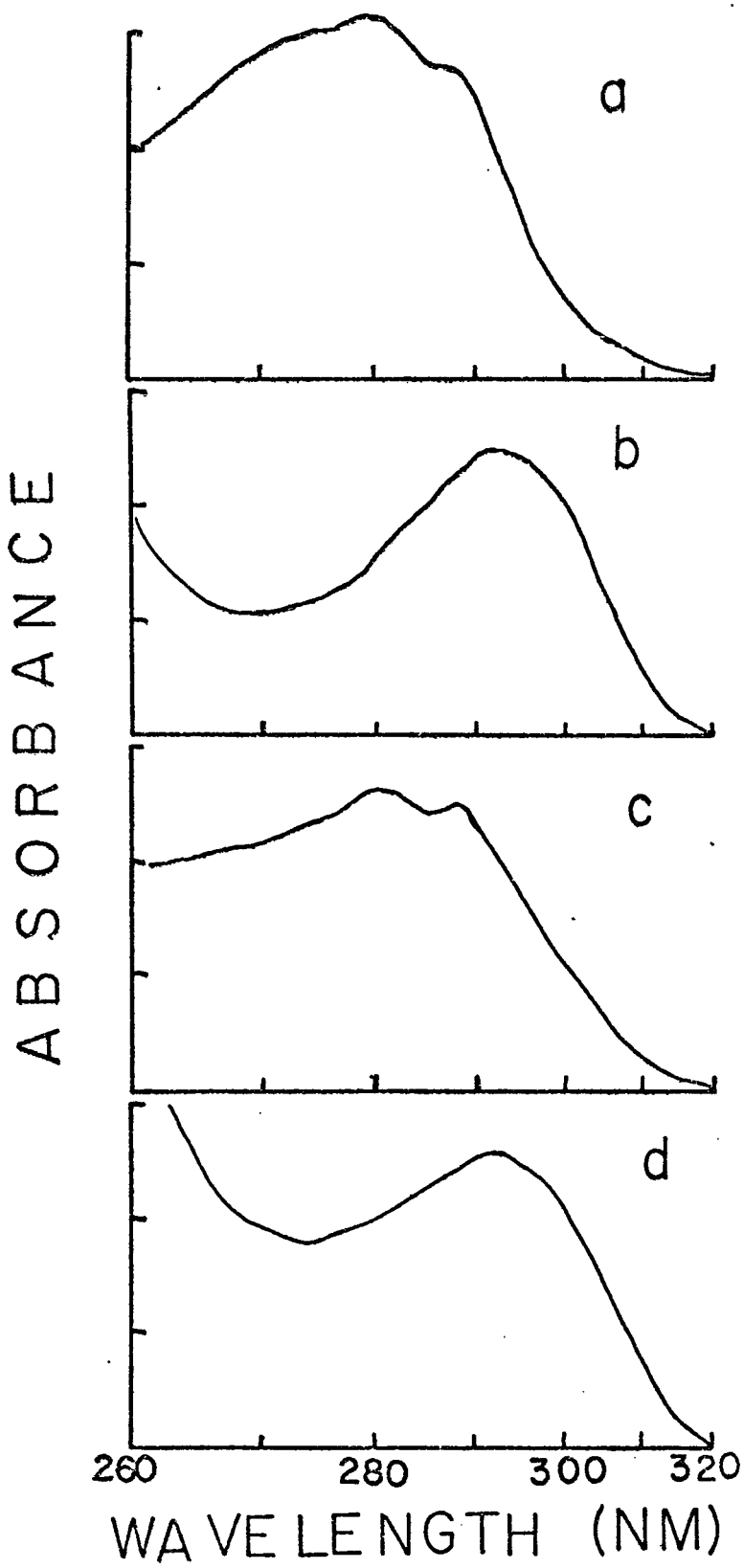
Fig. 17. Spectrophotometric scans for detection of tryptophan and tyrosine.

(a) Tryptophan alone with its maximum absorbance at 282 nm.

(b) Tyrosine alone with its maximum absorbance at 293 nm.

(c) Tryptophan + tyrosine at a molar ratio of 1:1. Both maximum absorbances are shifted. Tryptophan to 280 nm and tyrosine to 289 nm.

(d) Scan of nuclear protein from *P. flavicomum* that is void of the acidic material. Maximum absorbance is at 293 nm.


a
b
c
d
ABSORBANCE
260
280
300
320
WAVELENGTH (NM)

Peak 1 from Sephadex G-100 of the 2 M NaCl extractable protein from true chromatin is acidic in nature. This material upon pH 4.4 electrophoresis stays on top of the gel. The total amino acid composition as shown in Table VII resembles that of P. polycephalum plasmodial actin and miosin A. Upon SDS electrophoresis, a single band is detected. the molecular weight of this acidic material is found to be 41,000 $\pm$ 4,000 daltons. The results of the SDS gel electrophoresis is presented in Fig. 18.

TABLE VII

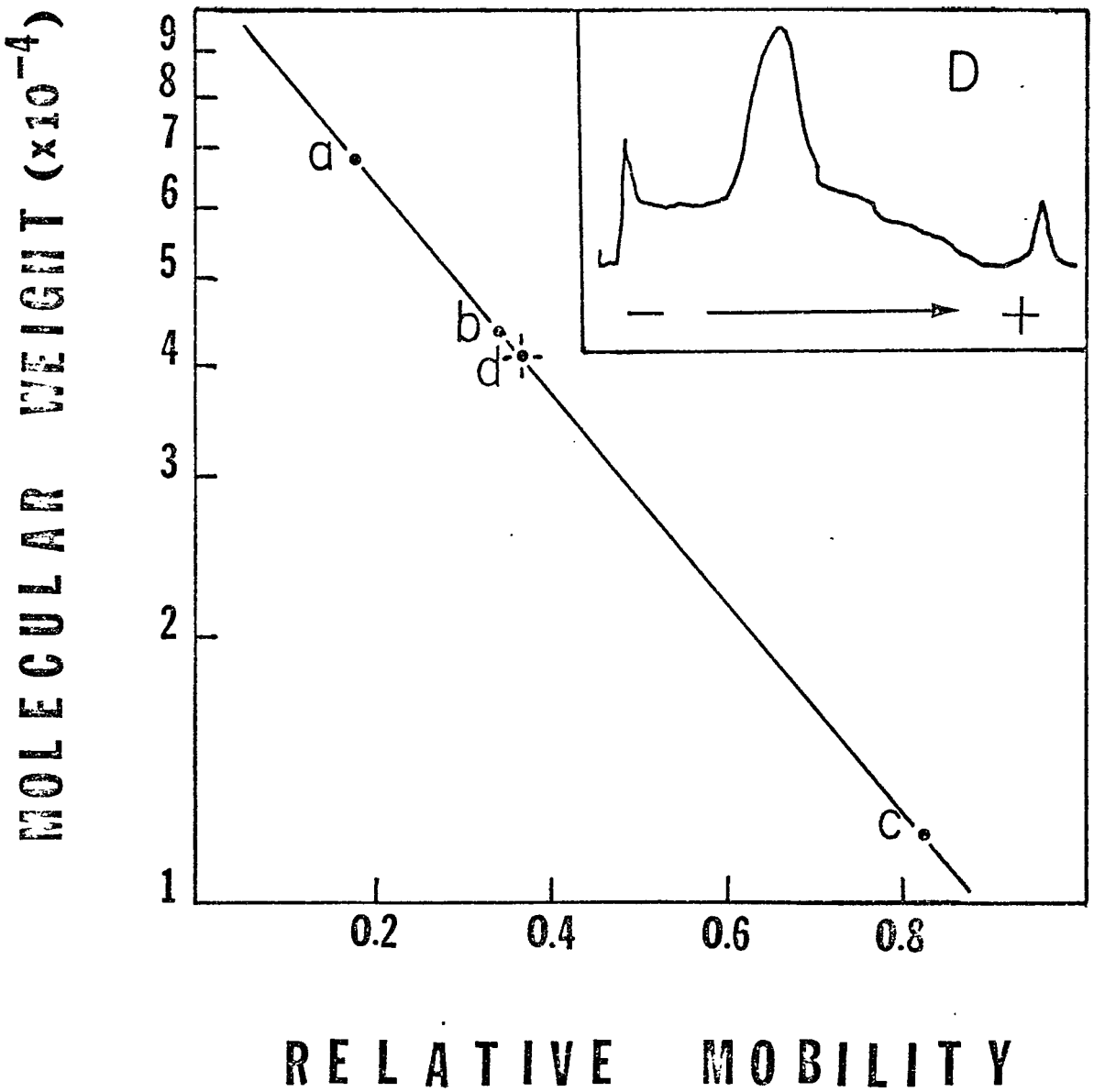
Amino acid composition of Peak 1 of the 2 M NaCl extractable protein from *Physarum flavicomum* fractionated by Sephadex G-100 as compared to the amino acid composition of actin and myosin A from *P. polycephalum*.

Amino acid	*P. flavicomum* Peak 1 (G-100) moles %	*P. polycephalum* Actin moles % (30)	*P. polycephalum* Myosin A moles % (31)
Aspartic acid (A)	10.0	10.0	11.0
Threonine	5.8	7.3	4.5
Serine	5.5	6.7	4.1
Glutamic acid (A)	11.6	13.7	18.0
Proline	5.5	7.3	5.0
Glycine	12.4	8.9	5.2
Alanine	8.5	8.2	8.8
Valine	3.6	3.8	4.6
Cystine/2	trace	trace	trace
Methionine	1.2	3.4	2.0
Isoleucine	5.0	4.1	3.8
Leucine	9.0	7.3	9.3
Tyrosine	2.6	3.8	1.8
Phenylalanine	4.4	3.5	2.8
Lysine	7.6	4.9	10.9
Histidine (B)	2.3	2.0	2.2
Arginine	4.8	4.8	6.2
Acidics (A)	21.6	23.7	29.0
Basics (B)	14.7	11.7	19.3
A/B	1.5	2.0	1.5

Fig. 18. Molecular weight determination by SDS gel electrophoresis. Peak 1 of the 2 M NaCl extractable protein fractionated by Sephadex G-100 is subjected to 10% acrylamide, 0.1% SDS gel electrophoresis at pH 7.2 Standards used as references are:

(a)	Bovine serum albumin	68,000 daltons
(b)	Ovalbumin	43,000 daltons
(c)	Cytochrome C	12,000 daltons

Unknown sample (d) is found to have a molecular weight of 41,000 $\pm$ 4,000 daltons. (D) is a densitometric tracing of the unknown sample gel.


MOLECULAR WEIGHT (x10^{-4})
9
8
7
6
5
4
3
2
1
a
b
d
c
D
−
+
0.2
0.4
0.6
0.8
RELATIVE MOBILITY

DISCUSSION

Rhythmical protoplasmic streaming is observed in the plasmodial stage of the Myxomycetes. Two contractile proteins, actin and myosin, are thought to be responsible for the cytoplasmic movements (32). Initial studies performed on plasmodial actin and myosin A by Hatano (30, 31) shows that these two proteins are easily extracted by use of a neutral salt solution. Whenever P. flavicomum nuclei are extracted with NaCl or $CaCl_2$, an acidic protein is co-extracted with histones. With NaCl or NaCl + urea, at least 50% of the extractable material is acidic in nature. With the $CaCl_2$ extraction, additional treatment by TCA and 0.04 N H_2SO_4 eliminates most but not all of the acidic protein.

This acidic protein which is slightly retarded by Sephadex G-100 has been found to have an molecular weight of 41,000 $\pm$ 4,000. This data agrees with the results obtained by Adelman (33) for molecular weight estimation on plasmodial actin (43,000 - 48,000 daltons) and myosin A (46,000 daltons). The amino acid composition is very close to the results reported by Hatano (30) on plasmodial actin except for concentrations of the amino acids glutamic acid, glycine, lysine and methionine which differ by 2 to 3.5%. The same similarities and differences hold true when these data are compared to Hatano's results on plasmodial myosin A (31). P. polycephalum plasmodial actin has an acidic/basic amino acid ratio of 2.0, and 1.5 for plasmodial myosin A. Acidic protein from P. flavicomum has an acidic/basic ratio of 1.5. The acidic

P. flavicomum protein therefore may be an actin-myosin like polymer, although the identification is only tentative.

The presence of this protein may very well explain the stability and resistance to breakage of isolated plasmodial nuclei. This contractile protein may be associated with the nuclear membrane thus giving the membrane a flexible nature. The nuclear membrane can therefore easily accomodate changes in osmotic pressure. Instead of bursting, the membrane has the capacity to swell or contract. This is indeed the case when nuclei are subjected to changes in osmotic pressure. Very high pressure, either by use of the French press or cell disruption bomb, is needed to get some breakage of the nuclei.

DTT, a reducing agent for disulfide bonds, is the only reagent capable of causing complete nuclear and nucleolar breakage. The incubation with DTT has to be at a relatively high pH of 9.2 and also at room temperature in order to achieve complete breakage. The contractile actin-myosin-like protein could be associated with the nuclear membrane protein by some disulfide linkage although the low cysteine content of the acidic protein argues against extensive disulfide crosslinking. Once this linkage is reduced by the presence of DTT, the nuclear membrane becomes fragile and bursts. Alternatively the stability of the nuclear membrane could be due to disfulfide linked membrane proteins, without involvement of the actin-myosin protein.

The exact role of this actin-myosin material has not been established. The synthesis of a nuclear protein in G2 phase is reported by Jockusch (34). By use of radioactive amino acids, this protein is found to be present in the nucleus of P. polycephalum only during the G2 phase. This protein is further characterized as an acidic protein with an molecular weight of 44,000 (35). This protein is synthesized all through the cell cycle, but can only be detected in the nucleus in the G2 phase. When compared with purified slime mold cytoplasmic actin, this G2 protein has the same electrophoretic mobility and both proteins are precipitated by vinblastine sulfate. This nuclear protein is therefore identified as actin-like material. This contractile protein could be synthesized in the cytoplasm then transported into the nuclei in the G2 phase just before mitosis. It is also possible that the nuclear actin-like material is synthesized exclusively in the nuclei during the G2 phase.

Recent finding by Jockusch et al (36) have demonstrated the presence of actomyosin filaments during the mitotic processes. Two theories have been hypothesized. One is that actomyosin may constrict the nuclear membrane during telophase in intranuclear mitosis and the other is that actomyosin may be involved in the movement of chromosomes during mitosis. Actomyosin is probably involved in the mitotic process but the exact role cannot be established until more specific data are obtained.

Of all the macromolecules present in the nucleus, the biosynthesis and turnover of DNA and histone has attracted the most attention. Preliminary studies by Stedman and Stedman (2) have shown that histones may function as gene repressors. Presently histones are considered to be non-specific repressors which can interact with any region of DNA to reduce the template activity of that region. They are considered to be structural elements important to the integrity of the chromosomes and they may serve to protect chromosomes from various types of damage. The attachment of histones to DNA has been found to occur principally through the formation of electrostatic linkages between the amino groups of histone and the phosphate group of DNA. In vitro, this linkage can be dissociated either by use of dilute acid or high concentration of salt.

Mohberg and Rusch (5) have demonstrated that the histone to DNA ratio remains constant during the cell cycle of P. polycephalum with slightly more histone present during mitosis. The biosynthesis of all the histones contained even after DNA replication is blocked by 5-flourodeoxyuridine. Histone synthesis in the absence of DNA replication continues for an extended period of time and results in a final three fold excess of histones over the DNA. The ratios of the individual histones change with the physiological and reproductive states of the culture.

No attempt was made to obtain synchronous cultures for the isolation of histone from P. flavicomum nuclei. Microscopic

examination of isolated nuclei indicated that only about 1% of the population is at mitosis. The presence of the G2 specific actin-myosin material would imply that many of the nuclei used must be in G2 phase. G2 phase is needed under normal conditions to permit time for substances essential for mitosis to accrue in the organism until they reach a level that induces mitosis.

Electrophoretic band 1 most probably is a lysine-rich histone. Mohberg and Rusch (6) have shown that the slow moving band 1 which is absent in Calf thymus histones, is the one with the observed change during the mitotic cycle of _P. polycephalum_. From their amino acid analysis (5) band 1 is shown to have a high lysine content, but differs quite a bit from the Calf thymus lysine rich fraction in the overall amino acid composition. Bradbury _et al_ (27) have shown that band 1 is phosphorylated during G2 phase just prior to mitosis. Phosphorlylation of band 1 histone occurs at times quite separate from DNA synthesis or the overall level of RNA synthesis but simultaneously with chromosone condensation. DNA synthesis follows division and the increase in phosphorlyation of band 1 occurs before division and clearly not at the same time as DNA synthesis. Phosphorylation of very lysine rich histone is temporarily coupled to division.

The very lysine rich histones have been shown to be highly tissue and species specific (37). Phosphorlyation of this

lysine rich fraction adds another dimension to the problem of heterogeneity and specificity of these proteins. Selective phosphorylation can introduce multiplicity in electrophoretic or chromatographic patterns of the phosphorylated protein (37). Modifications of amino acid sidechains in histones such as phosphorlyation, appear to be an important process in regulating histone function. Addition of a negatively charged phosphate group on the histone side chain would tend to neutralise the basic amino group on the histone thereby weakening its interaction with DNA. This might be a selective mechanism to free specific parts of the DNA, thus allowing it to be used as a template.

The slightly lysine rich fraction (IIb2) of P. flavicomum has a similar electrophoretic mobility as compared to the calf thymus standard. Until amino acid analysis and molecular weight determination is performed on a purified fraction, one cannot say if indeed they are similar.

Histones III (arginine rich) from P. flavicomum is composed of three bands instead of the one found in calf thymus. The major band most probably is the arginine rich histone, while the two minor bands are most probably lysine rich histones.

Fraction IV and IIb1 (slightly arginine and lysine rich) are both present in P. flavicomum histone but fraction IV is present in a relatively small amount.

P. flavicomum histone has the 5 basic fractions as found in calf thymus as indicated by the similar electrophoretic

pattern. However, the distribution of each band is different. Molecular weight and charge of each fraction most probably is not the same as calf thymus as indicated by the results obtained. It is evident that the P. flavicomum histone does not have properties identical to any of the mammalian histones. One of the major differences is the presence of slow moving electrophoretic band 1. The difference most probably arises as a result of change in the primary structure. This band 1 has also been found to be modified by phosphorylation. Modifications of other fractions are highly probable. These modifications most probably cannot be detected by electrophoresis but would greatly alter the charge or solubility of the different fractions. Future fractionation procedures must therefore be modified accordingly in order to achieve any kind of meaningful separation of the different fractions.

The amino acid analysis of the whole P. flavicomum histone shows that it is similar in composition to P. polycephalum histone (27). Any difference that might be present could be explained by two factors. First, asynchronous cultures are used for P. flavicomum histone isolation. Second, different media and growth conditions are used for the two different species. P. flavicomum histone also has a gross similarity to the Calf thymus and Tetrahymena histone. This is not too surprising since histones have been found to be conserved during the course of evolution (38).

Myxomycete whole histone has a relatively low basic/acidic amino acid ratio and a relatively high lysine/arginine ratio as compared to calf thymus. Tetrahymena whole histone also has a similar composition. This might be characteristic for the lower eucaryotes as compared to the higher organisms.

For Myxomycetes, histone IV is present in a relatively small amount. This could therefore lower the basic/acidic ratio and increase the lysine/arginine ratio. Histone IV has been shown to be highly conserved during evolution. As quoted by DeLange and Smith (39) this histone IV has a mutation rate of approximately 0.06 residues per 100 million years. This makes this fraction the most evolutionary stable protein known at the present time.

Recent X-ray diffraction studies by Skidmore and Walker (40) have demonstrated that while all histone fractions are involved in maintaining the conformation of DNA, histone IV has been found to be an absolute requirement in order to maintain the tertiary conformation of the DNA. In order to assure survival no change can be tolerated in this particular fraction. As the organisms become more complex in structure and function, rather than changing the chemical composition of this fraction, which cannot be tolerated, the relatively quantity of this frction is increased instead. This might be one mechanism for the evolution of the non-specific gene repressor histones.

SUMMARY

Physarum flavicomum plasmodial nuclei of high purity are exposed to different methods of breakage and extraction. The proteins are partially fractionated and characterized. An acidic protein which is co-extracted with basic proteins by salt at neutral pH is found to be present in the nucleus. From the amino acid composition and molecular weight determination this acidic protein is identified as an actin-myosin-like protein.

Histones extracted from plasmodial nuclei have the five basic electrophoretic bands as are found in calf thymus histone. Different methods of fractionation by use of column chromatography and solubility properties are applied to the isolated histone samples. Plasmodial histones behave quite differently from calf thymus histones during fractionation.

The amino acid composition of whole *P. flavicomum* histones agrees with the published results obtained on histones from the Myxomycete *P. polycephalum*. In general, the amino acid composition is also somewhat similar to published results on calf thymus and *Tetrahymena* histones.

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