et al., 1973). Furthermore, sequence studies on peptide 6 (Butler, W. T., and Miller, E. J., submitted for publication) indicate that its primary structure corresponds quite closely to that of residues 4–36 in α1-CB2 (Bornstein, 1967). These data strongly suggest that one of the tripeptides (peptide 2 or 3) comprises the α1(II) sequence corresponding to residues 1–3 in α1(I)-CB2. The most likely choice is peptide 3 (a glycine-proline-methionine sequence) corresponding to the sequence glycine-proline-serine in α1(I)-CB2.

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Isolation of the Nonhistone Proteins of Rat Walker Carcinoma 256. Their Association with Tumor Angiogenesis†

Dorothy Tuan, Sandra Smith, Judah Folkman, and Ezio Merler*

ABSTRACT: A procedure is described to isolate nonhistone proteins of malignant cell nuclei for the purpose of testing certain of its biologic activities. Walker rat carcinoma 256 cells were disrupted by N_2 cavitation, the nuclei isolated by differential centrifugation, the chromatin was purified, and the DNA separated from chromatin protein by chromatography on Bio-Gel A 5m in the presence of 4 M guanidine-HCl. Further separation of histone and nonhistone proteins was achieved with CM-Sephadex C-50, eluting alkylated nonhistones with 0.4 M guanidine-HCl in 0.01 M phosphate buffer and subsequent elution of histones in 2 M guanidine-HCl in 0.1 M acetic acid. Nonhistone and histone proteins were obtained in good yield and satisfactory purity. The histone and nonhistone proteins were characterized by amino acid analysis, macromolecular composition, and gel electrophoresis. A fraction containing tumor nonhistone proteins was mitogenic to endothelial cells, and, when implanted into the rabbit cornea, caused proliferation of vascular endothelium and new blood vessel formation. Tumor angiogenesis factor was thus demonstrated to be associated with a fraction also containing nonhistone proteins of malignant cell nuclei. Histone fractions had no activity in this bioassay.

The deoxyribonucleic acid in eukaryotic organisms is present in the cell nucleus not as free DNA but in the form of chromatin, a chemically complex material composed of nucleic acids, relatively constant amounts of histone, and a variable amount of nonhistone proteins. The nonhistone proteins, unlike histones, vary both qualitatively and quantitatively with developmental stages (Marushige and Ozaki, 1967; Hill *et al.*, 1971) and from tissue to tissue of a given organism (Dingman and Sporn, 1964; Elgin and Bonner, 1970; Chytil and Spelsberg, 1972). *In vitro* template assay of isolated chromatin has established a direct relationship between the amount of nonhistones present and the template activity of isolated chromatin (Bonner *et al.*, 1968). In general,

† From the Departments of Surgery and Medicine (Immunology), Children's Hospital Medical Center, and the Departments of Biological Chemistry, Surgery, and Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115. *Received February 5, 1973.* This work was supported by U. S. Public Health Service Grants CA-08185 and AI-05877, and a grant (IC-28) from the National Chapter of the American Cancer Society and the Alza Corporation.

chromatin of metabolically active tissues contained greater quantities of nonhistone proteins. Moreover, rapid turnover of nonhistones has been correlated with synthesis of DNA-like RNA in Ehrlich ascites and other cell types (Holoubek and Crocker, 1968). Direct evidence has been presented that nonhistone proteins of chromatin are necessary for the template activity of DNA (Paul and Gilmour, 1968). The nonhistone proteins as a class have, therefore, been proposed to play some central role in the regulation of chromosomal functions. Klein *et al.* (1971), using the technique of cell fusion, have suggested that malignancy might also be under chromosomal control, but the part played by the nonhistones in this is as yet unknown. Attempts to understand the molecular mechanism of nonhistone involvement in cellular processes, such as carcinogenesis and embryogenesis, would be elucidated by the successful isolation and purification of these proteins.

This report describes the isolation of nonhistone proteins from nuclei of Walker carcinoma 256 ascites tumor cells. It has been possible to isolate the nonhistone proteins from histones in good yield and in active form for a bioassay of tumor angiogenesis factor (Folkman, 1971). The nonhistones incited mitosis of vascular endothelial cells and new blood vessel formation in rabbit cornea.

Materials and Methods

Isolation of Walker Carcinoma Nuclei. Walker 256 ascites tumor cells were harvested from 21-day old Sprague-Dawley rats which had been injected with 2×10^6 tumor cells 4–5 days previously. To free the cells of contaminating red blood cells, they were washed twice with 1 vol of 0.87% NH_4Cl, pH 7.3, and once with 9 vol of medium 199 at 4°. Liberation of nuclei from the cells was achieved by N_2 cavitation (Ozer and Wallach, 1967). To do this, the cells were suspended in 10 vol of 0.15 M phosphate buffer, pH 7.2, containing 0.25 M sucrose and 10^{-4} M $MgSO_4$ (solution A) and exposed with constant stirring for 30 min at 4° to N_2 gas maintained at a constant pressure of 1500 psi in a closed stainless steel container (Parr cell disruption bomb). When the pressure was suddenly released, the suspension, which contained mostly intact nuclei, was ejected through a nozzle and collected in glass beakers. Two drops of decyl alcohol and 0.85 ml of 0.02 M Na_2EDTA were added for each 40 ml of suspension which was then centrifuged for 20 min at 500*g*. The nuclei in the pellet were washed three times in solution A, once in 1.23 M sucrose–0.005 M $MgCl_2$–0.005 M Tris (pH 8) and finally in 0.18 M NaCl–0.024 M Na_2EDTA (pH 8) and centrifuged at 12,000*g* for 10 min. The purity of the nuclear preparation was verified by electron microscopy. The nuclei were also cultured on blood agar to test for bacterial contamination. Only sterile preparations of nuclei were used for chromatin preparation.

Preparation of Chromatin. The general procedure of chromatin preparation described by Bonner *et al.* (1968) was followed with minor modifications. To liberate chromatin, the purified pellets of nuclei were resuspended in 10 vol of 0.25 M sucrose–0.05 M $MgCl_2$–0.005 M Tris (pH 8) and sonicated for 1.5 min at 40-W energy output at 20 kHz. Ten-milliliter portions of nuclear suspension in a 30-ml Corex tube were processed each time. To keep the content chilled during sonication, the tube was immersed in a NaCl ice bath. The chromatin suspension was then dialyzed against 20 vol of 0.005 M EDTA–0.005 M Tris (pH 8) for 4 hr. Twenty-milliliter portions of the dialyzed chromatin solution were layered on equal volumes of 1.7 M sucrose in 40-ml cellulose nitrate tubes, the chromatin–sucrose interphase was stirred to form a crude gradient, and the tubes were then centrifuged in a SW-27 rotor for 3 hr at 100,000*g*. The gelatinous pellets were dissolved in 0.005 M Tris, pH 8, to a concentration of 25 OD units/ml at 260 nm and sonicated for 30 sec at 40-W energy output at 20 kHz. The supernatants after centrifugation at 6000*g* for 10 min constituted purified chromatin.

Separation of Chromatin Protein from DNA on Bio-Gel. Purified chromatin was diluted to a concentration of 20 OD units/ml at 260 nm in 4 M guanidine-HCl (Heico Co.)–5.0 mM dithiothreitol–0.005 M Tris (pH 8). Twenty milliliters of it was layered on a 2.5 × 100 cm column containing Bio-Gel A 5m which was equilibrated and eluted with 4 M guanidine–2.5 mM dithiothreitol–0.005 M Tris (pH 8). The flow rate was 30 ml/hr. Effluent fractions (15 ml) were collected. The optical densities at 230, 260, and 320 nm of each fraction were read in a Zeiss spectrophotometer. The fractions were then combined into a nucleic acid pool and a protein pool based on the optical density readings. All operations were carried out at 4°.

Compositional Analysis of Chromatin and Its Subfractions. To determine the DNA and RNA content of chromatin and its subfractions, precipitation with 10% Cl_3CCOOH for 10 min on ice was followed by centrifugation at 12,000*g* for 10 min. The pellet was washed with ethanol–ether (3:1) (30 min at room temperature) and 0.2 N $HClO_4$ (15 min on ice). RNA was hydrolyzed with 0.3 N KOH for 1 hr at 37°. $HClO_4$ was then added to a final concentration of 0.20 N; after 10 min on ice, the solution was centrifuged. The supernatant was analyzed for RNA and the pellet hydrolyzed in 0.2 N $HClO_4$ (70°, 20 min) for DNA determinations. All centrifugations, if not specified, were carried out at 12,000*g* for 10 min. The DNA content was measured by either the diphenylamine reaction of Burton (1956) or by 260-nm absorption of the DNA nucleotides and the RNA content was measured by orcinol reaction (Dische and Schwarz, 1937) or 260-nm ultraviolet (uv) absorption. Standard curves for the above were constructed using a mixture of calf thymus DNA (1 OD_{260nm} unit = 47.6 μg/ml (Tuan and Bonner, 1969)), yeast RNA (1 OD_{260nm} unit = 32 μg/ml (Munro and Fleck, 1966), crystalline bovine serum albumin ($E_{280nm,1\%}$ = 5.8 (Sober, 1968), and calf thymus histones ($E_{230nm,1\%}$ = 42.5 (Tuan and Bonner, 1969) in the proportion of 1:0.05:1:1, a ratio comparable to the composition of chromatins. Bovine serum albumin was used in place of the nonhistone proteins.

To determine the histone and nonhistone contents, the sample was extracted with 0.4 N H_2SO_4 (20 min on ice) and centrifuged (17,300*g* for 10 min). The histone sulfates in the supernatant, after precipitation with 20% Cl_3CCOOH, and the nonhistone protein-containing pellets were dissolved in 0.5 N NaOH for analysis (Lowry *et al.*, 1951), using crystalline bovine serum albumin or IgG globulin as standard.

Separation of Histone from Nonhistone Proteins by CM-Sephadex. To the Bio-Gel protein pool containing approximately 100 μg of protein/ml of eluting buffer, Tris base, Na_2EDTA, and dithiothreitol were added to make their respective final concentrations 0.025 M, 0.015%, and 0.002 M. After incubation for 1 hr at 4° in the above solvent, iodoacetamide, 100 μM for each ml of incubation mixture was added. Incubation was continued for 2 hr in the dark under N_2 at 4°.

The alkylated protein pool was dialyzed to a final concentration of 0.4 M guanidine-HCl, 5 M urea (deionized im-

mediately before use through Rexyn I-300, Fisher Scientific Co.), and 0.01 M $Na_2P_2O_7H_2$, pH 7.8. A small aliquot (10–20 ml) was further dialyzed exhaustively against 0.05 M acetic acid, freeze dried, and reserved for gel electrophoresis. The remainder was applied to a 2.5 × 15 cm CM-Sephadex C-50 column (Pharmacia, Piscataway, N. J.) equilibrated in the same solvent and maintained at a flow rate of 30–40 ml/hr; effluent was collected in 5-ml fractions and monitored at 230 nm. Following the application of sample, the column was washed with several bed volumes of the sample buffer until the first broad peak was completely eluted and the base line steadied. The eluting buffer was then changed to 2.4 M guanidine-HCl–5 M urea–0.1 N HOAc, to elute histones. Two pools consisting of two protein peaks were dialyzed against 20 vol of chilled 0.05 N HOAc and freeze dried. The protein in this form could be stored in the freezer (−13°) for several weeks without noticeable changes in electrophoretic property.

Amino acid analysis was performed according to Spackman *et al.* (1958). Approximately 50 μg of the lyophilized protein fractions eluted from CM-Sephadex columns was dialyzed exhaustively against 0.05 N HOAc, freeze dried again, and hydrolyzed in 6.6 M HCl containing 1% phenol at 105° for 22 hr. The sample, dried and dissolved in 0.1 N HCl, was analyzed.

Quantitative Analysis of the Composition of the Histone and Nonhistone Fraction. The protein contents of the two protein pools were determined by either the Lowry reaction or the ninhydrin reaction in the amino acid analyzer. To determine the nucleic acid content of samples, the procedure in the section on Compositional Analysis of Chromatin and Its Subfractions was followed. Alternatively for RNA measurement, the sample in 0.005 M Tris (pH 8) was first digested with DNase I and II (Worthington Biochemicals Corporation) for 2 hr at 37° followed by a 4-hr incubation with nuclease-free Pronase (Calbiochem). The sample was then made into 0.5% sodium dodecyl sulfate–2 M NaCl, and shaken with an equal volume of chloroform–isoamyl alcohol (24:1). After centrifugation for 10 min at 12,000*g*, the aqueous layer was collected and the deproteinization procedure was repeated twice. The aqueous layer was dialyzed exhaustively against 0.01 M phosphate buffer, pH 6. The uv absorption at 230, 260, and 280 nm was recorded and the RNA content of the sample determined by using the formula of 1 OD unit at 260 nm/32 μg of RNA.

The carbohydrate content of the sample was measured by the α-naphthol reaction (Dische, 1955). To 0.5 ml of sample solution containing 5–25 μg of sugar was added with mixing 4.5 ml of 89% (v/v) H_2SO_4. The tube was transferred to tap water and then to a boiling water bath for 3 min and then cooled in tap water. Then, 0.2 ml of 2% α-naphthol (freshly made before use) was added and mixed. Optical absorption at 560 nm was read after 6 hr. Glucose as standards and water were run simultaneously.

Gel Electrophoresis of Histone and Nonhistone Proteins. Two gel systems were employed to study the electrophoretic pattern of the histones and nonhistones separated by the CM-Sephadex column chromatography. One is the gel system of Panyim and Chalkley (1969). The gel composition was 15% acrylamide, 0.1% bismethylene acrylamide, 2.5 M urea, 0.5% *N,N,N′,N′*-tetramethylenediamine, 0.125% ammonium persulfate, and 0.9 N acetic acid. The gel dimension was 0.6 × 7.5 cm. Preelectrophoresis was at 2 mA/gel for 3 hr. Samples in 5 M urea–0.005 M Tris (pH 8)–1% β-mercaptoethanol were electrophoresed in the cathodal direction for 2.5 hr at 2 mA/gel and stained for 4 hr with 0.25% Coomassie Brilliant Blue in 50% methanol–7.5% HOAc, or overnight in 0.1% Buffalo Black in 20% ethanol–7.5% HOAc. Destaining was done by diffusion in 5% methanol–7.5% HOAc, in an apparatus (Hoefer Scientific Instruments, San Francisco, Calif.) which contains a cartridge of Bio-Rex mixed bed resin (RG 501-X8) to absorb the diffused dye.

The sodium dodecyl sulfate gel system of Weber and Osborn (1969) was used to assess the molecular size distribution of the nonhistone and histone proteins. The gel composition was 10% acrylamide, 0.27% *N,N′*-bismethyleneacrylamide, 0.1% sodium dodecyl sulfate, 0.1 M sodium phosphate buffer (pH 7.2), 0.075% (v/v) *N,N,N′,N′*-tetramethylenediamine, and 0.075% ammonium persulfate. The electrophoresis buffer was 0.1 M sodium phosphate buffer (pH 7.2)–0.1% sodium dodecyl sulfate. The sample buffer was 0.2% sodium dodecyl sulfate–5 M urea–1% β-mercaptoethanol–0.005 M Tris (pH 8). The gel dimension was 0.6 × 8 cm. Electrophoresis was for 4 hr at 8 mA/gel. Staining and destaining were done as described above. The molecular weight markers used were phosphorylase A (mol wt 94,000), crystalline bovine serum albumin (mol wt 68,000), carbonic anhydrase (mol wt 29,000), and β-chain human globin (mol wt 15,000).

Assay of Tumor Angiogenesis Activity. Adult rabbits were anesthetized with intravenous Nembutal. Further local anesthesia was obtained by retrobulbar infiltration with 2% Xylocaine. A 1-mm pocket was made in the cornea with a spatula 1–2 mm from the limbus.

All fractions tested were checked for sterility prior to being embedded in 7% acrylamide buffered to pH 7.4 with Hepes (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid). Fractions (0.1 ml) containing 300 μg/ml of protein were mixed with 0.4 ml of acrylamide and the solution was polymerized by the addition of ammonium persulfate and sodium bisulfite. The polymer forms a translucent gel which can be passed through a no. 25 hypodermic needle. The gel containing the dispersed protein (20 μl) was embedded into the cornea of one eye and a control of acrylamide in the opposite eye.

Each fraction was tested in two rabbits. Growth of new corneal capillaries was recorded daily with a stereoscopic slit lamp.

Results

Composition of Chromatin. The Walker ascites chromatin prepared and analyzed by the present methods contained amounts of DNA, RNA, and proteins as presented in Table I. Although the composition of chromatin was observed to vary with methods of preparation, chromatin of constant composition was obtained by following the procedure described under Materials and Methods and by using fresh samples for analysis (less than 4 days following cell disruption). As the chromatin ages, some of its proteins were degraded and lost in the process of analysis, thus resulting in a lowered protein to DNA ratio. When chromatin had been stored in the refrigerator for 8–9 days after preparation, the histone to DNA ratio, as well as the nonhistone to DNA ratio, dropped to 80–90% of the original value.

Separation of Chromatin Protein from DNA by Bio-Gel A 5m. Chromatin dissociated into its macromolecular components in 4 M guanidine-HCl was sieved to separate its components on a Bio-Gel A 5m column. Following the emergence of DNA in the void volume, chromatin proteins were eluted in two separate peaks (peaks 2 and 3, Figure 1). Since gel

TABLE I: Composition of Chromatin and Peak 1 of Bio-Gel Column Chromatography.

	DNA	RNA	Histones	Nonhistones
Walker chromatin[a]	1.00	0.03 ± 0.006	1.09 ± 0.04	0.8 ± 0.06
Bio-Gel peak 1	1.00	0.018 ± 0.002	0.035 ± 0.01	0.25 ± 0.023

[a] Using the amount of DNA as a basis, contents of RNA, histone, and nonhistone proteins were expressed as weight ratios. The data reported are for freshly prepared chromatin.

electrophoresis on the two protein fractions demonstrated the presence in each of both histone and nonhistone proteins, they were routinely pooled for further separation on carboxymethyl Sephadex C-50 columns.

The distribution of chromatin components in the Bio-Gel eluate was as follows: 90–95% of chromatin DNA was recovered in the first peak, together with 30–40% of chromosomal nonhistones and approximately 50% of chromatin RNA (Table I); 60–70% of nonhistones and all of the histone components of the chromatin sample were recovered in the second and third peaks.

Fractionation of Bio-Gel Protein Pool on Carboxymethyl Sephadex C-50. CM-Sephadex gave satisfactory separation of chromatin proteins (Figure 2). Chromatography on this cation exchange resin resolved two classes of chromatin proteins. The anion exchange resins DEAE- and TEAE-cellulose gave low yields of nohhistones as has been also previously reported by Wang and Johns (1968).

After adjusting the Bio-Gel protein pool eluate to 0.4 M guanidine-HCl–5 M urea–0.01 M sodium pyrophosphate (pH 7.8), the sample was applied to a 2.5 × 15 cm CM-Sephadex column, and the nonhistones were eluted with equilibrating buffer whereas the histones were eluted with 2.4 M guanidine-HCl, 5 M urea, and 0.1 N acetic acid (Figure 2). Approximately 35–40% of the total sample protein was found in the first fraction and the remainder in the second peak, which contained all the histone. More than 95% of the nonhistone proteins were recovered in the first eluted peak as will be shown later by gel electrophoresis. When the guanidine-HCl concentration was lower than 0.4 M, the nonhistones were not completely eluted in the first peak and, as a result of this, the histone fraction was contaminated with nonhistone proteins to varying degrees, depending on the concentration of guanidine-HCl in the initial equilibrating buffer. A sample buffer with a guanidine-HCl concentration higher than 0.4 M resulted in the histones contaminating the nonhistone fractions.

Amino Acid Analysis of CM-Sephadex Fractions. The proteins eluted in each peak were assessed by amino acid analysis and gel electrophoresis. The amino acid compositions of the two protein fractions eluted from CM-Sephadex are given in Table II. The amino acid composition of histones extracted with 0.4 N H_2SO_4 is also shown in Table II. The histones eluted from CM-Sephadex had a ratio of basic to acidic amino acid residues of 2 and was very similar to acid-extracted histones in amino acid composition. The nonhistones eluted from CM-Sephadex had a ratio of basic to acidic residues of 0.83 and a distribution of amino acids markedly different from that of the histones.

Gel Electrophoresis. Gel electrophoresis patterns (pH 3.2 in 2.5 M urea) of chromatin protein before and after CM-Sephadex fractionation are shown in Figure 3. Chromatin protein before fractionation contained approximately 40 discernible bands (Figure 3a). Of these, the 13 most rapidly moving bands are found in the histone fraction (Figure 3c); the remaining bands, mostly of slower mobilities, represented

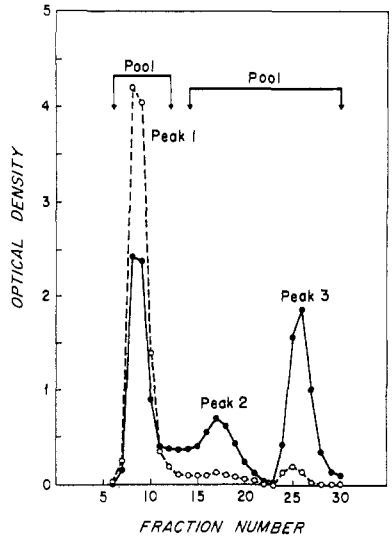

FIGURE 1: Elution profile of chromatin on Bio-Gel A 5m column chromatography: (●) absorption at 230 nm; (○) absorption at 260 nm.

TABLE II: Amino Acid Analysis of CM-Sephadex Column Fractions and Acid-Extracted Histones.

Mol %	Fraction 1 (Nonhistones)	Fraction 2 (Histones)	Acid-Extracted Histones
Lysine	9.1	13.6	14.1
Histidine	1.7	2.0	1.8
Arginine	6.7	10.3	8.1
Aspartic acid	9.0	4.7	5.3
Threonine	5.7	5.4	5.0
Serine	6.9	5.0	6.0
Glutamic acid	11.7	8.1	8.3
Proline	6.6	5.4	6.8
Glycine	7.5	8.8	8.7
Alanine	7.8	12.0	12.8
Valine	6.1	6.1	6.2
Methionine	2.0	0.9	0.5
Isoleucine	4.8	4.7	3.9
Leucine	8.7	8.4	7.7
Tyrosine	3.2	2.8	2.3
Phenylalanine	2.6	1.7	1.8
B/A[a]	0.83	2.0	1.8

[a] B/A = (Lys + His + Arg)/(Asp + Glu).

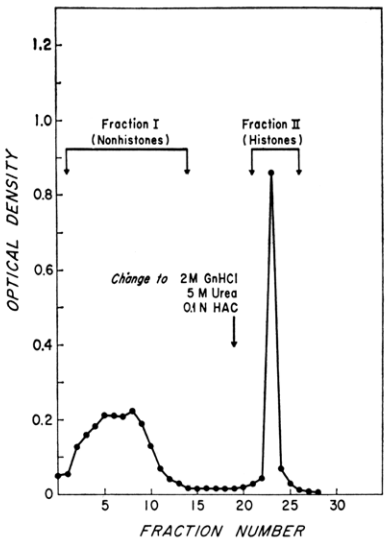

FIGURE 2: Elution profile of chromatin proteins on CM-Sephadex. Fractions were monitored at 230 nm.

the nonhistone fraction, which contained around 30 bands (Figure 3b). Except for the three very faint, slowly moving bands in the histone fraction (Figure 3c) and the four rapidly moving bands in the nonhistone fraction (Figure 3b), there is little overlap between the two. For purposes of comparison, the gel pattern of 0.4 N H_2SO_4 extracted histones is represented in Figure 4d. The electrophoretic patterns of the two were very similar, with only one notable difference—the acid-extracted histones seemed to contain more of the slow-moving nonhistone bands.

To establish the molecular weight distribution of the histones and the nonhistones, the sodium dodecyl sulfate gel system was employed which is known to resolve proteins by virtue of their molecular size (Shapiro *et al.*, 1967; Weber and Osborn, 1969). In this system, 18 bands of nonhistones were distributed between a mol wt range of 100,000–13,000 (Figure 4a). Quantitatively, the proteins were not evenly distributed among these bands. Eight out of the 12 darker staining bands ranged between 49,000 and 80,000 in mol wt; two, at around 15,000; one, at 25,000; and another, at 12,000. The histone fractions showed five bands within a molecular range of 30,000–12,000 (Figure 4b). There is, thus, no concordance in molecular weight distribution of the histones and the nonhistones, except for the histone component with a mol wt of 16,000.

Composition of the Nonhistone and Histone Fractions. The nonhistone fraction from CM-Sephadex chromatography was found to be composed of protein containing also around 1% DNA, 3% RNA, and 32% carbohydrate (Table III). The values of nucleic acids reported in Table III were obtained by uv absorption. In comparison, the histone fraction contained no RNA or DNA and possessed negligible amounts of carbohydrate.

Angiogenesis Activity of Nonhistone Proteins. New capillaries appeared in the rabbit cornea when tumor nonhistone proteins were admixed with the acrylamide (total of 15 separate experiments of which 13 were positive). In the presence of concentrations of nonhistone protein as low as 1 μg, new capillaries began to penetrate the cornea by the third day and continued to grow at 0.1 mm/day for approximately 10 days (Figure 5). Histologic sections showed these to be proliferating capillaries with endothelial cells in mitosis. There was evidence of inflammation in 1 of the 15 experiments. In six of seven experiments, the tumor histone fraction was found negative for new capillary growth. In six separate experiments, tumor proteins inactivated by heat (100°) were also found negative. Viable rat liver cells (three experiments) were found not to stimulate capillary growth and neither were nonhistone proteins obtained from rat liver cells (three experiments). Alkylation of the nonhistone proteins appeared

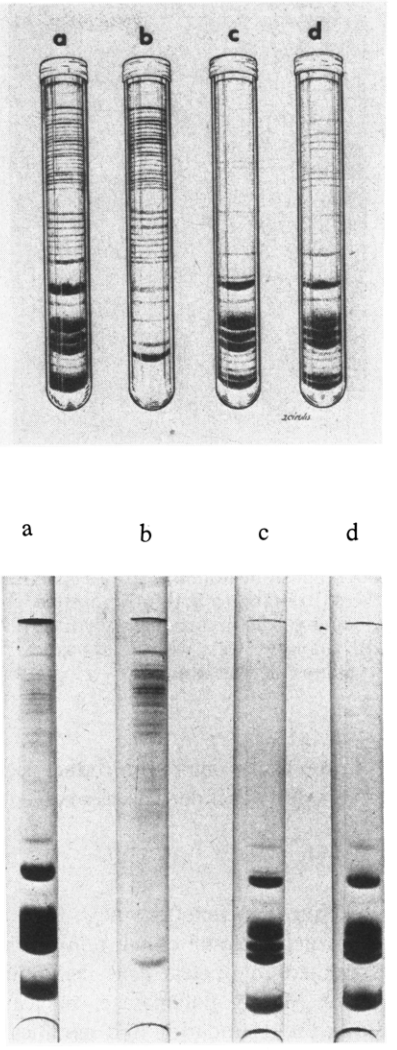

FIGURE 3: Gel electrophoresis (pH 3.2, 2.5 M urea): (a) ~150 μg of chromatin protein before CM-Sephadex fractionation; (b) ~100 μg of nonhistones (fraction I of CM-Sephadex chromatography; (c) 40 μg of histones (fraction II) of CM-Sephadex chromatography; (d) 50 μg of 0.4 N H_2SO_4 extracted histones. To facilitate comparison, a graphic rendition of gels was presented on top of each gel. The gels were stained with Buffalo Black and stored in 20% alcohol–7% acetic acid. Electrophoreis was from the top (+) to the bottom (−).

TABLE III: Composition of the Nonhistone and Histone Fractions of CM-Sephadex Chromatography.[a]

	Protein	RNA	DNA	Carbohydrate
Nonhistone fraction	1.00	0.03	0.01	0.32
Histone fraction	1.00	0	0	0.01

[a] Using the amount of protein as a basis, contents of RNA, DNA, and the carbohydrate were expressed as weight ratios.

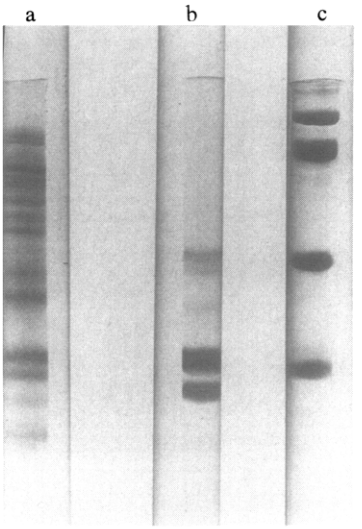

FIGURE 4: Gel electrophoresis (0.1% sodium dodecyl sulfate, pH 7.2): (a) 150 μg of nonhistone fractions; (b) 30 μg of histone fraction; (c) mol wt markers (from top to bottom): phosphorylase a (94,000), bovine serum albumin (68,000), carbonic anhydrase (29,000), β-chain globin (15,000). Staining was with Coomassie Brilliant Blue. Electrophoretic migration was from top (−) to bottom (+).

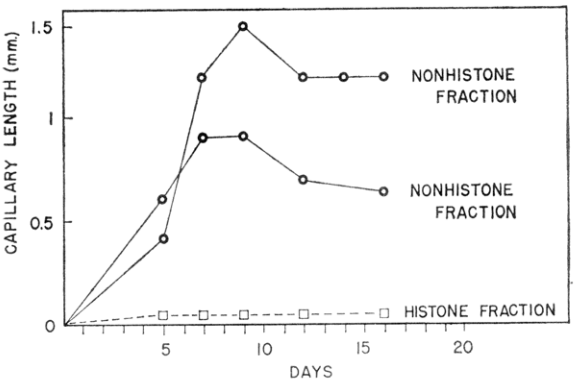

FIGURE 5: Capillary growth in rabbit cornea. Day 0 was the day of corneal implantation. The sample containing gel was inserted in cornea approximately 1 mm from the limbus. After 6 days, the longest capillary sprouting from the limbal vessel bed was measured every other day. Approximately 1–4 μg of proteins was used in each assay. The nonhistone samples for the two curves were from different preparations of chromatin.

to render replicate assays more reproducible, although it was not clear if this step was indeed necessary.

Discussion

A number of biological functions have been attributed to the nonhistone proteins. Some of the nonhistones are probably enzymes related to nucleic acid metabolism, such as RNA polymerases, DNA polymerase, nucleases, and enzymes involved in the degradation and modification of chromosomal proteins (Furlan and Jericijo, 1967; Gallwitz, 1971). Nonhistones have also been described as tissue-specific hormonal acceptors in estrogen-stimulated uteri of ovarectomized rat (Teng and Hamilton, 1969; Barker, 1971), in chick oviduct exposed to progesterone (Spelsberg *et al.*, 1971), and in aldosterone binding to renal chromatin (Swaneck *et al.*, 1970). A particular nonhistone protein has been shown to be differentially synthesized in cortisol-stimulated liver cells of adrenalectomized rat (Shelton and Allfrey, 1970). One possible function of these proteins may be structural, in changing or maintaining the conformation of chromatin at special sites (Kleinsmith and Allfrey, 1969; Mirsky and Osawa, 1961).

The nonhistone proteins of Walker ascites tumor cells prepared by us reveal yet another facet of biological activities. Implanted into the rabbit cornea, they elicited blood vessel growth from the limbus to the location of the implant. The histone fraction completely lacked this activity. When injected into a variety of tissues, the tumor nonhistones behave in a fashion similar to injected whole tumor cells (Cavallo *et al.*, 1972) and induced, within 6 hr, endothelial cell division in an area immediately surrounding the site of injection. Within 48 hr, the effect was manifested morphologically in the appearance of new blood vessels.

For separation of chromosomal nonhistones from histone proteins, the use of cation exchange resins, such as SP-Sephadex C-25 (Graziano and Huang, 1971), Bio-Rex 70 (Levy *et al.*, 1972), and SE-Sephadex C-25 (Elgin and Bonner, 1972), has been reported. CM-Sephadex was initially reported by Hill *et al.* (1971) to be useful for these separations. The present method of nonhistone protein preparation offers certain advantages, particularly in its yield. The conditions of separation were mild in that no exposure to extremes of pH occurred nor was detergent used. The temperature of the sample was carefully controlled at 4° at all times, which was essential for successful separations. The proteins, after Bio-Gel column chromatography, were alkylated, thus minimizing the possibility of degradation during further manipulations.

The nonhistone proteins, as prepared by the present method, revealed approximately 30 protein bands to pH 3.2 in 2.5 M urea gel electrophoresis. This might, however, represent an underestimate due to the inherent limitation of detection by gel electrophoresis. The maximum amount of nonhistones that can be analyzed on each gel is 100–150 μg, and the minimum amount that can be visibly stained is 1–0.5 μg; therefore, proteins present in the nonhistone population in amounts less than 1% would have escaped detection. In the sodium dodecyl sulfate gel system, Walker nonhistones displayed 18 bands similar in number to the values reported by others for such diverse tissues as liver and kidney (Levy *et al.*, 1972; Teng *et al.*, 1971), chick brain (Graziano and Huang, 1971), pea buds, chicken erythrocytes, liver, and kidney (Elgin and Bonner, 1970).

Unlike the histones, the nonhistones from CM-Sephadex were not pure protein in that they contained in addition to protein a small amount of RNA (approximately 3% by weight of the proteins), some DNA (1%), and quite substantial amounts of carbohydrates (32%). The nucleic acid component, as suggested by Levy *et al.* (1972), could be eliminated with DEAE-cellulose chromatography. According to them, the nonhistone proteins were eluted from DEAE-cellulose at 0.3 M NaCl in the equilibrating buffer, while the nucleic acids eluted with 0.6 M NaCl in the same buffer. In our hands under slightly different conditions, the nucleic acids were, however, eluted between 0.3 and 0.32 M NaCl from DEAE-cellulose, together with some nonhistones, as revealed by gel electrophoresis. Whether the nucleic acids and nonhistones were covalently linked or were physically separate entities, fortuitously eluted in the same fraction due to their similarity in charge and size, is currently under investigation. The substantial amount of carbohydrate may have its origin in the

nuclear membrane since it has been observed that chromatin fibers of mammalian nuclei converge and attach to the annuli of the nuclear membrane (Comings and Okada, 1970; Engelhardt and Pusa, 1972). Alternatively, the carbohydrate might be acquired by and necessary for some components of the nonhistones during their transport from the nucleus to the cytoplasm (Folkman *et al.*, 1971).

The implication in tumor growth of the blood vessel inducing ability of tumor nonhistones requires further definition to ascertain in which species of nonhistones this activity resides. The nonhistone proteins of nontumor origin, such as rat liver, which contain comparable amounts of RNA, DNA, and carbohydrates, do not possess this activity. The angiogenesis activity of a complex obtained from the cytoplasm of Walker cells was shown to be neutralized by treatment of the complex with either ribonuclease of trypsin (Folkman *et al.*, 1971). In a similar way, the angiogenesis activity of tumor nonhistone proteins was neutralized by treatment with either ribonuclease or proteases (Tuan *et al.*, 1973[1]). In order to identify further the active component for tumor angiogenesis activity in tumor nonhistones, further separation of each nonhistone protein from others is being pursued.

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