INVESTIGATIONS OF CHROMATIN BOUND ENZYMES

Thesis by

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In Partial Fulfillment of the Requirements
For the Degree of
Doctor of Philosophy

California Institute of Technology
Pasadena, California

(Submitted May 16, 1974)

To

My Parents and Wife

ACKNOWLEDGEMENTS

I am deeply grateful for the support and encouragement of Professor James Bonner. His contagious enthusiasm, and deep insight are a constant source of inspiration and an example worthy of following. The flexibility, generosity and freedom he permits in the lab has enabled me to pursue my individual scientific interest actively.

I am indebted to the members of my committee : Dr. Mitchell, Dr. Attardi, Dr. Hood and Dr. Russell, for their careful and constructive criticism.

I would especially like to thank Mrs. Bonner who kept me supplied with endless amounts of chemicals. It would be impossible to keep the lab going without her.

It is a pleasure to acknowledge the constant advice and assistance of Dr. Garrard and Dr. Wu, and helpful comments on this thesis from Dr. Douvas.

I would like to thank the California Institute of Technology for financial support from the Arthur McCallum Fund.

ABBREVIATIONS

DFP: diisopropylfluorophosphate

PMSF: phenyl methyl sulfonyl fluoride

GuCl: guanidine hydrochloride

SDS: sodium dodecyl sulfate

Na_2 EDTA: disodium ethylene diamine traacetate

TCA: trichloroacetic acid

TAME: α - tosyl arginine methyl ester

SSC: standard saline citrate

NHC Proteins: nonhistone chromosomal proteins

BSA: bovine serum albumin

TEMED: N,N,N',N' - tetramethylethylene diamine

C-RNA: chromosomal RNA

ABSTRACT

Part I. Rat liver chromatin contains a neutral protease with a marked preference for chromosomal proteins as substrates. The enzyme has been purified 705-fold from chromatin by salt extraction, chromatography on Bio-Rex 70, Sepharose 6B, calcium phosphate gel and QAE Sephadex. The enzyme has a molecular weight of 200,000 with two identical subunits of molecular weight 100,000. It attacks rat liver histones, NHC proteins and L-poly-lysine preferentially, is essentially inactive with rat liver cytosol proteins, and slowly degrades caseine, L-poly-arginine and protamines. The K_m for histones is 0.5 mg/ml; for NHC proteins K_m is 1 mg/ml. The enzyme is quite stable when stored at -20° for 4 months. Activity is diminished to 50% by heating to 62° for 15 min and totally destroyed at 70°. The enzyme has an optimal pH at 7.0 and a half maximal activity at pH 6.0, suggesting that a histidine residue is involved in catalysis. It is inhibited by DFP and PMSF, suggesting that a serine residue is involved. Hg^{++} inhibits enzyme activity, suggesting sulfhydryl group is important for enzyme activity. High salt (above 1M NaCl) inhibits enzyme activity completely but reversibly. The enzyme needs divalent ions as activators; especially potent is Mn^{++} (6-8 mM) which stimulates activity about 2 fold. The isolated enzyme appears to be similar to that responsible for the endogeneous degradation of histones in chromatin. The susceptibility of the five histone fractions to proteolysis is critically dependent upon whether or not the histones are complexed with DNA. In the intact nucleohistone four major histones are rather resistant to proteolytic attack, while histone I is rapidly attacked. If histones are freed from DNA all the histone molecules are attacked at about the same rate except histone I, which is relatively resistant.

Part II. Rat liver chromatin also contains a nonspecific esterase which cleaves the artificial substrate--α-tosyl arginine methyl ester (TAME). The enzyme has been purified to 510 fold from chromatin by salt extraction, chromatography on Bio-Rex 70, Sephadex G-200, calcium phosphate gel and SE Sephadex. The molecular weight of the purified enzyme is estimated to be about 15,000. The enzyme has an optimal pH at 8.2 and half maximal activities at 6.9 and 10.5, suggesting that histidine and lysine residue might be involved in catalysis. It is inhibited by DFP and PMSF, suggesting that a serine residue is involved. At high substrate concentrations inhibition is noted. The K_m for TAME is 0.16 mM.

Part III. Ferritin tagged c-RNA molecules were hybridized to rat ascites nuclear DNA in an effort to map the arrangement of these sequences in the rat genome by electron microscopy. The ferritin acts as an electron dense marker. The interferritin distance is also the inter RNA distance

and c-RNA hybridizes specifically to middle repetitive DNA sequences. Thus measuring the distances between ferritin molecules attached to c-RNA reveals that middle repetitive DNA sequences (about 300 nucleotides in length) were arranged either singly or in tandem and immediately followed by a unique sequence about 500-1500 nucleotides in length.

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CHAPTER I

GENERAL INTRODUCTION

The time has come for direct attack on one central problem of biology, the problem of how a fertilized egg can give rise to an adult organism made of many different kinds of cells. This development process has been described and thought about by biologists for a long time. Its nature has remained a mystery. Now it is clear that each cell in the adult organism has the same complement of genomal DNA sequences. (Gurdon, 1962; McCarthy and Hoyer, 1964; Davidson, 1968). Thus within the nucleus there must be some kind of programme which determines the proper sequences of repression and derepression which make development. What are the mechanisms of gene repression and derepression which make development possible.? Candidates for such control mechanisms include multiple RNA polymerases, multiple polymerase factors (Stein and Hausen, 1970; Seifert, 1970; Froehner and Bonner, 1973), and other components which interact with nuclear DNA. There is evidence which suggests that this latter class, namely the components of chromatin, play an important role in the control of gene expression.

Chromatin, the collective term for isolated interphase chromosomes, is composed of DNA, a set of basic proteins — the histones, a complement of less basic and acidic proteins — the nonhistone chromosomal proteins, and traces of RNA (Bonner et al, 1968). During the past decade a great deal of information has been accumulated on the nature of chromatin and its components. In spite of this information, little is known about how these components interact at the molecular level to lead to specificity in gene control. It is known that a remarkably constant pattern of histones has been found to be present in nearly all eukaryotes. Histones are tightly bound to chromosomes and are present in a mass ratio of histone: DNA about of 1 : 1 (a range of 0.7 to 1.3) (Bonner et al., 1968). Histones can be divided into five discrete fractions, identifiable by their amino acid compositions and molecular size. These five fractions have been found in virtually all organisms which possess a defined nucleus(Johnson et al., 1974). Each of the five principal subgroups of histones has now been found to possess some microheterogeneity resulting from small differences size and charge. This heterogeneity may be due to either slight differences in primary structure or to enzymatic modification of amino acid side chains in histone molecules (DeLange and Smith, 1974). Although histone I is a partial exception, the other histones appear to lack tissue (Panyim and Chalkley, 1969) and species specificity and show a high degree of conservation during the course of evolution. (Elgin and Bonner, 1973).

The precise biological roles of histones are not fully known. Two principal functions have been proposed; 1) that histones may be responsible for determining the structure of chromosomes during the cell cycle, and/or 2) that they may act as repressors of DNA transcription. The second can be further subdivided into specific repression, implying a selective recognition process between histones and other chromosomal components, and a nonspecific masking of the template capacity of DNA. Although much evidence has shown that histones render DNA inactive as a template for RNA synthesis(Bonner _et al._, 1968), it seems unlikely that the direct interaction of unmodified histones and DNA takes place with any sequence specificity(Leng and Felsenfeld, 1966; Johns and Butler, 1964). It is true, however, that a number of findings reported in the literature suggest the possibility is that the other chromosomal components, perhaps NHC proteins(Paul and Gilmour, 1968) or chromosomal RNA(Bekhor, Kung and Bonner, 1969; Huang and Huang, 1969) might participate in a tertiary complex with histones and DNA to confer specificity on their interactions. A second plausible mechanism for permitting specificity of base sequence interaction could involve reducing the electrostatic attraction between histone molecules and DNA via acetylation or phosphorylation of lysine, serine, threonine and histidine residues to partially neutralize their basicity(Allfrey, 1971). A third possibility is that specific proteolysis of histones might play a role in gene derepression. Special conditions altering the state of histones to render them susceptible to the protease at the appropriate gene loci would have to be invoked by such a model

(Garrels et al, 1972; Marushige and Dixon, 1971). Since previous studies (Garrels et al, 1972) have shown that there is in the nuclei of rat liver cells a protease which shows a high activity toward histones as substrates, I have purified this enzyme to homogeneity in an effort to contribute toward an understanding of its possible role in gene regulation.

Aside from a possible role for a protease in gene regulation, other important subsidiary reasons exist for the study of the enzyme. The possibility that some of the minor components in histone preparations are artifacts produced by degradation of the proteins during chromatin isolation, is an open question and one of increasing importance. It has become interesting to look for specific new components in chromatographic or electrophoretic patterns of histones isolated from different tissues. However, the identification of any component as an endogeneous histone is dependent upon freedom from minor degradative artifacts. Furthermore, investigations on the primary structure of histones could be easily misled by the presence of minor amounts of such artifacts. It is not clear how one could recognize such artifacts in the general, unknown situation, as for example the comparison of histones isolated from different sources. So the isolation, characterization of the protease(s) and the finding of potent inhibitors to prevent degradation become important.

Much research is currently directed towards understanding genetic control. Isolated chromosomal material is frequently used as a standard system in vitro. The protease(s) which degrades the histones presents a serious obstacle to many types of studies on the histones themselves and also of studies of their interaction with nonhistone chromosomal proteins and DNA as in reconstitution. How to inhibit the protease(s) seems to be a prerequisite for these kinds of studies.

An additional reason for characterizing a protease with specificity toward histones is its potential usefulness in protein sequencing analysis. Although the amino acid sequences for histones II_{b1}, II_{b2}, III and IV isolated from a variety of sources (DeLang and Smith, 1974) have been completed, this is not the case for histone I. This latter protein has presented difficulties because of the necessity to obtain pure subfractions and because of technical difficulties resulting from the unusual composition of the C-terminal half of histone I, 80 mole percent of this fragment consists of the amino acids lysine (40 mole per cent), alanine and proline (DeLang and Smith, 1974). A protease which specifically cleaves this C-terminal portion could thus be very beneficial in regard to the determination of amino acid sequence of histone I and also for sequence analysis of the other proteins which present difficulties when routine methods are used.

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CHAPTER II

PURIFICATION AND PROPERTIES OF A NEUTRAL PROTEASE FROM RAT LIVER CHROMATIN

INTRODUCTION

Earlier workers pointed out the possibility that some of the histone fractions obtained by column chromatography might be products of enzymatic degradation (Crampton _et_ _al_., 1957; Moore, 1959; and Satake _et_ _al_., 1960). Phillips and Johns (1959) showed that calf thymus histones prepared by acid extraction of deoxynucleoprotein contained a protease, which gave rise to extra N-terminal alanine, lysine and glycine residues in histones, especially at pH 7-8. Sarker and Dounce (1961) provided evidence for the existence in calf thymus nuclei of four proteases, which exhibited maximal activity towards hemoglobin substrate at pH 3.8 and 5.6, and towards serum albumin substrate at pH 7.0 and 9.0. However, Furlan and Jericijo (1967 a & b) using deoxynucleohistone as substrate, demonstrated the presence in calf thymus nuclei of only two proteases which hydrolyzed nucleoprotein substrate at pH 4.4 and 7.8. Their results suggested that the neutral protease was located exclusively in nuclei, whereas the presence of the acid protease might be ascribed to contamination of the nuclear preparation by whole cells. Later, Furlan and Jericijo (1968) enriched the neutral protease from calf thymus nuclei. The protease had a molecular weight of approximately 24,000, a pH optimum of 7.8 and activity maxima at 0.1 M NaCl and 1 M NaCl. The enzyme activity was inhibited by p-chloromercuribenzoate and by DFP, suggesting a serine residue at the active site. Deoxyribonucleoprotein was 6-7 times more susceptible as substrate than other proteins. That the enzyme was normally a constituent of chromatin was indicated by the fact that it coprecipitated with DNA and histones at sodium chloride concentrations between 0.1 and 0.3 M. As shown by reconstitution and acid extraction experiments, the interaction between the protease and DNA appeared to be quite similar to that between DNA and histone I. Thus the characteristics of this enzyme suggested that it might be responsible for part or all of the histone degradation observed by Panyim Jensen and Chalkley (1968).

More recently Bartley and Chalkley (1970) reported that in calf thymus nucleohistone, histones I and III were attacked most rapidly by endogenous protease. In contrast, if histones were used as substrate in solution (without the presence of DNA), only histone I was resistant and all the other histone fractions were rapidly degraded. They also found that the enzyme activity was maximal at pH 8 and was relatively inactive below pH 7 and at lower ionic strengths. The proteolytic enzyme was firmly bound to nucleohistone. There seems little doubt that a histone protease is a normal constituent of chromatin.

The biological role of such an enzyme has been a topic of speculation. Bartley and Chalkley (1970) found a much greater protease activity in chromatin from thymus and intestinal mucosa cells than in that isolated from lung or Ehrlich ascites cells, suggesting that the protease may function principally during autolysis. A subsequent report of relatively high levels of protease activity in rat liver chromatin (Garrels et al.,1972) makes this generalization less tenable. A possible role for chromatin-bound protease in removing histone from DNA during spermatogenesis has been suggested by Marushige and Dixon (1971). High substrate specificity is implied in this case since the protease would have to degrade histones without affecting protamines which replace histones in the sperm nucleus. Small heterogeneous acid soluble protein fragments were observed in trout testis chromatin late in the transformation from nucleohistone to nucleoprotamine; these could be the breakdown products of histones, which are rapidly being replaced by protamines. It was also shown that histones became acetylated and phosphorylated during the time of their replacement. This evidence suggested that in spermatogenesis, histones might be removed from DNA by proteolytic degradation; the minor modifications presumably might be necessary to render the histones available and susceptible to the protease digestion. If the same hypothetical mechanism should be operative in the removal of histones during gene derepression, then such a protease would be an enzyme of considerable biological significance.

Therefore this enzyme has been purified to homogeneity in an attempt to set the background for future studies on its role in gene regulation.

MATERIALS AND METHODS

Materials

Frozen rat liver was purchased from Pel-Freeze Biologicals , Inc. Poly-L-arginine (MW 40,000) was purchased from Pilot Chemicals, Inc. Poly-L-lysine (MW 5,900) was obtained from Miles-Yeda Ltd. Salmon protamine sulfate, egg white lysozyme and bovine serum albumin were obtained from Sigma Co. Bio-Rex 70 (200-400 mesh, sodium form), Bio-Gel A-50 (50-100 mesh), calcium phosphate gel, acrylamide, Bis (N,N'-methylene- bis-acrylamide), TEMED, and ammonium persulfate were purchased from Bio-Rad Lab. Sepharose 6B, Sephadex G-100 and QAE Sephadex A-25 were obtained from Pharmacia, Fine Chemicals. Human γ-globulin (fraction II), ovalbumin (non-enzymic protein molecular weight markers) were from Schwartz/Mann. _E_. _coli_ β-galactosidase was obtained from Worthington Biochem. Corp. Amicon PM-10 ultrafiltration membrane was purchased from Amicon Corp. Dansyl chloride was purchased from Pierce Chem. Co. Polyamide layer sheet was obtained from Gallard-Schlesinger Chem. Corp.

Precycling of Bio-Rex Resin. One hundred grams of Bio-Rex 70 (200-400 mesh sodium form) was suspended in 3 liters of 2 N HCl and allowed to settle. After removal of the fines, the resin was washed extensively with water and suspended in 3 liters of 2 N NaOH. After excess base was removed by filtration and washing with water, the material was resuspended in 2 N HCl and washed further with water. Finally the resin was suspended in 2 M NaCl; titrated to pH 7 with 2 N NaOH and collected by filtration. Before using, the resin was washed several times with the desired buffer and the pH was adjusted by titration.

Treatment of Dialysis Tubing. Dialysis tubing was allowed to soak in acetic anhydride overnight at room temperature; washed extensively with water for 1 day, and boiled for 30 min in a solution of 10 mM di-sodium EDTA - 0.1 M $NaHCO_3$. After cooling, the tubing was washed extensively with deionized water; soaked overnight in ethanol; and washed again with deionized water. The treated tubing was stored in 50% glycerol at 4°.

Preparation of Nuclei. Nuclei were prepared by a modification of the method of Blobel and Potter (1966). Sprague-Dawley rats (150-250 g) were sacrificed. Livers were removed quickly and chilled immediately in two volumes of ice-cold 0.25 M sucrose in TKM buffer (50 mM Tris, pH 7.5 at 20° - 25 mM KCl - 5 mM $MgCl_2$). All subsequent operations were performed at 4°. Livers were blotted, weighted, and minced with scissors. After homogenization using a Teflon homogenizer (10-15 strokes at 100 V), the homogenate was filtered through four layers of cheesecloth. One part of filtrate was mixed with two parts of 2.3 M sucrose in TKM buffer. The resulting mixture was overlaid on 0.25 volumes of 2.3 M sucrose in TKM buffer in

SW 25.2 certrifuge tubes and centrifuged for 1 hr at 76,900 g in a SW 25.2 rotor. After decanting the supernatant, material adhering to the wall of the tube was removed prior to harvesting the pellet. The yield of nuclei based on the recovery of DNA was 50 to 70%.

In some instances as indicated in the text, the resulting nuclei were washed once with non-ionic detergent by suspension in 0.5% (v/v) Triton X-100 - 0.25 M sucrose - TKM buffer. After stirring at 4° for 30 min, the material was pelleted by centrifugation at 1,500 g for 10 min. The resulting pellet was washed twice with 0.25 M sucrose - TKM buffer by centrifugation as above. The chromatin was prepared from purified nuclei after washing with saline - EDTA as outlined in the "Preparation of Chromatin" section.

Isolation of Nuclear Membrane. The method of Kashnig and Kasper (1969) was adopted to isolate nuclear membrane. Nuclei were isolated as described above, but without detergent washing. After suspending nuclei in 30 ml of TKM buffer to give a concentration 1 mg per ml protein, the material was sonicated with a Branson Sonifier at a setting of 6.5 for 10 to 15 sec at 4°. This procedure was repeated until microscopic examination revealed over 90% nuclear lysis. Solid potassium citrate was added with mixing to a final concentration of 10% (w/v) and the solution was centrifuged at 39,000 g for 45 min at 4°. The resulting pellet was suspended in 10 ml of TKM - 10% potassium citrate - sucrose solution (density 1.22 g per cm^3) by vortex mixing and transferred to a nitrocellulose tube (Spinco SW 25.1 rotor). The membrane suspension was overlayered, in a stepwise manner, with 7 ml each of TKM buffered sucrose solutions containing 10% potassium citrate with densities of 1.20, 1.18, and 1.16 g per cm^3, respectively. After centrifugation at 100,000 g for 10 hr at 4°, nuclear membrane was harvested from the interfaces of densities 1.18 to 1.20 and 1.16 to 1.18 g per cm^3.

Preparation of Chromatin. Chromatin was prepared from frozen rat liver (Pel-Freeze Biologicals, Inc.) as described by Elgin and Bonner (1970). Sucrose purified chromatin was washed with 10 mM Tris (pH 8) and sheared in a Virtis homogenizer at 30 V for 90 sec. After centrifugation at 12,000 g for 15 min, the supernatant was used as the starting material for the preparation of chromatin-bound protease.

Treatment of Chromatin with PMSF to Inactivate Endogenous Protease. Purified sheared chromatin was adjusted to 10 A_{260} per ml in 10 mM Tris (pH 8) and PMSF (stock solution 50 mM in isopropyl alcohol) was added to a final concentration of 1 mM. The resulting solution was stirred overnight at 4°; dialyzed against 0.1 mM PMSF - 10 mM Tris (pH 8) for 24 hr; then dialyzed extensively against 10 mM Tris (pH 8). This procedure was effective in inactivating endogenous protease since subsequent incubation of chromatin for 8 hr at 37° followed by analysis of the disc electrophoretic patterns of histones and NHC proteins revealed no detectable breakdown.

Assay of Chromatin-bound Protease Activity. Protease activity was assayed as previously described (Garrels et al., 1972). The complete assay mixture contained the following components in a final volume of 0.2 ml: NaCl, 40 μ moles; Tris (pH 8), 2.0 μ moles; histones, 0.2 mg; and enzyme. After incubation in sealed test tubes for 20 hr at 37°, 0.5 ml of ninhydrin reagent was added to each assay sample. (Ninhydrin reagent is 0.4 gm ninhydrin, 80 ml of 95% ethanol, 1 g $CdCl_2$, 10 ml acetic acid and 20 ml water.) Similar assay mixtures but without addition of either substrate or enzyme serve as controls. The assay tubes were capped, placed in a boiling water bath for 4 min, cooled quickly and absorbance at 506 nm was measured. The sum of the absorbance values of minus enzyme and minus substrate controls was subtracted from the absorbance values of the complete reaction mixtures. One unit of protease activity is arbitrarily defined as the amount of enzyme that causes a change in absorbance at 506 nm of 0.01 above control values after 20 hr incubation.

Purification of Chromatin-bound Protease. All subsequent operations were carried out at 4°, unless otherwise mentioned. To purified sheared chromatin (20 A_{260}/ml in 10 mM Tris, pH 8) was added crystalline sodium chloride with stirring to achieve a final concentration of 0.7 M. After additional stirring for 4 hr, the resulting solution was subjected to gel filtration by chromatography on Bio-Gel A-50 (4 x 100 cm column) equilibrated with and eluted by 0.7 M NaCl - 10 mM Tris (pH 8). Ten milliliter fractions were collected. Fractions of the included protein peak were pooled , mixed with Bio-Rex 70 resin (2 mg of protein per gram of resin) previously equilibrated with 0.4 M NaCl - 10 mM Tris (pH 8) and the slurry was dialyzed against the same buffer. Columns were poured and washed with the same buffer. Nonabsorbed protein was collected; and concentrated by ultrafiltration (Amicon PM 10 membrane). The concentrated protein solution was then applied to a Sepharose 6B column (2.5 x 116 cm) equilibrated and eluted by 0.4 M NaCl - 10 mM Tris (pH 8). Fractions containing the protease were pooled and mixed with calcium phosphate gel at the ratio of 1 mg protein per ml gel. After stirring for 15 min, the resulting slurry was centrifuged at 10,000 g for 10 min. The pellet was collected and extracted with 0.4 M NaCl - 10 mM sodium phosphate buffer (pH 8) and centrifuged as above. The resulting clear supernatant was dialyzed against 10 mM Tris (pH 7) and was applied to a QAE Sephadex A-25 column (0.9 x 25 cm) previously equilibrated with 10 mM Tris (pH 7). The column was washed with the same buffer extensively and eluted with a linear salt gradient from 0 to 0.3 M NaCl - 10 mM Tris (pH 7) in a total volume of 300 ml. The purified enzyme was eluted at 0.1 M NaCl concentration.

Sucrose Gradient Centrifugation. Linear sucrose gradients (5% to 20%) in 0.1 M NaCl - 10 mM Tris (pH 8) were prepared according to the method of Martin and Ames (1961). Approximately 200 units of protease enzyme in 50 μl was layered on a 5 ml gradient.

Human γ-globulin (1 mg in 50 μl) served as a standard (sedimentation coefficient = 7.0 S, MW = 160,000).

Gradients were centrifuged at 116,000 g in the SW 50 rotor for 10 hr at 3°. Samples were collected by punching a hole in the bottom of each tube with a needle and collecting drops; 8 drops per fraction were collected.

Enzyme activity was assayed in each fraction and human γ-globulin was measured by A_{280}. The sedimentation coefficient(s) was calculated using the formula

$$\frac{S_1}{S_2} = \left(\frac{MW_1}{MW_2}\right)^{2/3} \qquad \text{(Schachman, 1959)}$$

Purification of Histones by Acid Extraction. For protease assays, disc electrophoresis and column chromatography, histones were prepared by acid extraction of purified sheared chromatin followed by precipitation of the extracted histones with ethanol (Bonner et al., 1968). Chromatin solutions, in 10 mM Tris (pH 8), were adjusted to 10 A_{260} per ml and 1/4 volume of cold 2 N sulfuric acid was added dropwise with stirring at 4°. After 30 min of additional stirring, the solution was centrifuged at 15,000 g for 15 min. To the supernatant, four volumes of cold absolute ethanol was added.

After storage at -20° for a minimum of 24 hr, histones were pelleted by centrifugation at 15,000 g for 15 min. The white pellets were resuspended in cold absolute ethanol and centrifuged at 15,000 g for 15 min. This step was repeated three times. Traces of ethanol were removed by lyophilization.

Preparation of Cytosol. Rat liver cytosol was prepared according to the method described by Kadenbach and Urban (1968). Rat liver was harvested, minced, homogenized and filtered identically to that described above (see Preparation of Nuclei section). The filtrate was centrifuged for 1 hr at 130,000 g at 4° using a SW 39 rotor. The resulting supernatant (cytosol fraction) was treated with 5 M urea - 0.125 M NaOH overnight at 4° to inactivate endogenous proteolytic enzymes. The denatured cytosol was dialyzed exhaustively against 10 mM Tris (pH 7).

Preparation of NHC Proteins. NHC proteins were prepared from rat liver chromatin according to the method of van den Broek *et al*., (1973) with a slight modification. Sucrose purified chromatin was adjusted to 10 A_{260} per ml with 10 mM Tris (pH 8). To the chromatin solution, crystals of sodium chloride were added with vigorous stirring to achieve a final concentration of 4 M. The mixture was sheared in a Virtis homogenizer (30 V - 90 sec) and stirred at 4° for 1 hr. DNA was removed by centrifugation at 215,000 g for 18 hr at 4° in a fixed angle rotor. The supernatant was collected and mixed with Bio-Rex 70 resin previously equilibrated with 0.4 M NaCl-10 mM Tris (pH 7). One gram of resin was used for each 5 mg of protein. The resulting slurry was dialyzed extensively against 0.4 M NaCl- 10 mM Tris (pH 7) and a column was poured. The protein that was not bound to the resin and which was eluted upon washing with 0.4 M NaCl- 10 mM Tris (pH 7) was collected and consists of the NHC proteins.

Urea Gel Disc Electrophoresis of Histones. Histones were dissolved in deionized 5 M urea- 0.9 N acetic acid- 1% (v/v) 2-mercaptoethanol at a concentration of about 1.0 mg per ml. Traces of insoluble material were removed by centrifugation. The protein concentration of the supernatant was determined by a modification of method of Kuno and Kihara (1967) using 25% trichloroacetic acid for precipitation and 0.5 mg per ml amidoschwartz in the staining solution. Bovine serum albumin was used as standard.

Disc electrophoresis was performed by the method Panyim and Chalkey (1969) using 15% polyacrylamide gels and 2.5 M urea at a final pH of 3.2 (after pre-electrophoresis). Approximately 30 μg of histones were applied to the gels, which were run under constant current (2 mA per gel) for 3.5 hr. At the end of electrophoresis, the gels were immersed in ice and removed by rimming tubes with a syringe containing a 2% SDS solution. After staining for 2 hr with 1% amidoschwartz in 40% ethanol - 7% acetic acid, the gels were destained by diffusion against 20% ethanol - 7% acetic acid. Gels were scanned at 600 nm using a Gilford 2000 spectrophotometer equipped with a linear transport. Pictures were taken using a Polaroid camera with an orange filter.

Preparation of Nonhistone Chromosomal Protein for Disc Electrophoresis. Nonhistone chromosomal protein was prepared by a modification of a previously described technique (Elgin and Bonner, 1970). Histone was removed from sheared chromatin preparations by two cycles of acid extraction. The residue was rinsed with 0.1 M Tris (pH 8) to remove traces of acid, and dissolved by homogenization in 2% SDS - 65 mM Tris (pH 6.8) to yield approximately 60 A_{260} units per ml final concentration. When necessary the pH of the resulting solution was adjusted to neutrality by the addition of crystals of Tris base. After stirring for 18 hr at 37°, the samples were centrifuged at 180,000 x g for 24 hr at 20° to pellet DNA. The resulting supernatants were dialyzed against the above pH 6.8 buffer. Prior to electrophoresis, 5% 2-mercaptoethanol was added and samples were heated for 1.5 min at 100°. After samples had cooled, glycerol was added to 10% (v/v). Protein concentrations were determined by a dye-binding assay (see Urea Gel Disc Electrophoresis section) using crystalline bovine serum albumin as standard.

SDS Disc Electrophoresis of NHC Protein. Protein samples, in 2% SDS - 55 mM Tris (pH 6.8) - 4.5% 2-mercaptoethanol - 10% glycerol - 0.001% bromophenol blue, were subjected to disc electrophoresis according to the method of King and Laemmli (1971). However, 0.384 M glycine - 0.1% SDS (titrated to pH 8.3 with crystals of Tris base) was used as the tray buffer. Polyacrylamide gels (10% w/v) of 0.6 x 12 cm dimensions were run at constant current (1.5 mA per gel until the sample had entered the stacking gel, 3 mA per gel thereafter) until the tracking dye had migrated 10 cm. Gels were stained 3 hr with 0.25% Coomassie brilliant blue R-250 in 5:5:1 water - methanol - acetic acid, and destained by diffusion against 10% acetic acid - 5% methanol. Gels were scanned and photographed as described above.

Isolation of Nuclear DNA from Rat Liver. Chromatin was partially purified from 40 g of rat liver by the method of Elgin and Bonner (1970) up to the sucrose step. The resulting chromatin solution (50 ml in 10 mM Tris, pH 8) was added to 100 ml of 1% SDS - 0.1 M Tris (pH 8) and stirred at room temperature until a homogeneous solution was obtained. An equal volume of freshly distilled phenol was added and the mixture was shaken gently at room temperature for 15 min. The solution was centrifuged at 10,000 g for 15 min and the aqueous phase was collected with a large mouth pipette. The resulting material was shaken gently with an equal volume of 24:1 chloroform - octanol, centrifuged at 1,000 g for 15 min. The aqueous phase was collected and reextracted as before. To the resulting aqueous phase was added two volumes of cold absolute alcohol. DNA was spooled out and dissolved in 50 ml of 1 x SSC by shaking slowly overnight at 4°. The resulting DNA solution was subjected to RNase (50 μg/ml) and α-amylase (200 μg/ml) treatments for 1 hr at 37°. (RNase was pretreated by boiling for 5 min to denature DNase.) This incubation mixture was followed with 3-hr treatment with pronase at 100 μg/ml (pronase was preincubated for 1 hr at 37° in 10 mM Tris, pH 8). The DNA solution was then extracted with phenol as above. DNA was spooled out of the aqueous phase after addition of ethanol. The resulting DNA material was dissolved in 50 ml of 1 x SSC and dialyzed extensively against $\frac{1}{100}$ x SSC.

Purification of Histone I. Total histones (150 mg) were prepared by acid extraction of rat liver chromatin dissolved in 8% GuCl - 0.1 M sodium phosphate buffer (pH 7) and applied to a Bio-Rex 70 column (2.5 x 70 cm) previously equilibrated with the same buffer. After washing the column extensively with the same buffer, protein was eluted with a linear 2 liter gradient of 8% to 13% GuCl - 0.1 M sodium phosphate buffer (pH 7). Ten milliliter fractions were collected. Protein was measured by turbidity at 400 nm following the addition of 1.1 M TCA (Bonner et al., 1968). The elution profile and disc electrophoretic patterns of the fractions are shown on Figure A. Fractions

60 to 105 were pooled, dialyzed extensively against 0.1 M acetic acid and lyophilized. The resulting material (25 mg protein) was dissolved in 10 ml of 0.01 N HCl and chromatographed at room temperature by gel filtration on Sephadex G-100 column (5 x 150 cm) equilibrated with an eluted by 0.01 N HCl. Ten milliliter fractions were collected. The elution profile is shown in Figure B. The first protein peak containing 10 mg of protein was judged to be pure histone I by disc gel electrophoresis.

Preparation of DNA - Histone I Complex. Reconstitution was performed by a modification of the procedure of Shih and Bonner (1970). Five milliliters of DNA (1.85 mg/ml in 5 M NaCl - 5 M urea - 10 mM Tris, pH 8) was mixed by vigorous stirring with an equal volume of histone I solution (0.74 mg/ml in 10 mM Tris, pH 8). The histone: DNA mass ratio was thus 0.4. All the following steps were carried out at 4°. The mixture was dialyzed successively against a step gradient of NaCl of concentrations 2 M, 1.5 M, 1.0 M, 0.8 M, 0.6 M, 0.4 M, and 0.2 M; all solutions contained 5 M urea - 10 mM Tris (pH 8). The resulting material was then dialyzed against 5 M and 2.5 M urea - 10 mM Tris (pH 8). Each of the above dialysis steps was for 4 hr. The urea was removed by exhaustive dialysis against 10 mM Tris (pH 8). The resulting complex was pelleted by centrifugation at 200,000 g for 15 hr at 4°, yielding a transparent gel-like material with a histone I: DNA mass ratio 0.4

FIGURE A

Chromatography of rat liver histones on Bio-Rex 70. Total histones (150 mg) were dissolved in 8% GuCl - 0.1 M sodium phosphate buffer (pH 7) and applied to the column (2.5 x 70 cm). Histones were eluted with a linear gradient of 8% to 13% GuCl in 0.1 M sodium phosphate buffer (pH 7). Ten milliliter fractions were collected. Protein was measured by turbidity at 400 nm following the addition of 1.1 M TCA.

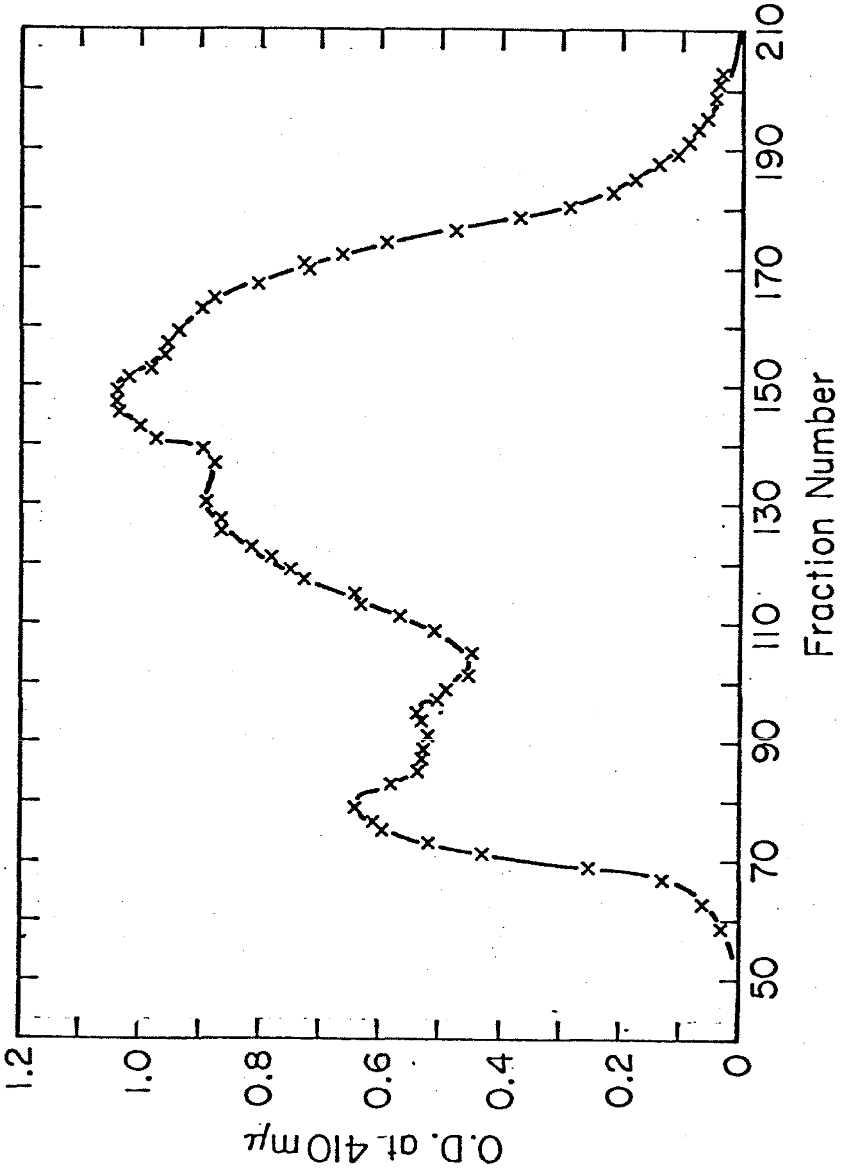
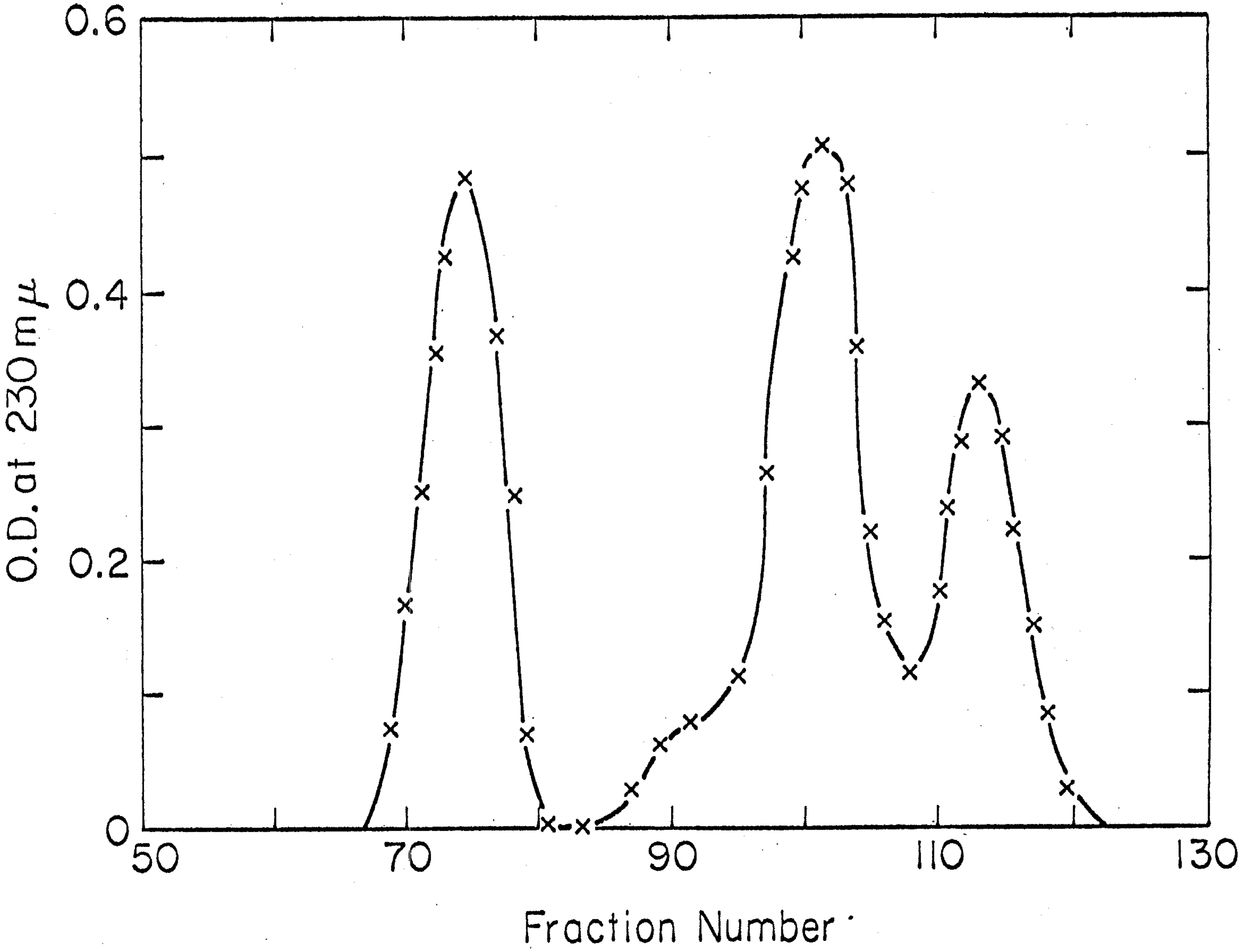

O.D. at 410 mμ
1.2
1.0
0.8
0.6
0.4
0.2
0
Fraction Number
50
70
90
110
130
150
170
190
210

FIGURE B

Chromatography of rat liver histones on Sephadex G-100. Histones (25 mg) was dissolved in 10 ml of 0.01 N HCl and applied to the column (5 x 150 cm). Histones were eluted with 0.01 N HCl. Ten milliliter fractions were collected, protein was measured by O. D. at 230 m .


O.D. at 230 mμ
0.6
0.4
0.2
0
50
70
90
110
130
Fraction Number

and a recovery of 70%.

N-Terminal Analysis. Qualitative N-terminal analysis was carried out by the method of Woods and Wang (1967). Approximately 20 µg of protein in 20 µl of 0.1 M $NaHCO_3$ contained in a 6 mm x 15 mm test tube was dansylated by the addition of 20 µl of dansylchloride in acetone (1 mg/ml). The tube, covered with parafilm, remained at 37^o for 2 hr; the cover was removed, and the contents were evaporated to dryness. A 50 µl volume of 6 M HCl was added; the tube was sealed and placed in a 105^o oven for 16 hr, then cooled and opened; its contents were evaporated to dryness. The reaction products were dissolved in 50% of pyridine and analyzed by thin layer chromatography on polyamide sheets in a series of solvents (Woods and Wang, 1967). The dansyl amino acids were identified by their migration positions relative to the known pattern and to standards spotted on the back of the same polyamide sheet.

Chemical Composition. Histone was determined by measurement of absorbance at 230 nm (ϵ = 3.5 l per cm g; Bonner et al., 1968) and by the method of Lowry et al., (1951), using crystal bovine serum albumin as standard. The two methods agreed well. NHC protein was determined by a dye-binding assay, see "Urea Gel Disc Electrophoresis" section and by the method of Lowry et al., (1951). DNA was determined by measurement of absorbance at 260 nm (ϵ = 20 ℓ per cm g).

RESULTS

Subnuclear Distribution of Protease Activity. The protease activity toward histone substrate, which has been reported by others (Garrels et al., 1972) to be in either nuclei or chromatin of rat liver, is not a contaminant. Washing nuclei with Triton X-100 to remove the outer nuclear membrane together with cytoplasmic contamination does not reduce the yield of protease activity (Table I). Furthermore approximately 88% of this activity is localized in the chromatin fraction; the remaining activity is associated with the nuclear membrane fraction. The specific activity of protease is slightly higher in the chromatin fraction compared to that of nuclear membrane fraction (see Table I) suggesting that the activity associated with the membrane fraction may be due to residual chromatin contamination. Since no activity could be detected in the nucleoplasmic fraction, the possibility remained that this fraction contained an inhibitor of the protease activity. However, no evidence for the presence of an inhibitor was found by mixing experiments (Table I).

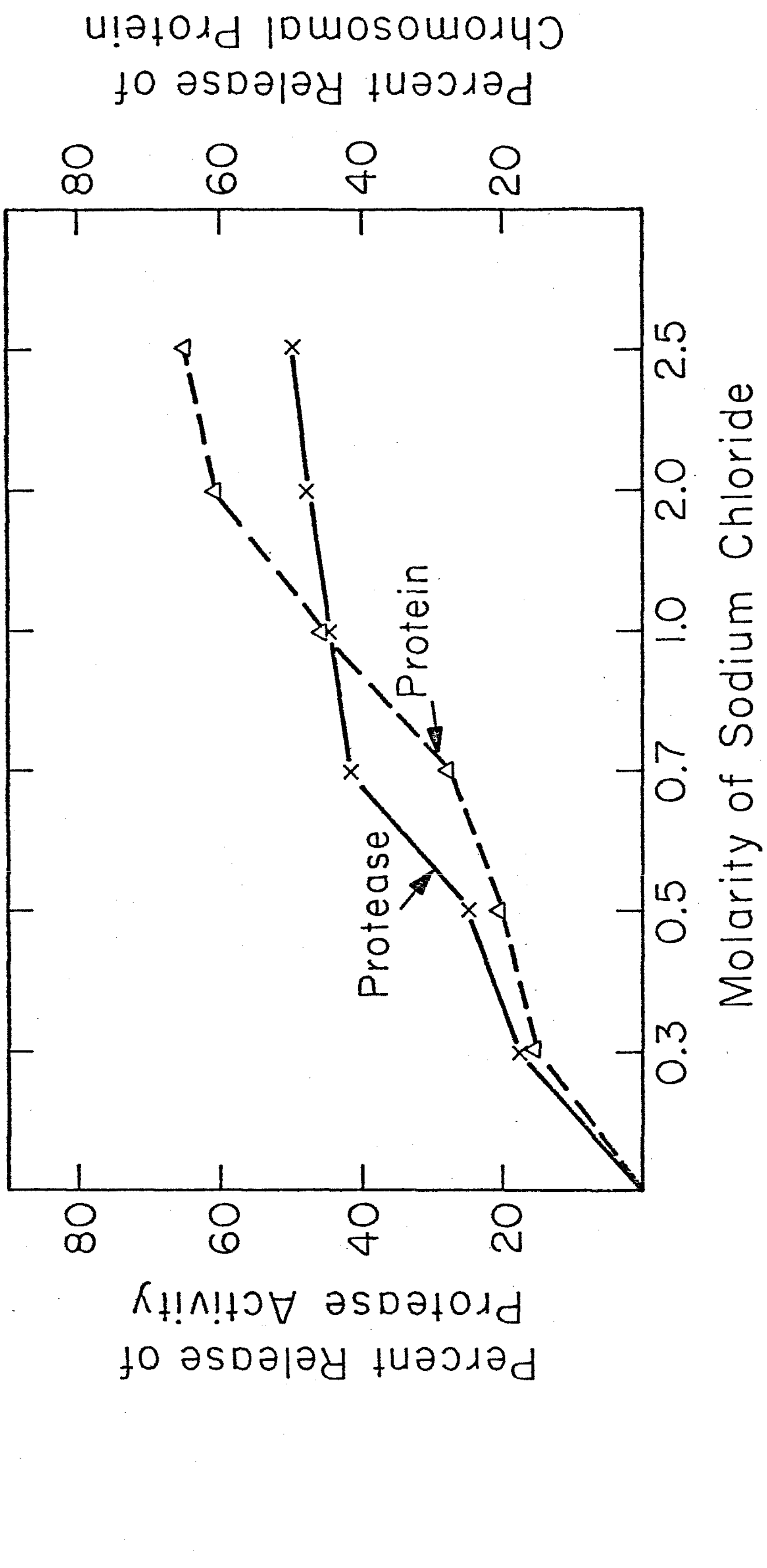
As shown in Figure 1, the protease activity is primarily bound to isolated chromatin. When chromosomal protein was released from DNA by treatment with increasing concentrations of sodium chloride, only 18% of protease activity was liberated at 0.3 M salt. Even at 2.5 M sodium chloride only

TABLE I: Subnuclear Distribution of Protease Activity.

Experiment #	Nuclear Fraction	Total Protein (µg)	Total Units of Protease Activity	% of Total Protease Activity	Specific Activity (units/µg Protein)
1	Whole nuclei	8,150	3,200 ± 160	-	0.40 ± 0.02
	Triton X-100 washed nuclei	7,700	2,920 ± 160	-	0.38 ± 0.02
2	Nucleoplasm	1,800	0	0	0
	Nuclear membrane	850	391 ± 18	12	0.46 ± 0.02
	Chromatin	5,500	2,860 ± 100	88	0.52 ± 0.02
3	Chromatin alone	95	50	-	-
	Nucleoplasm alone	35	0	-	-
	Chromatin & nucleoplasm	130	50	-	-

FIGURE 1

Effects of different salt concentrations on the extraction of protease from chromatin. Chromatin (10 A_{260}/ml in 10 mM Tris) was treated with different concentrations of sodium chloride overnight. The resulting solutions were centrifuged at 177,700 g for 8 hr. The protease activities and protein concentrations were measured for the supernatants and pellets separately.


Percent Release of Protease Activity
80
60
40
20
Percent Release of Chromosomal Protein
80
60
40
20
0.3
0.5
0.7
1.0
2.0
2.5
Molarity of Sodium Chloride
Protease
Protein

50% release of protease activity could be achieved. Routinely, the enzyme was released from chromatin by use of 0.7 M NaCl for reasons clear from Figure 1.

Purification of Chromatin-bound Protease. Table II summarizes the steps utilized to purify chromatin-bound protease to homogeneity. The procedure adopted resulted in a 705-fold purification over total chromosomal protein with a yield of activity of 1.2%. Standard methods were used; these included chromatography on Bio-Rex, Sepharose 6B (Figure 2) and QAE - Sepahadex A-25 (Figure 3). The single most effective purification step, based in the criteria of fold purification and recovery, was batchwise calcium phosphate gel adsorption (Table II).

Molecular Properties of Purified Protease. The molecular weight of the native purified enzyme was estimated to be approximately 200,000 by gel filtration (Figure 4) and about 190,000 by sucrose gradient centrifugation (sedimentation coefficient = 7.8 S, see Figure 5). A single symmetrical peak of protein was obtained upon subjecting the purified protease to SDS disc electrophoresis in the presence of reducing agent (Figure 6). Densitometry indicated that over 95% of the applied protein migrated in this band, which had an estimated molecular weight of 100,000 relative to the mobilities of standards (Figure 7). It is thus concluded that the native enzyme is composed of two identical subunits. Upon the basis of the fold-

TABLE II: Purification of Chromatin-bound Protease.

Purification Step	Volume (ml)	Protein Concentration (μg/ml)	Total Protein (μg)	Activity (units/ ml)	Total Activity (units)	Yield (%)	Specific Activity (units/μg Protein)	Fold of Purification
Total chromatin	600	950	570,000	500	300,000	100	0.52	1
Bio-Gel A-50	600	200	120,000	210	126,000	42	1.05	2.1
Bio-Rex 70	12	2,500	30,000	3,600	44,000	14.6	1.46	2.8
Sepharose 6B	38	64	2,430	540	20,500	6.8	8.44	16.2
Calcium phosphate gel	15	8	120	800	12,000	4	100	192.3
QAE Sephadex	22	0.45	10	165	3,630	1.2	366.6	705.0

FIGURE 2

Chromatography of the protease activity on Sepharose 6B. Enzyme (44,000 units; 29,000 μg) in 12 ml of 0.4 M NaCl - 10 mM Tris (pH 8) buffer was applied to the column (2.5 x 116 cm). Elution was with the same buffer; 3.8 ml fractions were collected; flow rate was adjusted to 20 ml/hr; all procedures were carried out at 4°.

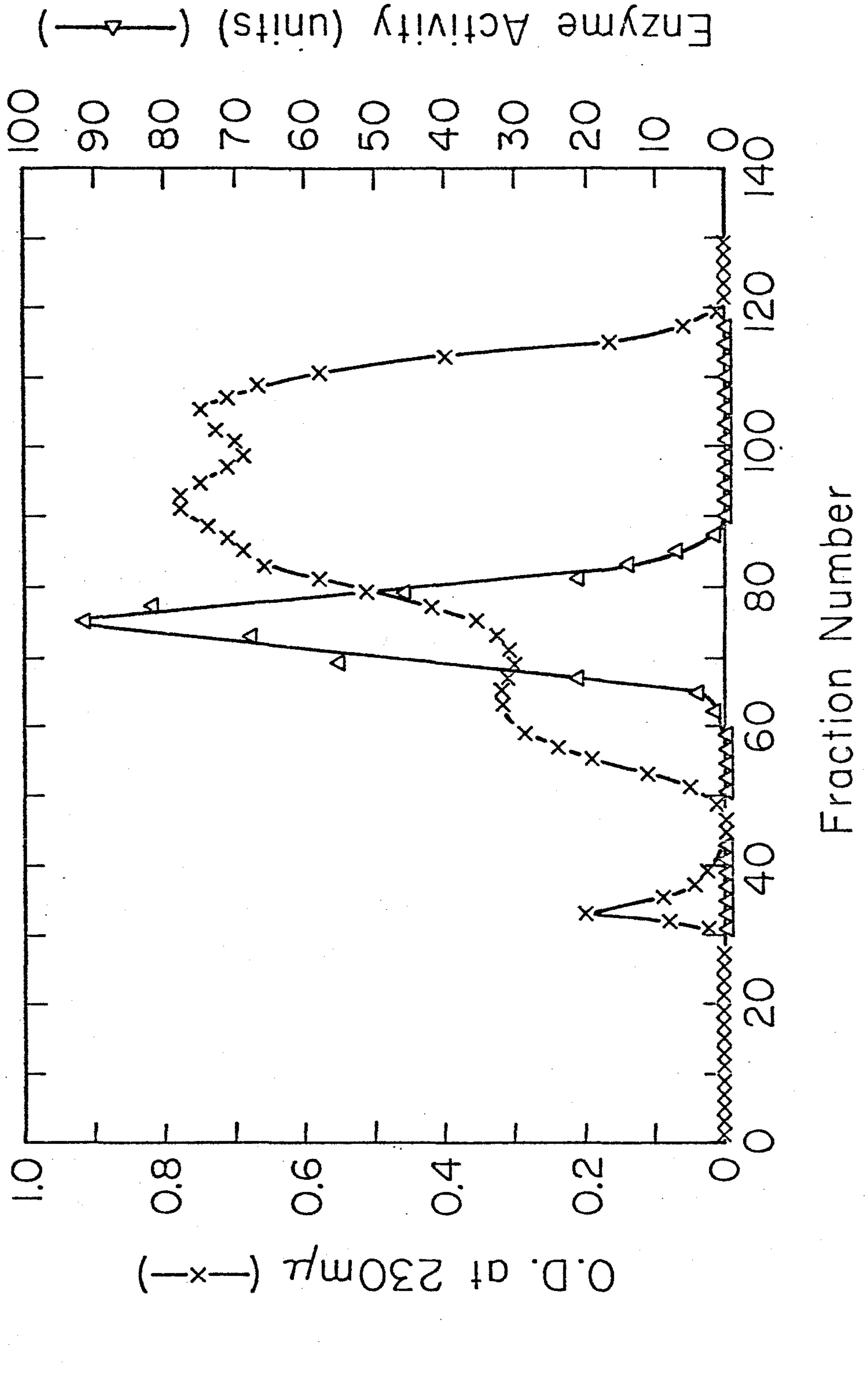

Enzyme Activity (units) (—▽—)
100
90
80
70
60
50
40
30
20
10
0
O.D. at 230mμ (—×—)
1.0
0.8
0.6
0.4
0.2
0
Fraction Number
0
20
40
60
80
100
120
140

FIGURE 3

Chromatography of the protease activity on QAE Sephadex A-25. Enzyme (12,000 units; 120 μg protein) in 15 ml of 10 mM Tris (pH 7) was applied to the column (0.9 x 25 cm). Elution was with a linear sodium chloride gradient from 0 to 0.3 M in 10 mM Tris (pH 7). 2.5 ml fractions were collected; flow rate was adjusted to 15 ml/hr; all procedures were carried out at 4°.

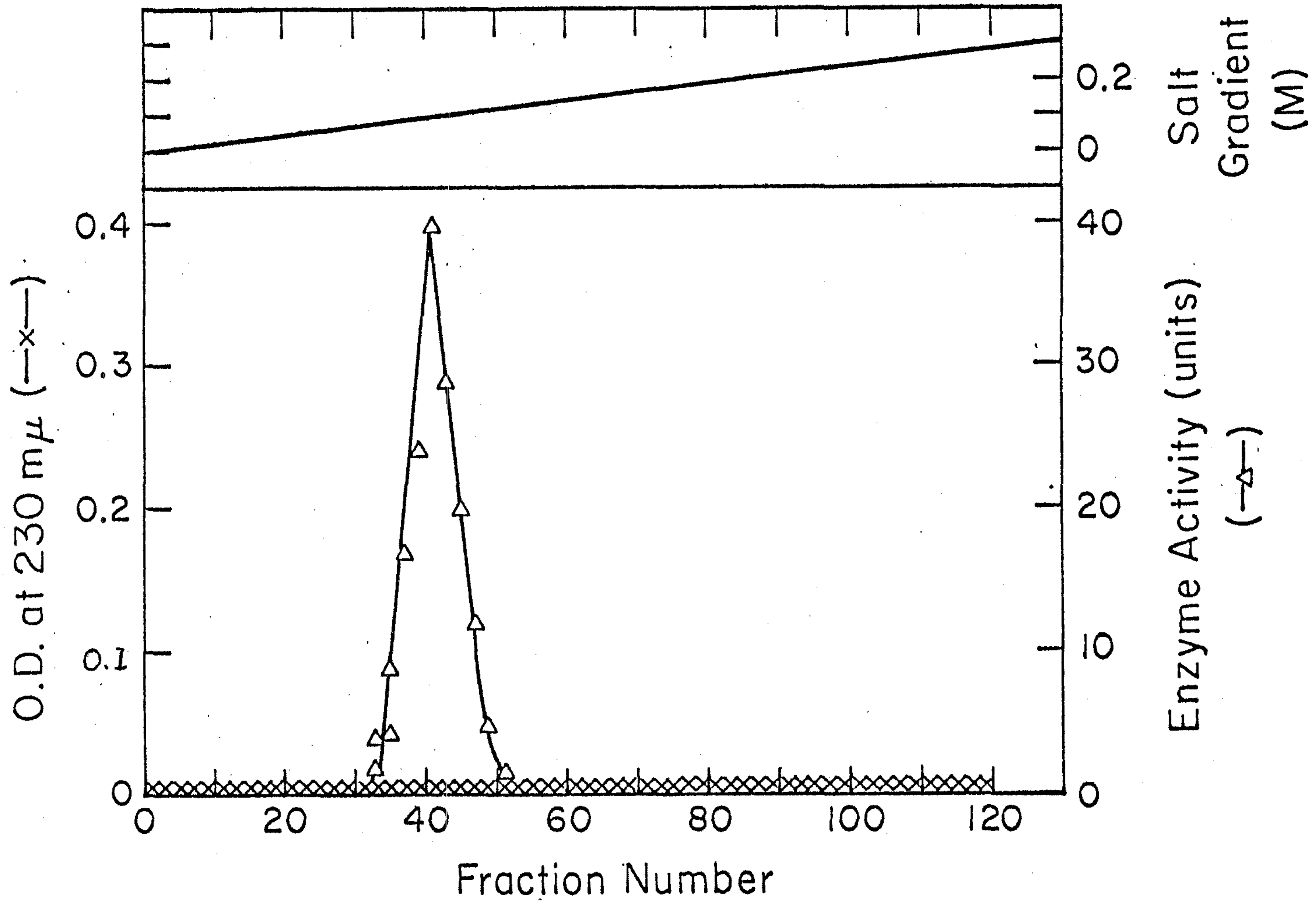

O.D. at 230 mμ (—x—)
0.4
0.3
0.2
0.1
0
0
20
40
60
80
100
120
Fraction Number
Salt Gradient (M)
0.2
0
Enzyme Activity (units) (—△—)
40
30
20
10
0

FIGURE 4

Estimation of the molecular weight of rat liver chromatin-bound protease by exclusion chromatography. The Sepharose 6B column was calibrated with proteins of known molecular weight.

The Kav

$$Kav = \frac{V_e - V_o}{V_t - V_o}$$

is plotted against the log of the molecular weight. The intercept for chromatin-bound protease activity (arrow) indicates a molecular weight of about 200,000.

V_e = elution volume

V_o = void volume

V_t = total volume of gel bed

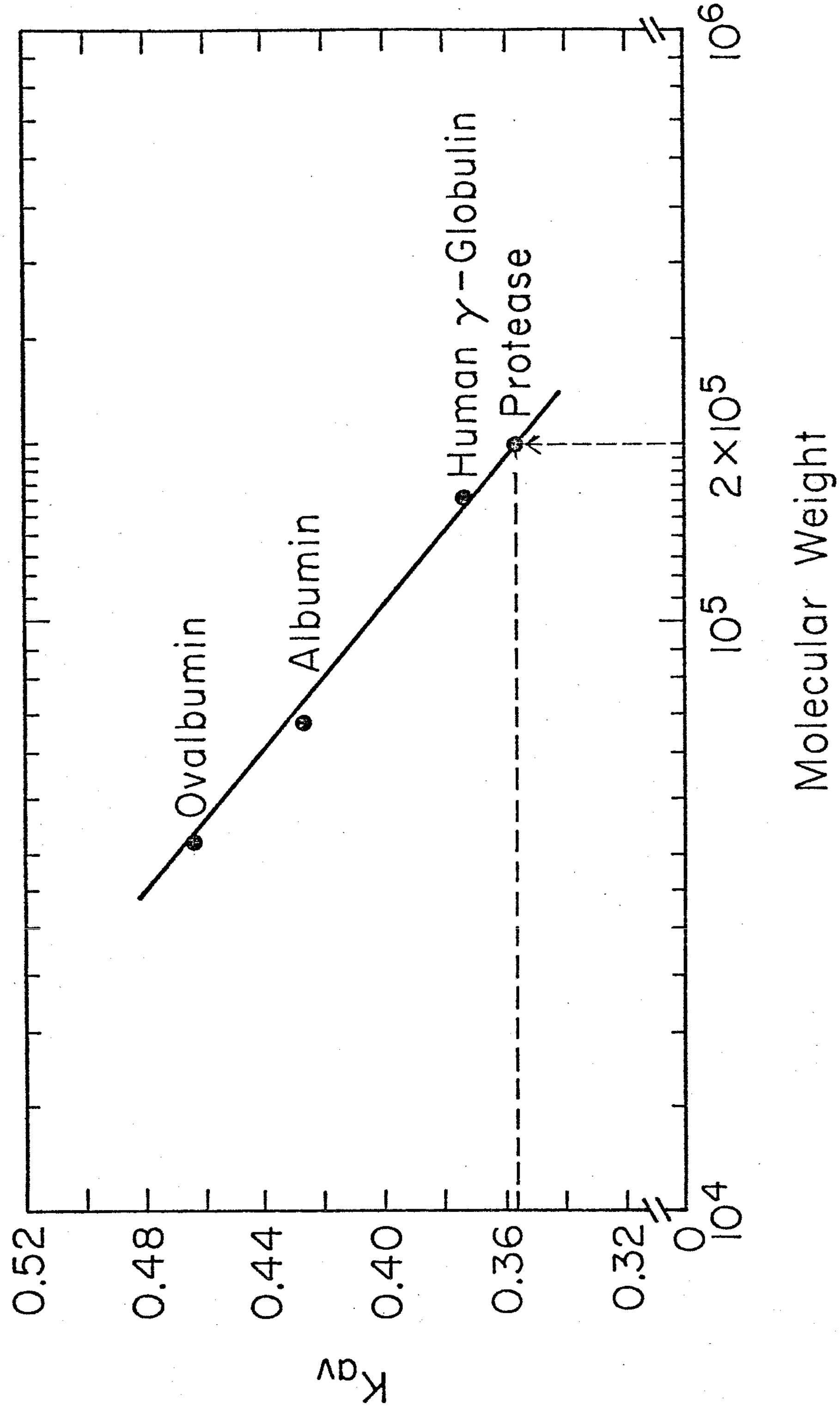
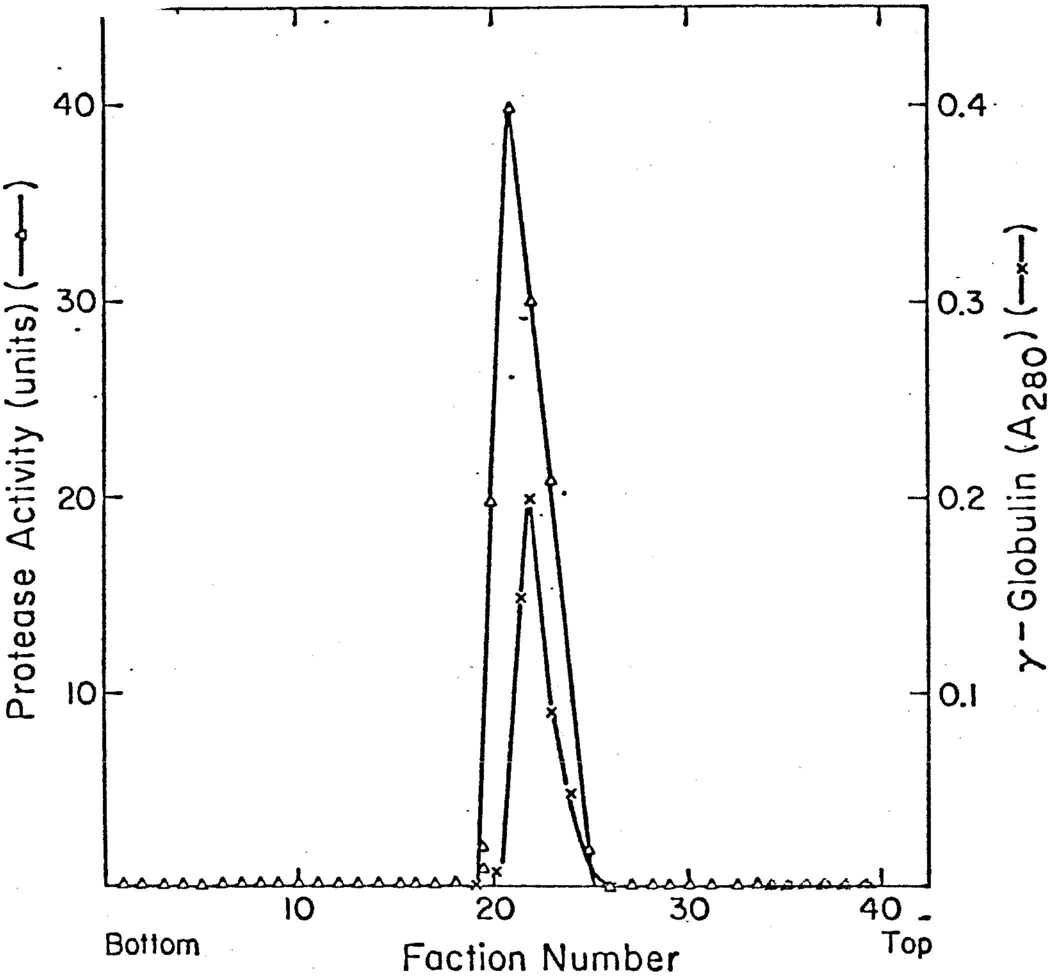

Kav
0.52
0.48
0.44
0.40
0.36
0.32
0
10^4
10^5
2×10^5
10^6
Molecular Weight
Ovalbumin
Albumin
Human γ-Globulin
Protease

FIGURE 5

Estimation of the molecular weight of rat liver chromatin-bound protease by sucrose gradient sedimentation. Enzyme (200 units; 0.5 μg protein in 50 μl of 0.1 M NaCl - 10 mM Tris, pH 7). Human γ- globulin (1 mg in 50 μl of 0.1 M NaCl - 10 mM Tris, pH 7) was used as an internal standard. Experiments were performed as given in Materials and Methods.



Protease Activity (units) (—△—)
γ – Globulin (A_{280}) (—×—)
40
30
20
10
0.4
0.3
0.2
0.1
10
20
30
40
Bottom
Top
Faction Number

FIGURE 6

Analysis of purified protease activity by SDS polyacrylamide gel electrophoresis. The enzyme was analyzed in 10% acrylamide gels in the presence of 0.1% SDS. The major band represents over 95% of the total protein applied to the gel (0.3 x 10 cm).

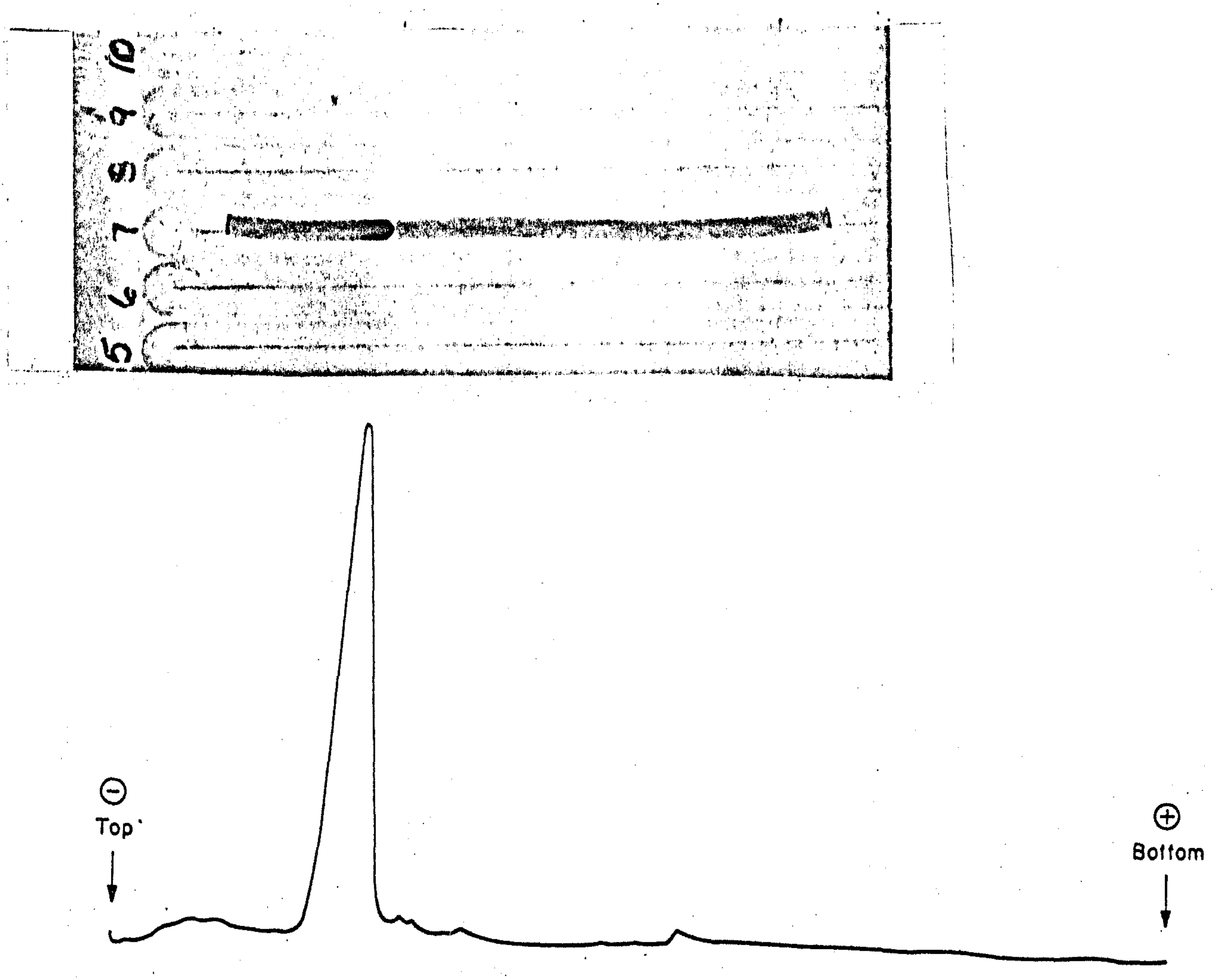
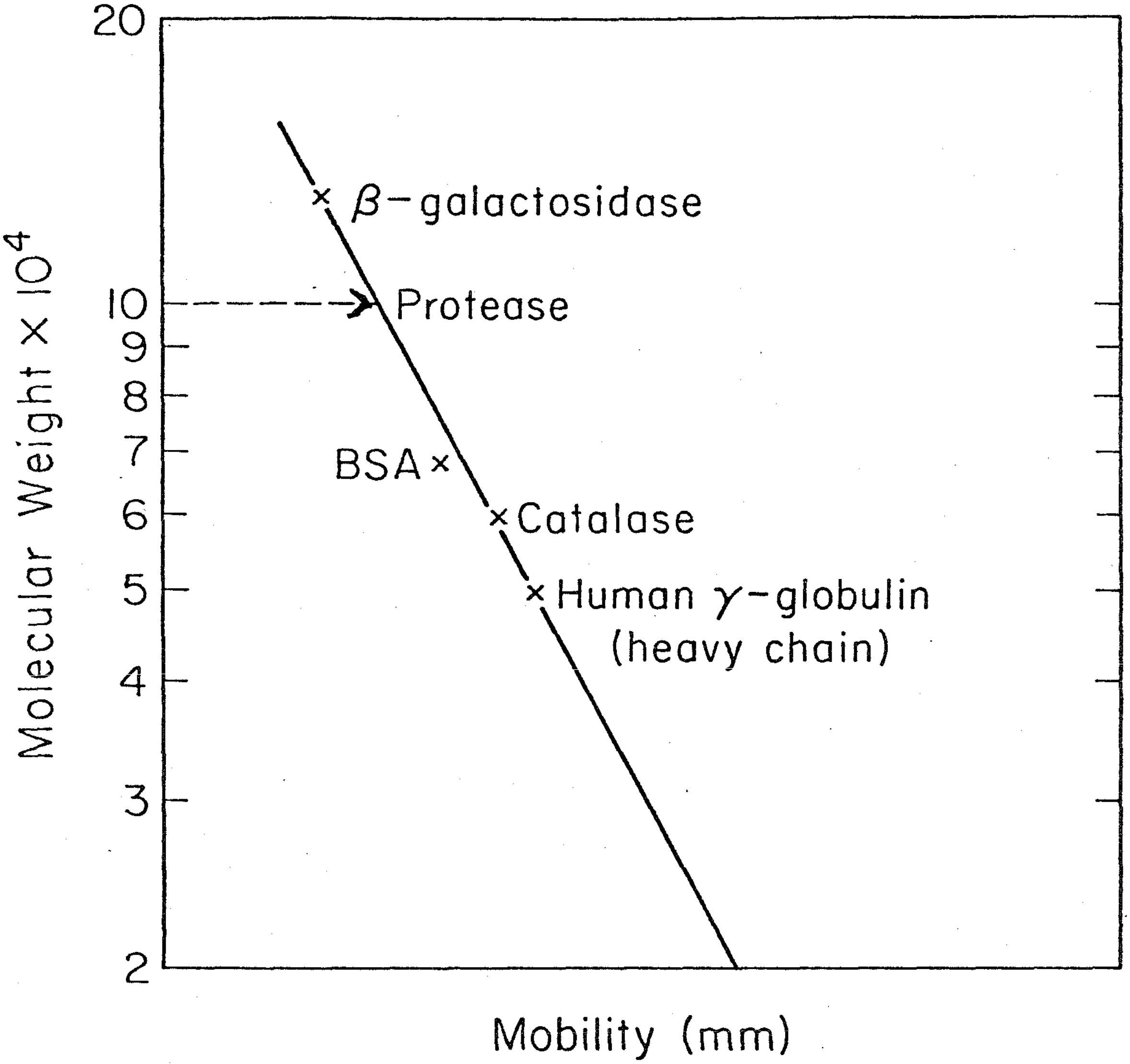

5
6
7
8
9
10
⊖
Top
⊕
Bottom

FIGURE 7

Estimation of the molecular weight of rat liver chromatin-bound protease by SDS polyacrylamide gel electrophoresis. The mobilities of different standard proteins are plotted against the log of the molecular weight. The intercept for chromatin-bound protease (arrow) indicates a molecular weight of about 100,000.


20
10
9
8
7
6
5
4
3
2
Molecular Weight × 10^4
Mobility (mm)
× β-galactosidase
Protease
BSA ×
× Catalase
× Human γ-globulin
(heavy chain)

purification data (Table II), assuming a molecular weight of the active enzyme of 200,000 and that the rat haploid genome size is 1.8 x 10^{12} daltons (Britten and Davidson, 1971), isolated rat liver chromatin would contain approximately 2.2 x 10^4 molecules of this enzyme per haploid genome. This estimate is subject to the further caveat that upon enzyme purification no activation or inactivation has occurred.

Effect of pH, Ionic Strength, Divalent Cations, and Inhibitors on Protease Activity. The purified enzyme, assayed using total histone substrate, shows a pH optimum of 7.0, and has a half maximal activity pH 6.0 suggesting that a histidine residue is important for activity (Figure 8). The enzyme is essentially inactive below pH 5.0 and above pH 10.0, presumably due to denaturation. The activity is maximal at 0.2 M sodium chloride, about twice the level observed at low ionic strengths (Figure 9). Above 1.0 M sodium chloride, the enzyme is inactive, but this effect is reversible. The enzyme is stable to storage for 4 months at -20°. However, as shown in Figure 10, 50% of the activity is lost after heating at 62° for 15 min.

Since 1 mM EDTA was found to be inhibitory to protease activity, the effect of divalent metal ions on reactivation was investigated (Table III). Except for Mg^{++}, all divalent metal ions tested were effective in reactivating the enzyme, Mn^{++}being the most efficient one. As shown in Figure 11, Mn^{++} even stimulates untreated enzyme preparations 1.95-fold

FIGURE 8

Effect of incubation pH on the protease activity. 50 units of enzyme in 0.2 M NaCl were incubated under different pH conditions. Enzyme activity assay was performed as given in Materials and Methods.

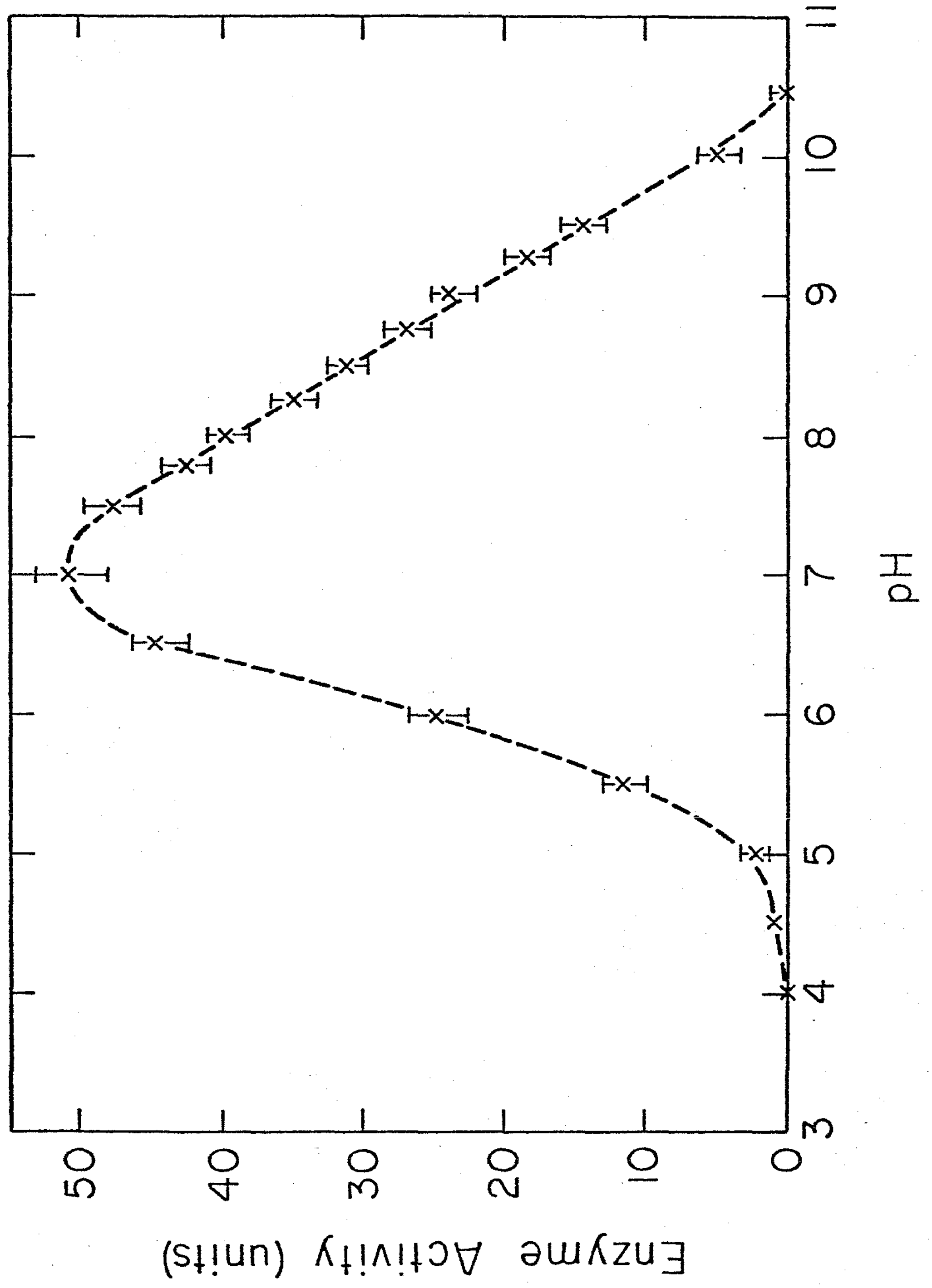

Enzyme Activity (units)
0
10
20
30
40
50
pH
3
4
5
6
7
8
9
10
11

FIGURE 9

Effects of salt concentration on the activity of chromatin-bound protease. Enzyme (40 units in 100 μl of 10 mM Tris, pH 7) was incubated with total histones (200 μg) as substrate and different salt concentration. Total volume of incubation mixture was 200 μl. Activity assay was performed as given in Materials and Methods.

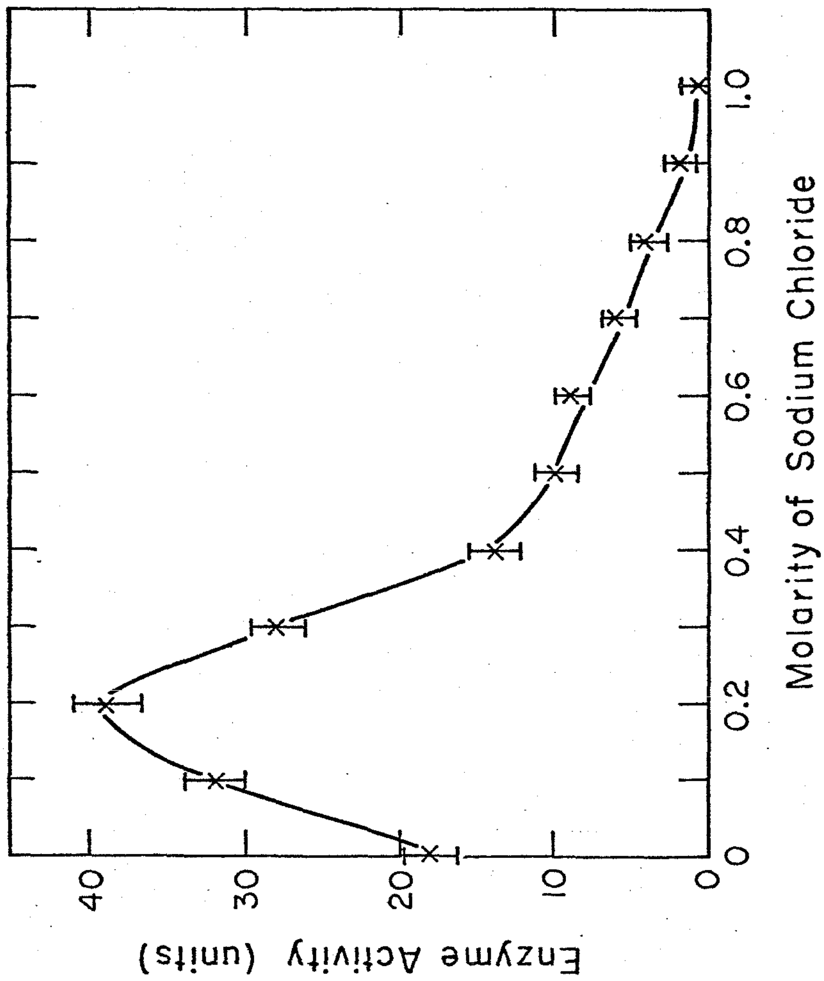

Enzyme Activity (units)
0
10
20
30
40
Molarity of Sodium Chloride
0
0.2
0.4
0.6
0.8
1.0

FIGURE 10

Temperature stability range of rat liver chromatin-bound protease. The enzyme was heated at the indicated temperature for 15 min and then assayed the activity at 37°. The activity is expressed relative to that of the unheated enzyme as 100%.

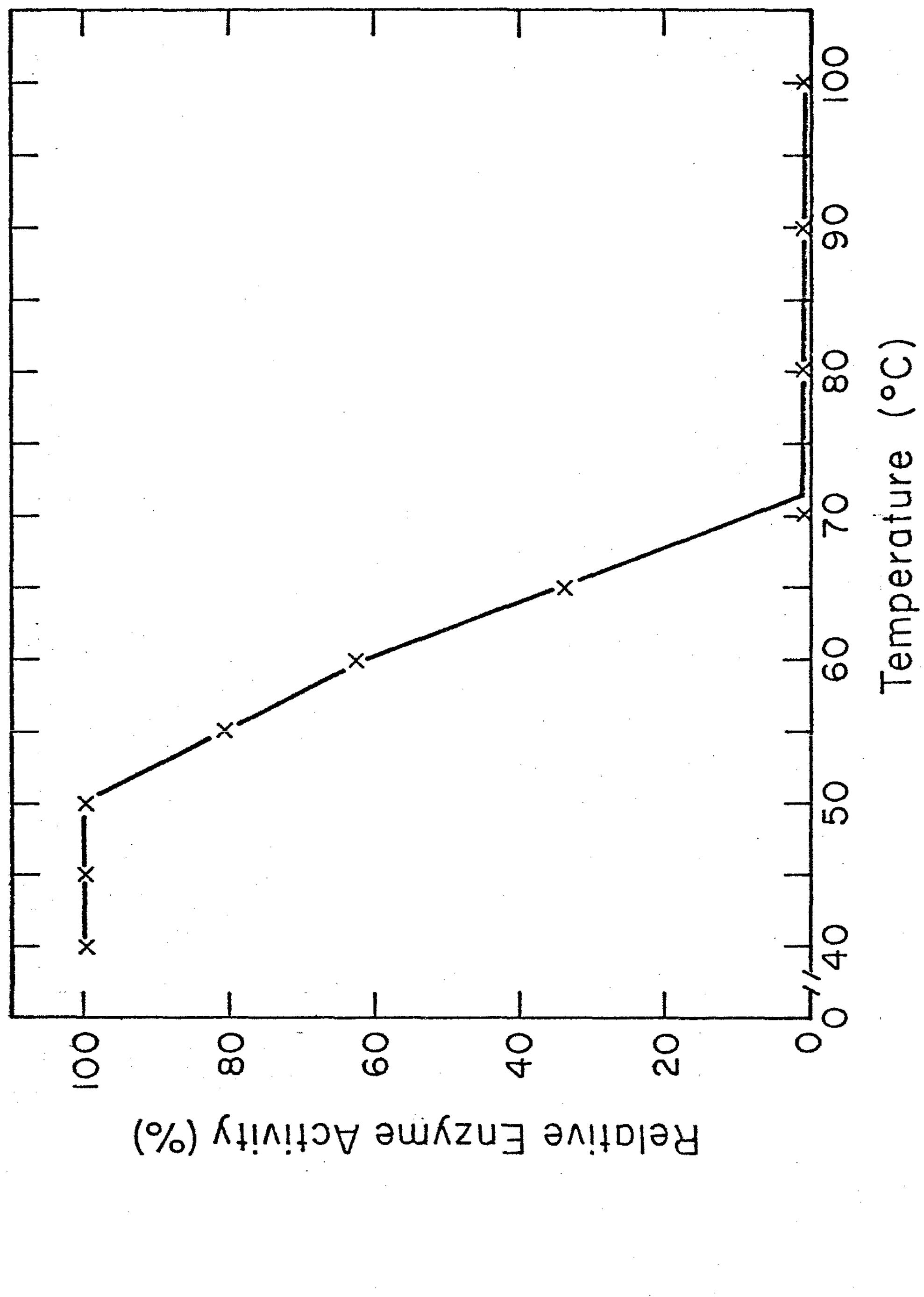

Relative Enzyme Activity (%)
100
80
60
40
20
0
Temperature (°C)
0
40
50
60
70
80
90
100

TABLE III: Effect of Divalent Metals on Protease Activity.

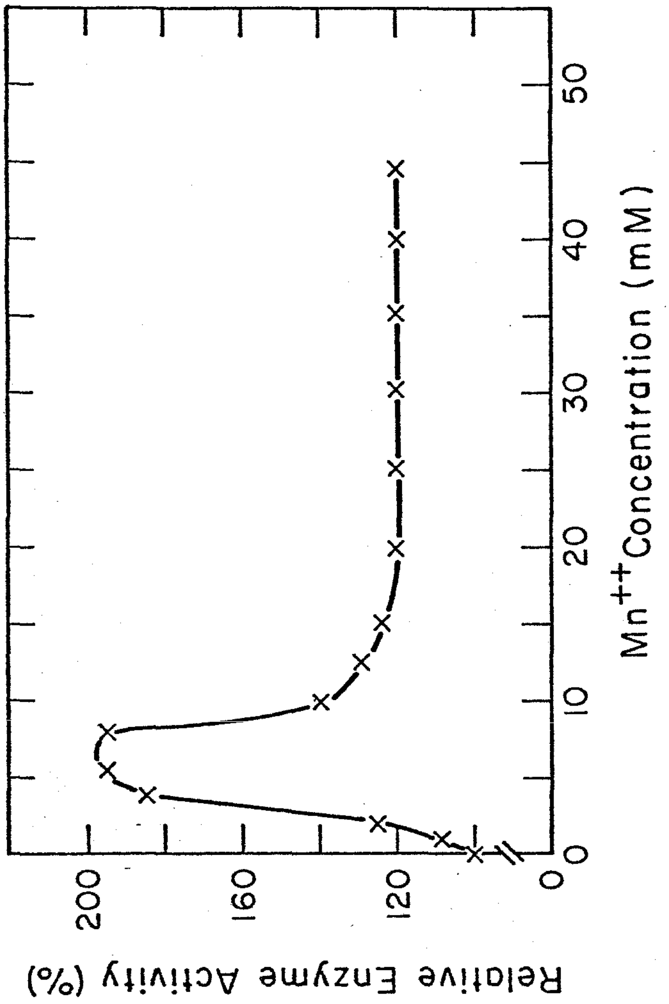
	Percent of Initial Activity	
Addition[a]	Control	Pretreated with 1 mM EDTA[b]
None	100	37
$CaCl_2$	100	100
$CdCl_2$	95	96
$MgCl_2$	42	37
$MnCl_2$	127	137
$ZnCl_2$	94	100

[a] All additions were at 1 mM final concentration.

[b] Protease was dialyzed against 1 mM EDTA - 10 mM Tris (pH 7) overnight; then the enzyme was dialyzed extensively against 10 mM Tris (pH 7) to remove excess EDTA.

FIGURE 11

Optimal concentration of Mn^{++} ion to activate chromatin-bound protease activity. The activity is expressed as relative to that of enzyme without addition of Mn^{++}.


Relative Enzyme Activity (%)
200
160
120
0
0
10
20
30
40
50
Mn^{++}Concentration (mM)

at concentrations of 6-8 mM. The inhibitory effects of Mg^{++} are likely due to precipitation of the histone substrate under the assay conditions used.

The effects of various inhibitors and denaturants of protease activity are shown in Table IV. High concentrations of reducing agents, and fairly low concentrations of heavy metals are inhibitory, suggesting that both free sulfhydryl groups as well as disulfide linkage are important for activity and structure function relationships. The enzyme activity is partially inhibited by 2 M urea, but completely abolished by 1% SDS or 0.85 M guanidine hydrochloride. The serine hydroxyl group reagents, DFP and PMSF, are potent inhibitors of the enzyme; sodium bisulfite is also effective but requires higher concentrations.

Substrate Specificity. Table V summarizes the substrate specificity of the purified protease. Both histones and NHC proteins were attacked preferentially compared to the other substrates tested, including rat liver cytosol. It is clear that the enzyme prefers nuclear proteins as substrates. In addition, it is interesting that L-poly-lysine was a very good substrate for the enzyme, but L-poly-arginine was a rather poor one. Denaturation of lysozyme and bovine serum albumin improved their abilities to serve as substrates, yet only to an efficiency of about 1/10 that of histones.

Figure 12 shows substrate saturation curves for enzyme activity on histones, nonhistones and cytosol of rat liver;

TABLE IV : Effect of Inhibitors and Denaturants on Chromatin-bound Protease.

Additions	Concentration	Percent of Initial Activity
None	-	100
DFP	0.1 mM	0
PMSF	1 mM	0
$NaHSO_3$	10 mM	0
Urea	2 M	40
SDS	1 %	0
GuCl	0.85 M	0
Mercaptoethanol	0.14 M	0
$HgCl_2$	1 mM	8.4
$CuSO_4$	1 mM	0
EDTA	1 mM	15.5

TABLE V: Substrate Specificity of Chromatin-bound Protease

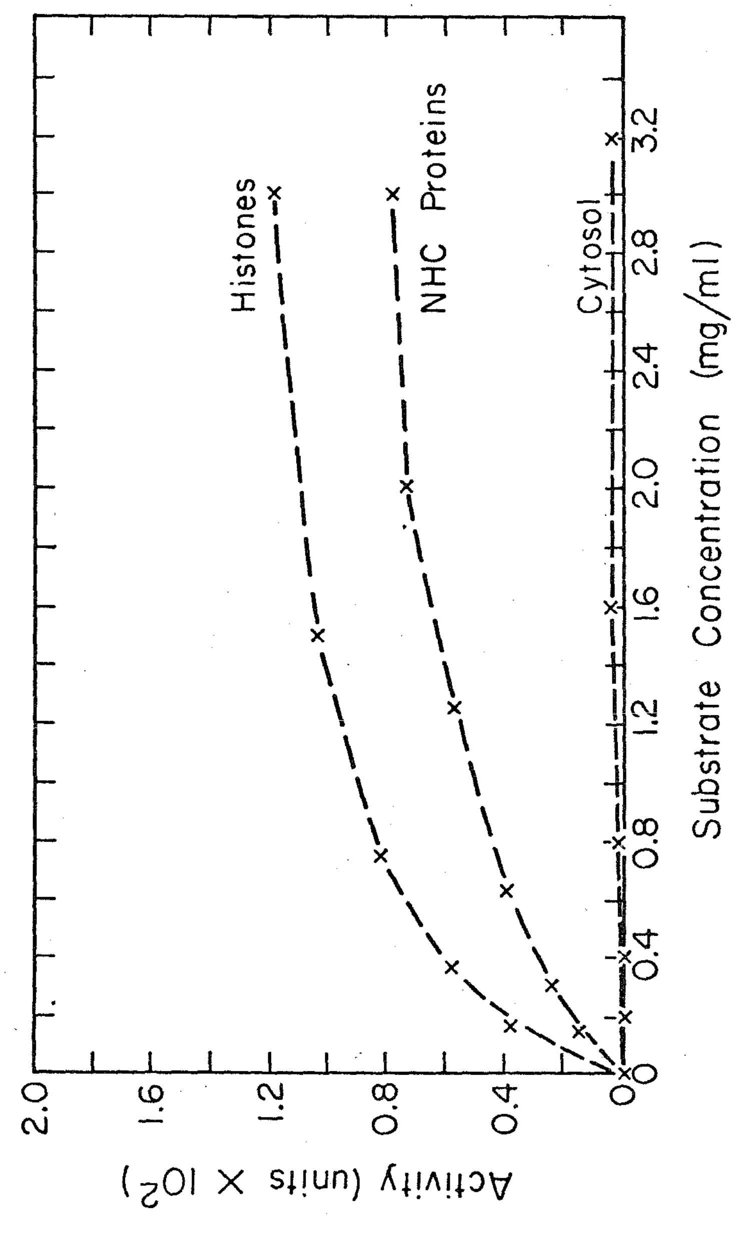
Substrate	Vmax	Vmax/Vmax of Histones
Histones	144	1.00
NHC protein	105	0.73
Cytosol[a]	3	0.02
Native chromatin-bound protein	78	0.54
L-polysine (MW = 5900)	150	1.04
L-polyarginine (MW = 40,000)	11.5	0.08
Protamines	10	0.07
Casein	30	0.21
Lysozyme (in native form)	0	0
Lysozyme (denatured)[b]	15	0.104
BSA (in native form)	0	0
BSA (denatured)[b]	10	0.07
Hemoglobin (denatured)[b]	0	0

[a] Rat liver cytosol was denatured in 0.125 M NaOH - 5 M urea overnight; dialyzed extensively against 10 mM Tris (pH 7) and assayed as indicated in Materials and Methods.

[b] The substrates were denatured in 0.125 M NaOH - 5 M urea for 1 hr, neutralized and assayed in 2.5 M urea, 0.2 M NaCl and 10 mM Tris (pH 7).

FIGURE 12

Kinetic study of chromatin-bound protease using rat liver histones, NHC proteins and cytosol as substrates. Rat liver cytosol was denatured in 0.125 M NaOH - 5 M urea overnight; dialyzed extensively against 10 mM Tris (pH 7) and assayed as indicated in Materials and Methods.


Activity (units X 10^2)
2.0
1.6
1.2
0.8
0.4
0
Substrate Concentration (mg/ml)
0
0.4
0.8
1.2
1.6
2.0
2.4
2.8
3.2
Histones
NHC Proteins
Cytosol

these data again indicate a strong preference of the enzyme for nuclear protein substrates. A modified Lineweaver-Burk plot of these data reveals that the K_mS for histones and NHC proteins are 0.5 mg/ml and 1 mg/ml respectively (Figure 13).

The Difference in Digestion of Histones in Solution Compared with Histones Bound to DNA. The ability of the purified chromatin-bound protease to fragment histones, when used as substrates either in solution or complexed to DNA, was assayed by disc electrophoresis after incubation of samples with enzyme. As shown in Figure 14, when the substrates used were not complexed to DNA, all the histone molecules were attacked at about the same rate except histone I. If on the other hand, histones were used as substrates when bound to native chromatin (endogenous protease being inactivated), histone I appeared to be most sensitive to degradation. Whether this is due to histone I having a more sensitive conformation in chromatin than in solution, or to the other histones having a more resistant conformation in chromatin is uncertain at present. In order to test the first possibility, purified histone I was reconstituted to DNA by gradient dialysis. The breakdown products resulting from enzyme digestion of this complex were then compared to those resulting from enzyme fragmentation of histone I in solution and histone I in native chromatin (endogenous protease being inactivated) by ex-

FIGURE 13

A modified Lineweaver-Burk plot of chromatin-bound protease acting on histones and NHC proteins. The equation

$$\frac{S}{V} = \frac{Km}{V_{max}} + \frac{1}{V_{max}} \, S$$

was used to calculate Km and V_{max}.

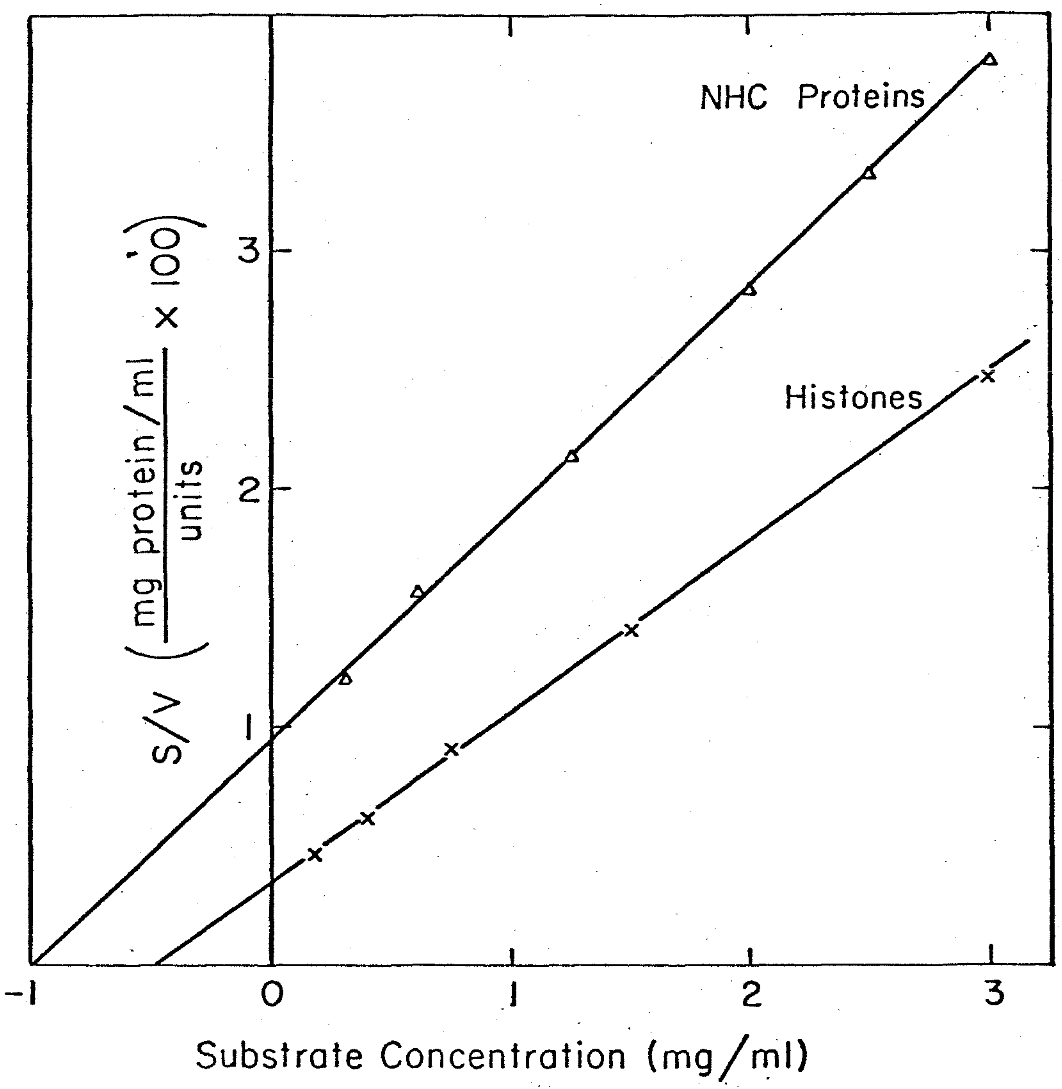
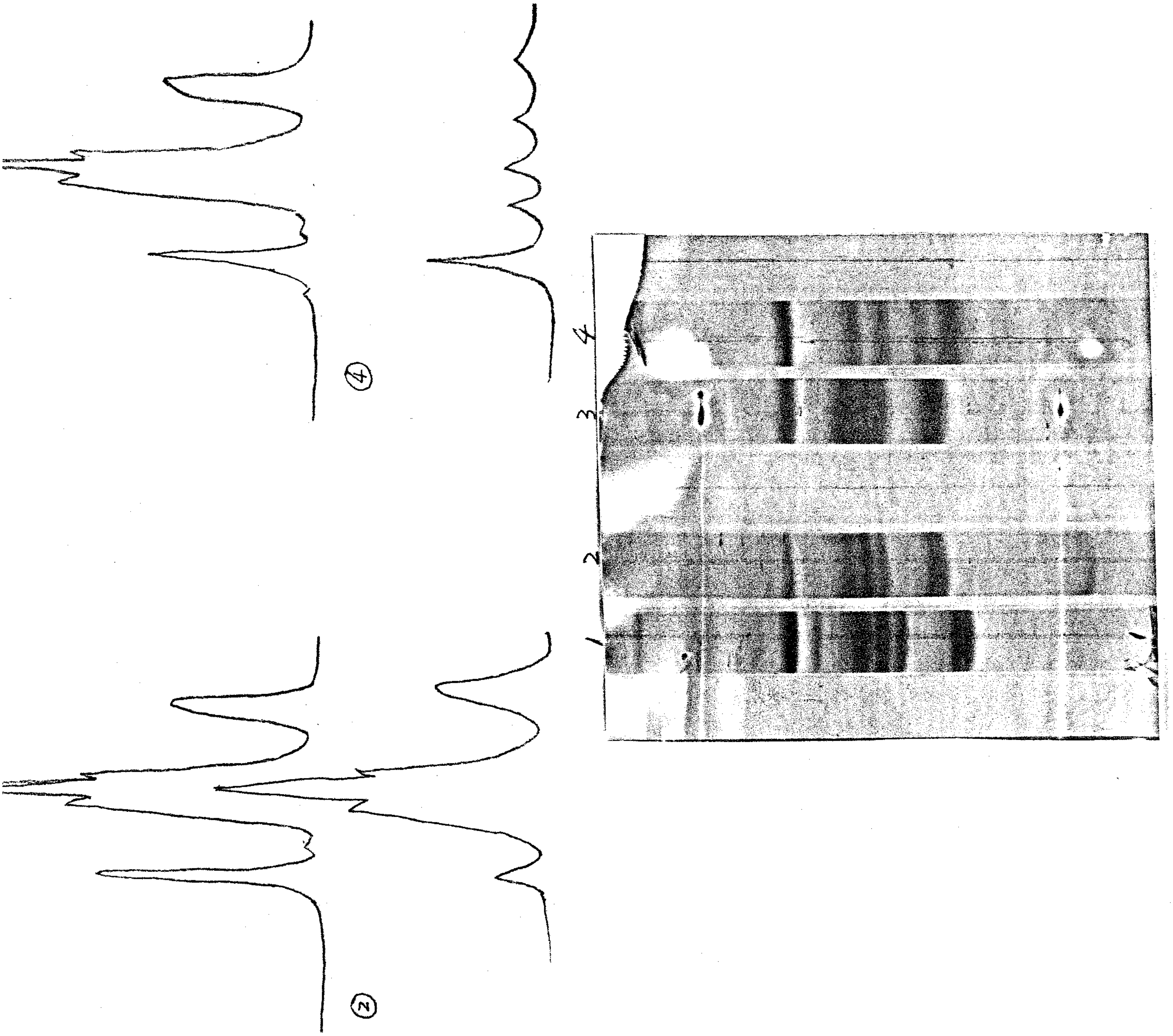

NHC Proteins
Histones
S/V $\left(\frac{\text{mg protein/ml}}{\text{units}} \times 100\right)$
1
2
3
-1
0
1
2
3
Substrate Concentration (mg/ml)

FIGURE 14

The difference in digestion of histones in solution compared with histones in chromatin. PMSF treated chromatin was digested with exogenous purified protease (gel 2) and without protease (gel 1) for 4 hr. Incubation at 37°, 0.04 M NaCl - 10 mM Tris (pH 8), chromatin concentration was 10 A_{260}/ml. PMSF treated total rat liver histones were dissolved in 0.04 M NaCl - 10 mM Tris (pH 8) and digested with exogenous purified protease (gel 4) and without protease (gel 3) for 4 hr at 37°.

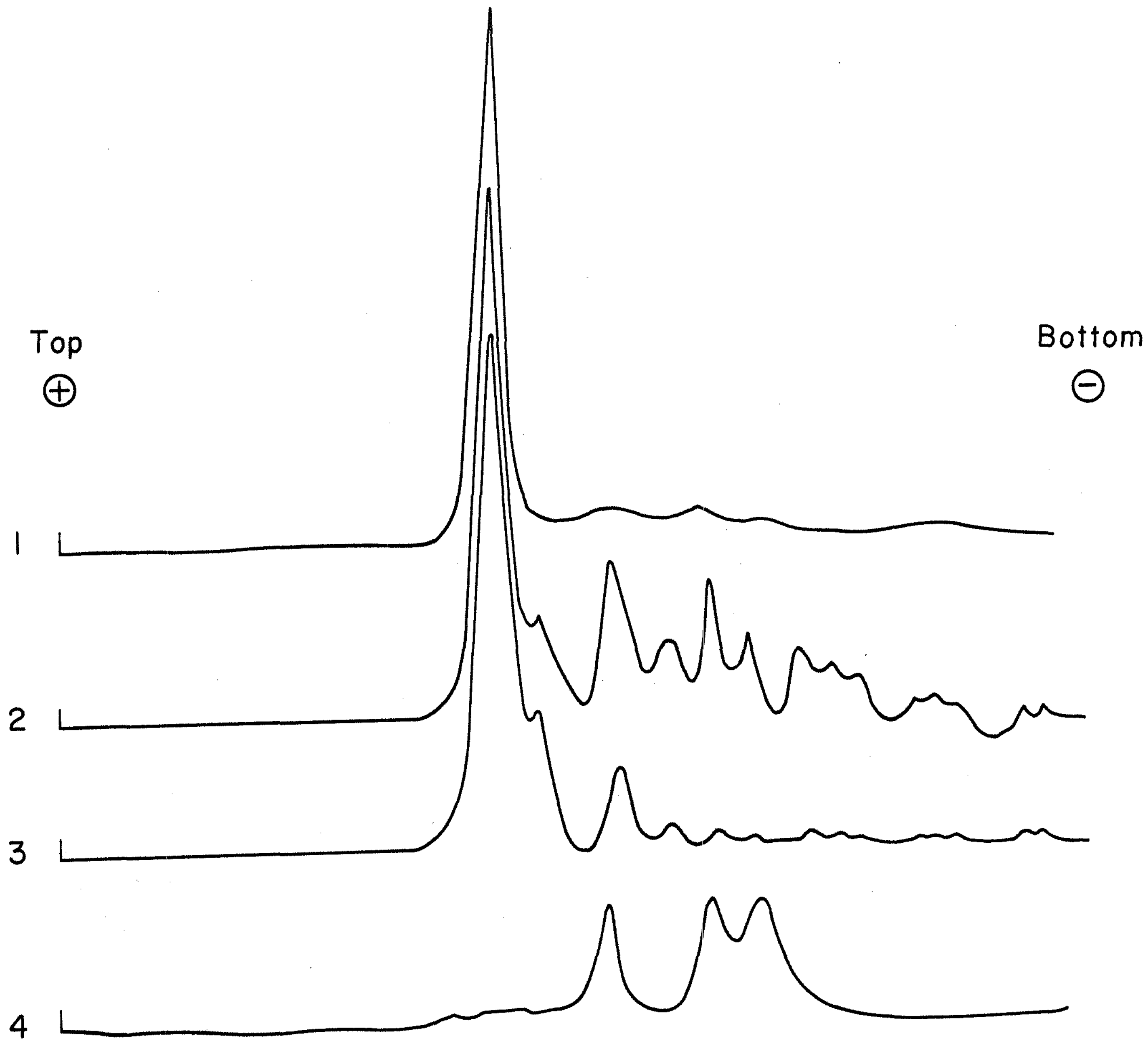

④
②
1
2
3
4

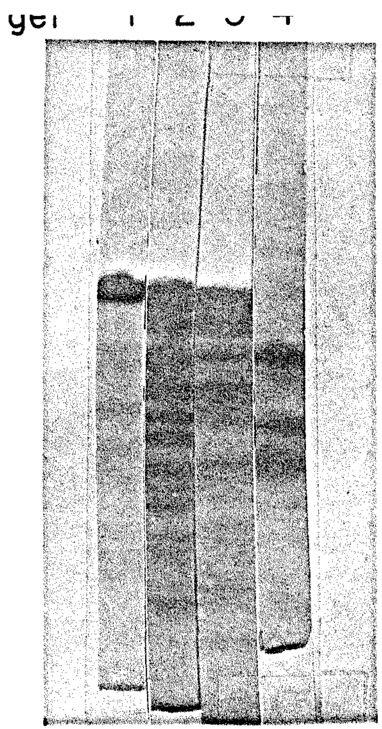
tracting histone I selectively by the method of Johns (1964). As shown in Figure 15, three principal products can be detected from fragmentation of histone I of native chromatin, while much more complex patterns of breakdown products were observed upon digestion of histone I in solution or histone I reconstituted to DNA. It seems clear that histone I of native chromatin exhibits a conformation which favors limited and specific sites of attack by the enzyme and that the technique used to reconstitute histone I to DNA failed to achieve this conformation. In order to test if the three principal products derived from histone I bound to native chromatin were the result of true proteolysis, rather than the effect of a change in the charge distribution on the histone molecules as a result, for example, of dephosphorylation or deacetylation, both intact and degraded samples of histone I were dansylated by the method of Woods and Wang (1967). The interpretation of the results was facilitated by the observation that intact histone I possesses no free N-terminal groups (Bustin *et al.*, 1969). Indeed, intact histone I isolated had no free N-terminal group. On the other hand, degraded products of histone I showed two new N-terminal groups (lysine and alanine). This result argues forcibly that the faster moving electrophoretic bands were the result of true proteolysis.

Similarity of Purified Protease to Endogenous Chromatin-bound Protease Activity. Since only 1.2% of the total

FIGURE 15

Degradation of histone I in solution, reconstituted to DNA and in native chromatin by exogenous purified protease. Histone I in solution or histone I reconstituted to DNA (2 mg/ml) was digested with exogenous purified protease (50 units in 0.1 ml of in 0.04 M NaCl - 10 mM Tris, pH 8) for 20 hr at 37°. PMSF treated chromatin (10 A_{260}/ml in 0.04 M NaCl - 10 mM Tris, pH 8) was digested with exogenous purified protease (500 units/ml) for 20 hr at 37°. At the end of incubation, histone I and its degradation products were extracted with 5% perchloric acid and subjected to disc gel electrophoresis as indicated in Materials Methods. (1) intact histone I (2) degraded histone I in solution (3) degraded histone I reconstituted to DNA (4) degraded histone I in native chromatin.


Top ⊕
Bottom ⊖
1
2
3
4


gel
1
2
3
4

chromatin-bound protease activity was recovered after purification of the enzyme to homogeneity (Table II), the possibility remained that the purified enzyme might not be representative of the major activity bound to chromatin. To test this possibility, native rat liver chromatin samples were incubated at 37^o for various periods of time, after which histones and NHC proteins were isolated and separated by disc electrophoresis. The same assay procedure was used for purified protease; in this case the endogenous activity was first inactivated by PMSF before addition of purified protease. As shown in Figures 16 and 17, the orders of breakdown of various chromosomal proteins catalyzed by the purified enzyme were essentially the same as those catalyzed by the endogenous activity. It is concluded that the purified protease is representative of the endogenous activity of chromatin.

To assay more carefully the breakdown of histones, histone I and its breakdown products were separate from the other histone species by the method of Johns (1964). As shown in Figure 18, breakdown products from histone I run in the same position as histone II_{b2} and histone IV.

FIGURE 16

Degradation of histones in chromatin by exogenous purified protease (A) and endogenous protease (B). Incubation at 37°, 0.04 M NaCl - 10 mM Tris (pH 8), chromatin concentration was 10 A_{260}/ml for 4 and 8 hr in each case. PMSF treated chromatin (in case A) was digested by exogenous purified protease (500 units per ml of chromatin). PMSF treated chromatin (C) without adding exogenous chromatin was incubated for 8 hr to serve as control.

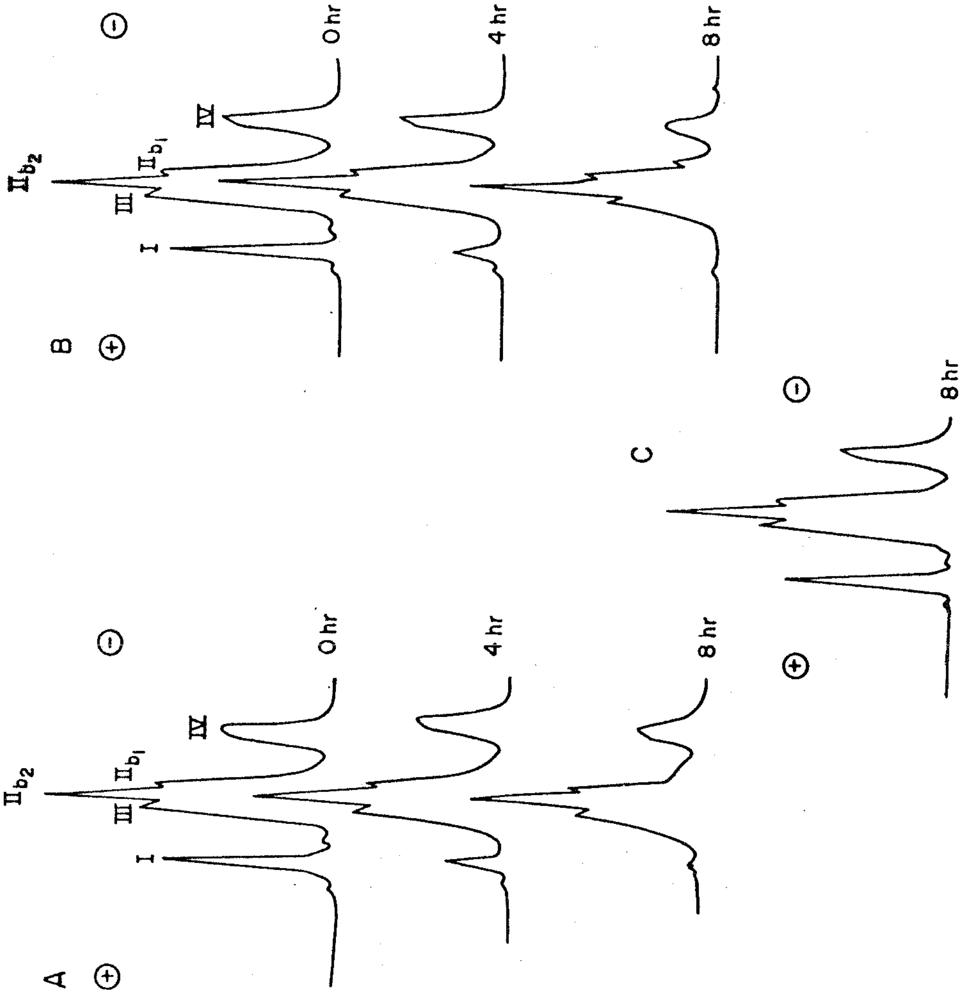

A
B
C
⊕
⊖
I
III
IIb2
IIb1
IV
0 hr
4 hr
8 hr

FIGURE 17

Degradation of NHC proteins in chromatin by endogenous protease (A) and exogenous protease (B). Incubation at 37°, 0.04 M NaCl - 10 mM Tris (pH 8), chromatin was 10 A_{260}/ml for 8 and 16 hr in each case. PMSF treated chromatin (in case B) was digested by exogenous purified protease (500 units per ml of chromatin). PMSF treated chromatin (in case C) without adding exogenous chromatin was incubated for 16 hr to serve as control.

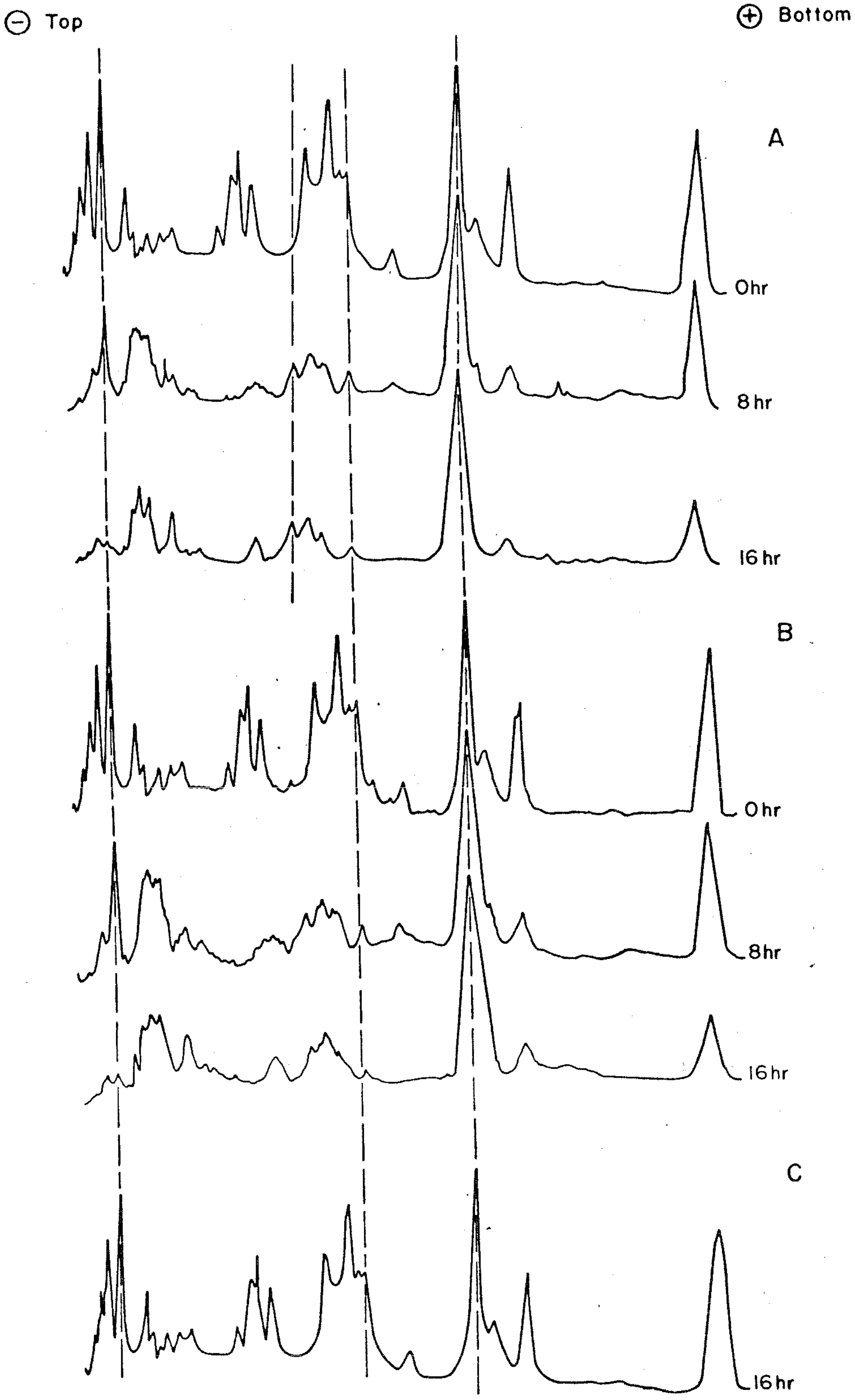
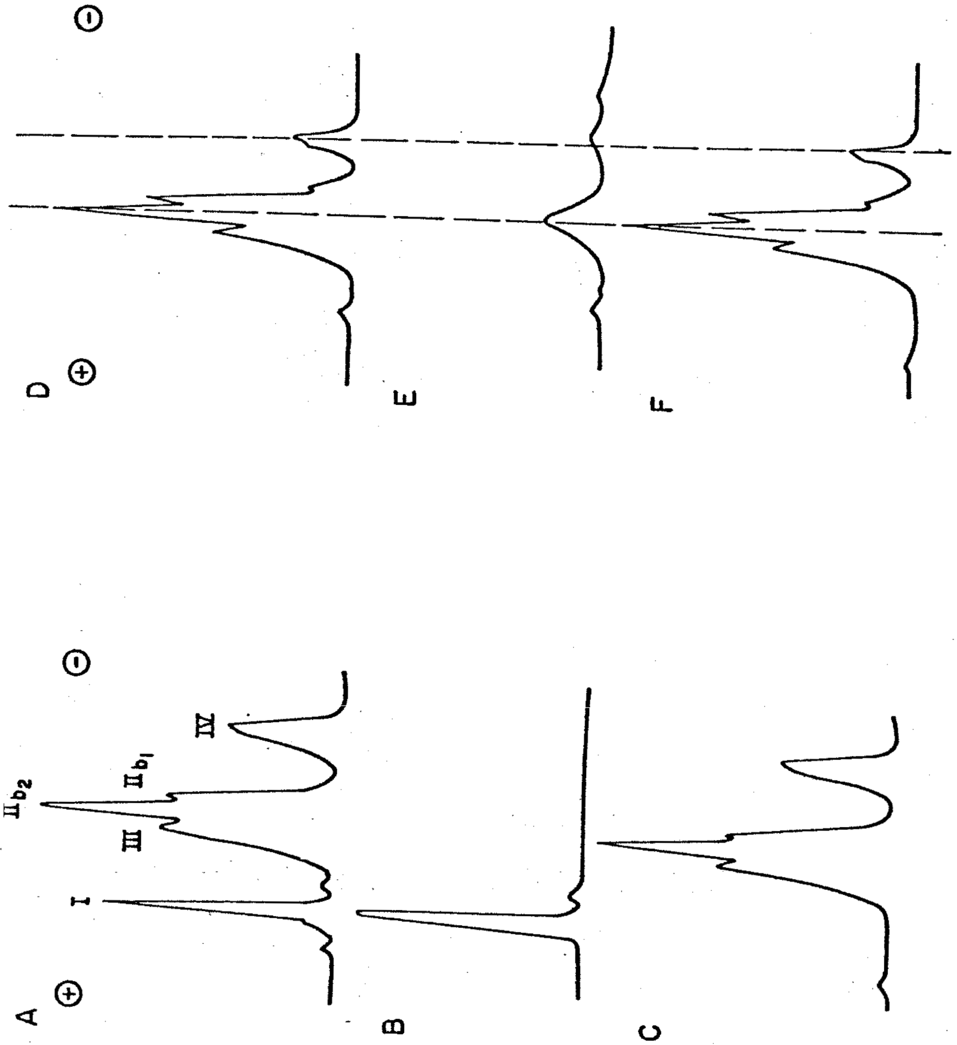

⊖ Top
⊕ Bottom
A
0hr
8hr
16hr
B
0hr
8hr
16hr
C
16hr

FIGURE 18

Degradation patterns of histones in chromatin. Chromatin was extracted with 5% perchloric acid to yield intact histone I (B); a sulfuric acid extract of the perchloric acid-insoluble material yielded the intact residual histones (C). Total intact histone is shown in (A). A similar John's fractionation on PMSF treated chromatin digested with exogenous purified protease yielded degraded histone I (E), and degraded residual histone (F). Unfractionated, degraded histones are shown in (D).


A
⊕
⊖
I
III
II_{b_2}
II_{b_1}
IV
B
C
D
⊕
⊖
E
F

DISCUSSION

The protease we have isolated is somewhat different from the neutral protease isolated from calf thymus by Furlan and Jericijo (1968). For example, the molecular weight is about 9 times bigger, optimal pH is 7 instead of 7.8, the enzyme needs divalent ions as activator and is inhibited by mercuric ion. However, there are some properties shared by both enzymes, such as, the enzyme activity is salt-dependent, thermolabile and inhibited by DFP, histone I is the first protein attacked by the enzyme when histone I is bound to chromatin, but once it is dissociated from chromatin, it is the last one being degraded.

It is of interest to consider the basis of specificity of chromatin-bound protease. The high activity with L-polylysine may explain why the enzyme prefers histones as substrate. Next possibility is that a small section of protein, such as the classical sequence of four basic amino acid residues followed by one aliphatic residue, which occurs in all histones, is the signal for specificity. However, it should be noted that this sequence also occurs frequently in the protamines. The protease shows only 7% activity with protamines relative to histones. This sequence could not be the key to specificity. Alternatively, the specificity observed could be the consequence of the relatively open structure of histones as compared to globular proteins. This possibility is supported by the evidence that the enzyme preferred the denatured forms of lysozyme and BSA as substrates to the native forms. However, this possibility still cannot explain why the enzyme preferred the chromosomal proteins to cytosol, even the denatured cytosol has been used as the substrate. The other possibility may be that there are specific sequences in the chromosomal proteins, which are sensitive to the enzyme digestion. This possibility can be tested by determining the amino acid sequences of the three major degraded products from histone I bound to the native chromatin.

The environment of histones undergoing proteolysis clearly has a profound effect upon the specificity of the protease. Thus, the initial effect of the protease upon histones in the intact nucleoprotein complex is seen primarily in its rapid attack upon histone I. However, if the entire histon complement is removed from its association with DNA, the protease attacks four of the five histone groups leaving histone I rather intact. Thus, the enhanced resistance of these histone fractions to the proteolytic enzyme probably lies in a protective effect of their interaction with DNA, indicating that these fractions are most likely in a much more intimate association with DNA than are histone I.

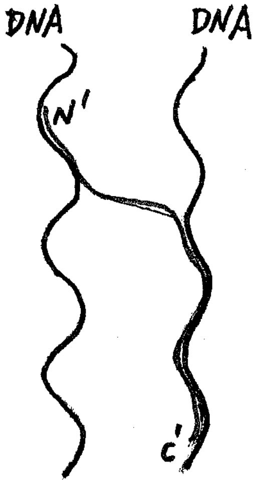
Several isolated facts suggest that histone I is deposited on DNA chain in such a manner that it is much more exposed to the aqueous environment than are the other four histone fractions. Brutlag et al., (1969) have observed that histone I is the first histone fraction to be fixed to the DNA when chromatin is exposed to increasing concentrations of formaldehyde. Jensen and Chalkley (1968) observed that when precipitation of chromatin in 0.15 M NaCl is conducted in the presence of DNA or RNA of molecular weight greater than 3×10^4, the extent of chromatin aggregation is greatly reduced. They showed that this is due to an increased negative charge on the chromatin molecules following a shift of a portion of histone I to the free nucleic acid. From optical rotatory dispersion data, Tuan and Bonner (1969) have calculated that the various histone fractions complexed with DNA can contain (upper limit) the following percentages of α-helix: histone I, 15%; histone II, 58%; histone III and IV, 42%. All of the ionic dissociating agents employed for the selective removal of histones from chromatin dissociate histone I at the lowest concentrations of dissociating agent (Ohlenbusch et al., 1967; Fambrough and Bonner, 1968). All of these properties of histone I (early removal from chromatin by increasing concentrations of dissociating agents, susceptibility to attack by proteolytic enzymes, proportionately large contribution to the aggregation of partially dehistonized chromatin in 0.15 M NaCl, ability to shift to free nucleic acids of moderately large size, and apparent low content of α-helix) can be accounted for by assuming that histone I is more exposed to and/or interacts more with the aqueous environment than are, and/or do, the other histone fractions.

From the partial sequence of three subfractions of histone I, two from rabbit thymus and one from calf thymus, the first 40 N-termial residues have 75 mole percent of amino acids being lysine, alanine and proline. This overall composition is similar to that of the last C-terminal 110 residues of the whole molecule (Bustin et al., 1969,1970) (80 mole percent being lysine, alanine and proline). Both basic regions of histone I (the first 40 residues and the last 110) may be sites for DNA binding. It has been suggested (Littau et al., 1965) from physical studies of nucleohistones that histone I cross-links chromatin; one basic region could be combined with one DNA molecule while the other basic region is combined with a second DNA molecule, a process which could lead to the formation of nucleohistone complexes of any size. NMR study on the reconstituted DNA-histone I complex (Lee, 1972) suggests that part of peptide chain in the N-terminal half (residue 1-51)of the protein is relatively free in the complex state. The second region (residues 51-100) of the peptide chain is relatively restricted in motion, because it is adjacent to the C-terminal half molecule (residue 100-219), which is strongly bound to DNA.

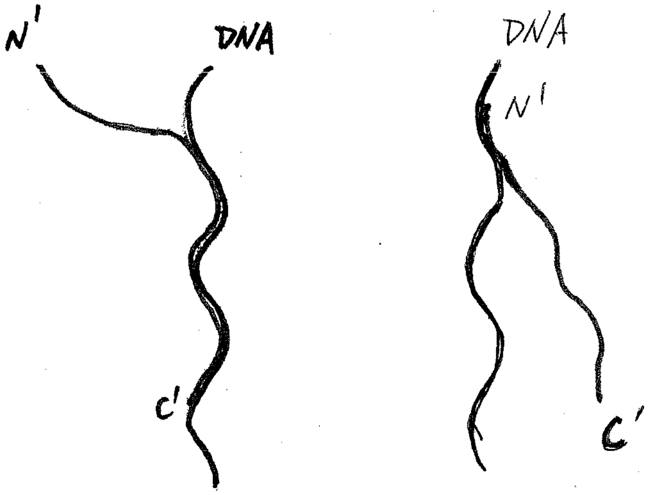
The schematic summary of histone I in native chromatin and reconstituted DNA-histone I complex is present as in Fig. 19. This model of histone I may explain why histone I

FIGURE 19

Schematic summary of histone I in native chromatin and reconstituted DNA-histone I complex.



Histone I in Native Chromatin


N'
DNA
C'
DNA
N'
C'

Reconstituted DNA-Histone I Complex

in chromatin subject to the enzyme digestion produced limited specific products, while more degraded products were detected when histone I in solution and DNA-histone I complex were subject to the protease digestion.

Because the enzyme purified in this study yields identical breakdown products to those produced by the endogenous chromatin-bound activity, we believe the two are one and the same enzyme. The fact that the enzyme prefers nuclear protein substrates suggests a physiological role in the turnover of nuclear proteins. Earlier, Bartley and Chalkley (1970) suggested that such an activity probably played a role in the reutilization of amino acids in populations of cells undergiong autolysis. On the contrary, we speculate that this activity plays a major role in the turnover of NHC proteins, which have been shown to have high turnover rates (Dice and Schimke, 1973). The pronounced susceptibility of histones to the attack of the enzyme, which was supposed to be a normal constituent of chromatin, is hardly compatible with the low turnover of histones in the cell nuclei. We could not find any inhibitor present in the nucleoplasm. Further experiments are necessary to investigate whether the chromatin-bound protease exists in the intact nuclei either as a precursor similar to proenzymes of the gastrointestinal proteases or being localized.

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CHAPTER III

PURIFICATION AND PROPERTIES OF A NONSPECIFIC ESTERASE FROM RAT LIVER CHROMATIN

INTRODUCTION

During attempts to purify a neutral protease from rat liver chromatin, an esterase incidentally was isolated. Many proteases such as trypsin, chymotrypsin and thrombin, use α-tosyl-arginine-methyl ester (TAME) as a substrate. Therefore TAME was tried as a substrate for protease activity. It ultimately became clear that the neutral protease isolated from rat liver chromatin could not use TAME. However, rat liver nuclei does contain a nonspecific esterase, which splits the ester bond of TAME. In the following, the procedures used to isolate the esterase and some of its properties are described.

MATERIALS AND METHODS

Assay of Esterase Activity by the Isotopic Procedure. The method of Roffman *et al.*, (1970) was used with slight modification. Each assay was performed in duplicate. The incubation mixture contained: 5 μl of [^{3}H]- TAME (0.005 μCi, approximately 0.033 nm; Biochemical and Nuclear Corp.) with unlabeled TAME (1 mmole), 50 μl of enzyme solution. The mixture was incubated at room temperature for the desired length of time in a scintillation vial with 10 ml of a counting solution consisting of Spectrafluor Butyl-PBD (70 ml) (Amersham/Searle) and toluene (948 ml). The vials were well shaken and counted immediately for 1 min in Beckman Model LS-200 B scintillation spectrometer. This served as zero time control. Then the vials sat at room temperature. At desired time intervals, the vials were reshaken well and counted. The reaction rate was linear up to 2 h of incubation. Routinely 1 h incubation was used to measure enzyme activity. In this biphasic system only the fraction of radioactive material extracted by toluence is counted since the scintillant Spectrofluor Butyl-PBD is totally absent from the water phase. Thus, at zero time, only the ester fraction extracted by toluene is measured. During the ester hydrolysis the counts per minute increase proportionally to the rate of hydrolysis as a result of the increased extraction of [^{3}H] methanol.

The rates of spontaneous hydrolysis of TAME were determined from vials containing substrate without enzyme. At pH 8, the spontaneous hydrolysis during 1 h was 4% for TAME. The values of enzyme activity presented were those after the values of spontaneous hydrolysis had been subtracted for each time interval.

Purification of Esterase from Chromatin. Sheared purified chromatin was adjusted to 10 A_{260} per ml. Solid sodium chloride was added to a final concentration of 0.7 M. The mixture was stirred overnight at 4°. The clear solution was centrifuged at 177,700 g for 8 h. The supernatant was collected and dialyzed against 0.3 M NaCl, 10 mM Tris (pH 8). The solution was then applied to a Bio-Rex 70 column (2.5 cm x 30 cm) equilibrated with 0.3 M NaCl, 10 mM Tris (pH 8). The column was washed extensively with the same buffer until the A_{230} reading was nearly zero. Then a linear salt gradient from 0.3 M NaCl to 1.0 M NaCl in 10 mM Tris (pH 8) (total 300 ml) was started. Three ml fractions were collected. Fractions containing the esterase activity were pooled and concentrated by ultrafiltration (Amicon PM 10 membrane). The concentrated protein solution was then applied to a Sephadex G-200 column (3 cm x 100 cm), equilibrated and eluted with 0.3 M NaCl - 10 mM Tris (pH 8). Three ml fractions were collected. Fractions containing the esterase activity were pooled and mixed with calcium phosphate gel. The mixture was centrifuged at 12,000 g for 10 min. The supernatant contained the enzyme activity and was applied to a SE-Sephadex C-50 column (0.9 x 25 cm) pre-equilibrated with 0.3 M NaCl - 10 mM Tris (pH 8). The column was washed with 50 ml of 0.3 M NaCl - 10 mM Tris (pH 8). Then a linear salt gradient from 0.3 M to 1 M NaCl in 10 mM Tris (pH 8) was started to elute the enzyme. The pure enzyme eluted at 0.7 M NaCl.

RESULTS

Distribution of Esterase Activity in Rat Liver Nuclei. Table I shows the distribution of esterase activity in different nuclear components. 41% of the activity is located in nucleoplasm, 33% is in the nuclear membrane and the remaining 26% is associated with chromatin. The specific activity in different nuclear components is also shown in the same table, nuclear membrane has the highest specific activity, followed by nucleoplasm and chromatin. At present, it is unclear whether different nuclear components have the same or different esterases. We have studied that associated with chromatin.

Extraction of Esterase from Chromatin with Different Concentrations of Sodium Chloride. The sheared chromatin was treated with increasing concentrations of sodium chloride to extract esterase. Fig. 1 shows that 33% activity was released from chromatin with 0.3 M NaCl and 56% activity was extracted with 0.7 M NaCl. However, higher salt concentrations did not extract more enzyme from chromatin although more protein was released. So, 0.7 M NaCl was routinely used to extract esterase from chromatin.

Purification of Esterase. Table II summarizes the steps utilized to purify esterase. The procedure adopted resulted in 510-fold purification over total chromosomal protein with a yield of 2% of the activity. Standard

Table I

Subnuclear Distribution of Esterase Activity

Subnuclear fraction	Total protein (µg)	Total esterase activity µmole/h	% of total esterase activity	Specific activity (µmole/h/µg)
Nucleoplasm	1,800	450	41%	0.25
Nuclear membrane	850	360	33%	0.42
Chromatin	5,500	275	26%	0.05

FIGURE 1

The effect of different salt concentrations on extraction of esterase from chromatin. The sheared chromatin was adjusted to 10 A_{260}/ml and solid sodium chloride was added to the desired salt concentration. The mixture was stirred at $4^{o}C$ for 4 h and centrifuged at 177,700 g for 8 h. The supernatant and pellet were collected. The esterase activity and protein concentration was determined as shown in Materials and Methods.

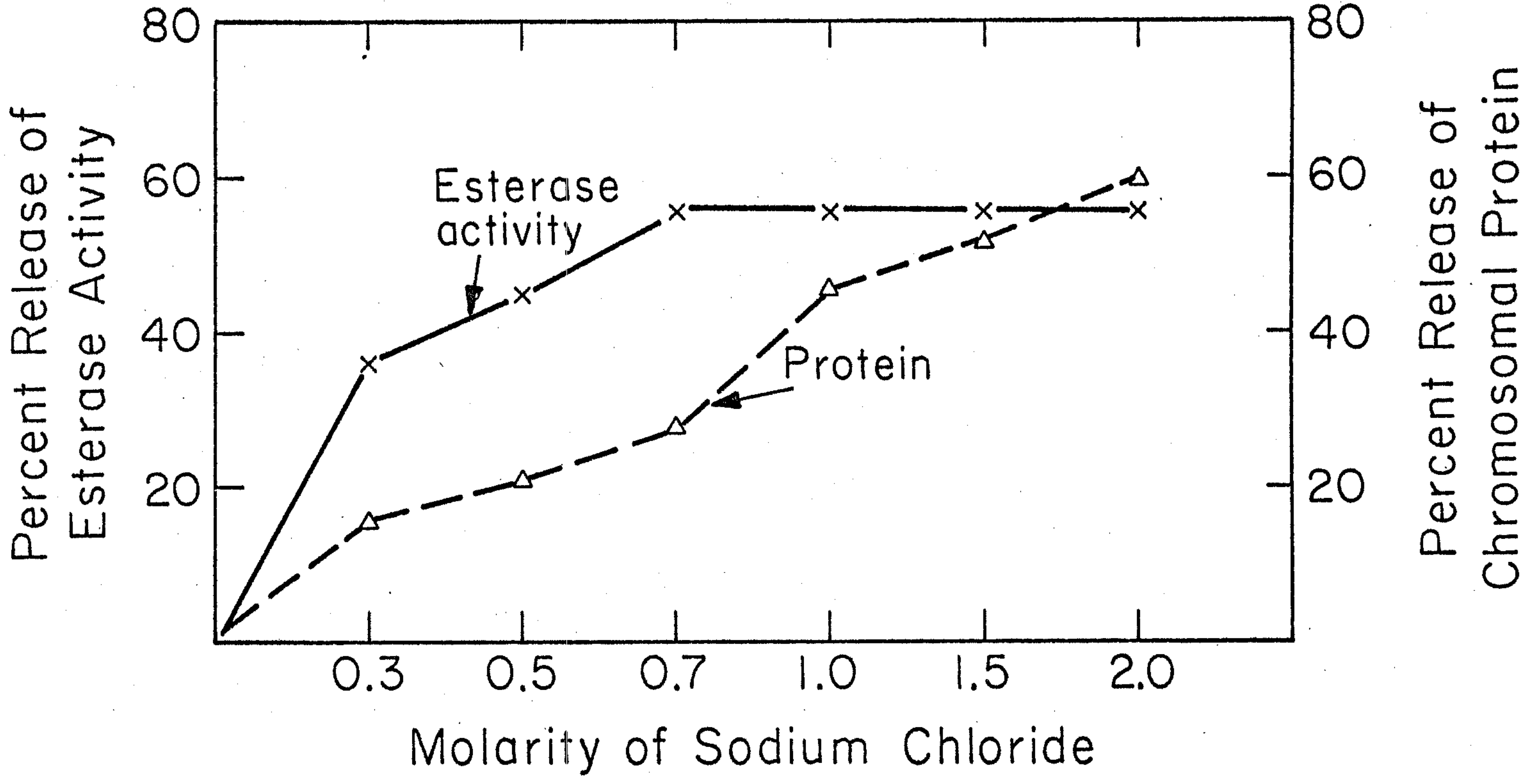

80
60
40
20
Percent Release of Esterase Activity
Esterase activity
Protein
0.3
0.5
0.7
1.0
1.5
2.0
Molarity of Sodium Chloride
80
60
40
20
Percent Release of Chromosomal Protein

Table II

Purification of Esterase

Purification step	Volume (ml)	Protein concentration (μg/ml)	Total protein (μg)	Activity (μmol/ml/h)	Total Activity (μmol/h)	Yield (%)	Specific activity (μmol/h/μg)	Fold of purification
Total chromatin	300	950	285,000	5,200	156×10^4	100	5.4	1
50K supernatant	300	200	60,000	2,900	87×10^4	56	14.5	2.7
Bio-Rex 70	30	73	2,190	20,000	60×10^4	38.4	274.0	50.7
Sephadex G-200	27	8.15	220	6,600	18×10^4	11.5	810.0	150
Calcium phosphate gel	20	3.2	64	4,500	9×10^4	5.7	1406.0	260.4
SE Sephadex	30	o.38	11.5	1,052	3.16×10^4	2	2750.0	510

methods were used, which included chromatography on Bio-Rex 70 (Fig. 2), Sephadex G-200 (Fig. 3) and SE Sephadex C-50 (Fig. 4). The most effective purification step, based on the criteria of fold purification and recovery, was Bio-Rex 70 column chromatography.

Molecular Properties of Purified Esterase. The molecular weight of the purified esterase was estimated to be approximately 15,000 by sucrose gradient centrifugation (sedimentation coefficient = 1.9 S) and about 15,000 by SDS disc electrophoresis (Fig. 5). A single peak of protein was obtained upon subjecting the purified esterase to SDS disc electrophoresis and urea gel electrophoresis in the presence of reducing agent (Fig. 6). Based on the fold-purification data (Table II), assuming a molecular weight of the active enzyme of 15,000 and that the rat haploid genome size is 1.8×10^{12} daltons (Britten and Davidson, 1971), the isolated rat liver chromatin would contain approximately 2×10^5 molecules of this enzyme per haploid genome. This estimate is subject to the further assumption that only one esterase is present in chromatin and no activation or inactivation of enzyme activity has occurred.

Effect of pH and Inhibitors on Esterase Activity. The purified esterase shows a pH optimum at 8.2 and half maximal activity at pH 6.9 and 10.5, suggesting that histidine and lysine residue might be involved in its activity. The enzyme

FIGURE 2

Chromatography of esterase activity on Bio-Rex 70. Enzyme (87 x 10^4 μmol/h, 60 mg) in 0.3 M NaCl - 10 mM Tris (pH 8) buffer was applied to the column (2.5 x 30 cm). Elution was with a linear sodium chloride gradient from 0.3 M to 1 M in 10 mM Tris (pH 8). 3 ml fractions were collected; the flow rate was adjusted to 15 ml/h; all procedures were carried out at 4^o.

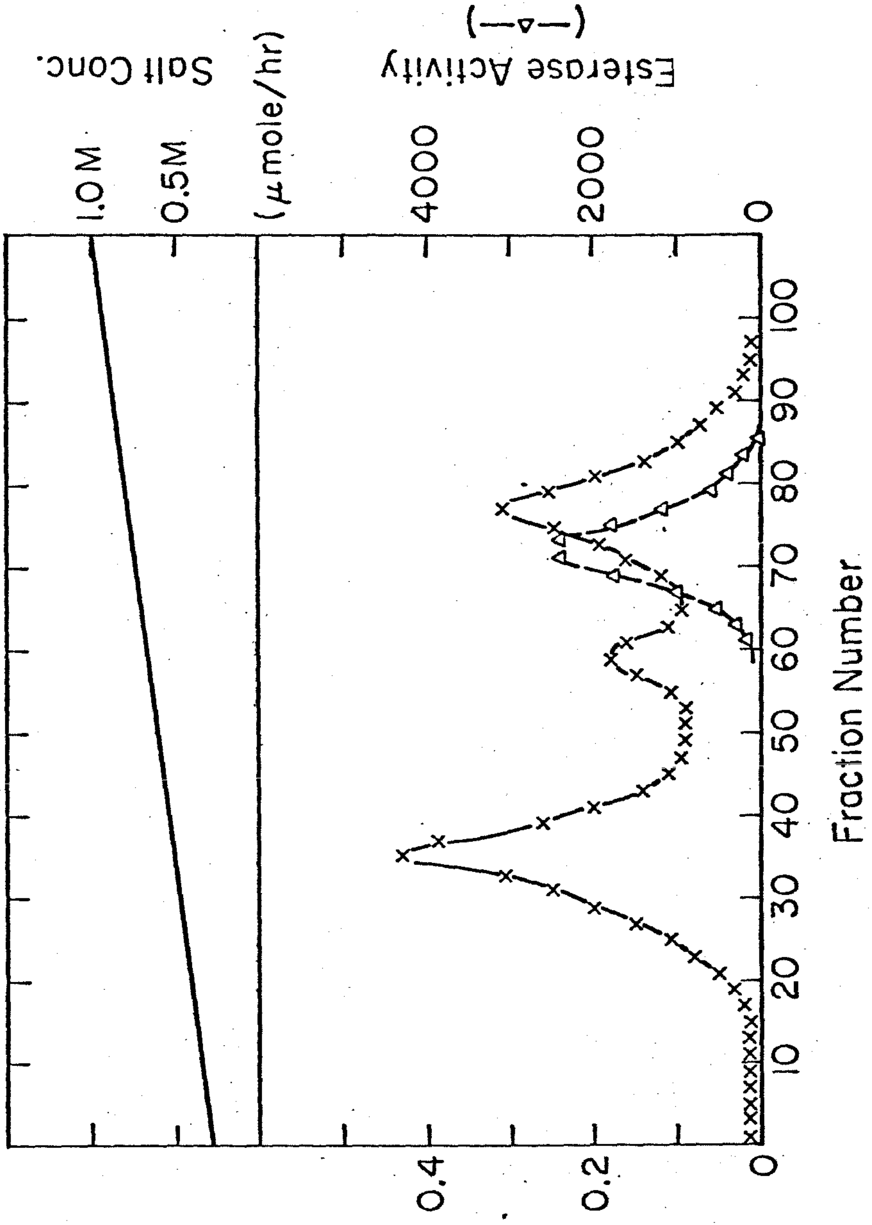

Salt Conc.
1.0M
0.5M
Esterase Activity (μmole/hr)
(—▵—)
4000
2000
0
O.D. at 230mμ (—×—)
0.4
0.2
0
10
20
30
40
50
60
70
80
90
100
Fraction Number

FIGURE 3

Chromatography of esterase activity on Sephadex G-200. Enzyme (60 x 10^4 μmol/h, 2,190 μg) in 0.3 M NaCl - 10 mM Tris (pH 8) buffer was applied to the column (3 x 100 cm). Elution was with the same buffer; 2.7 ml fractions were collected; flow rate was adjusted to 10 ml/h; all procedures were carried out at 4°.

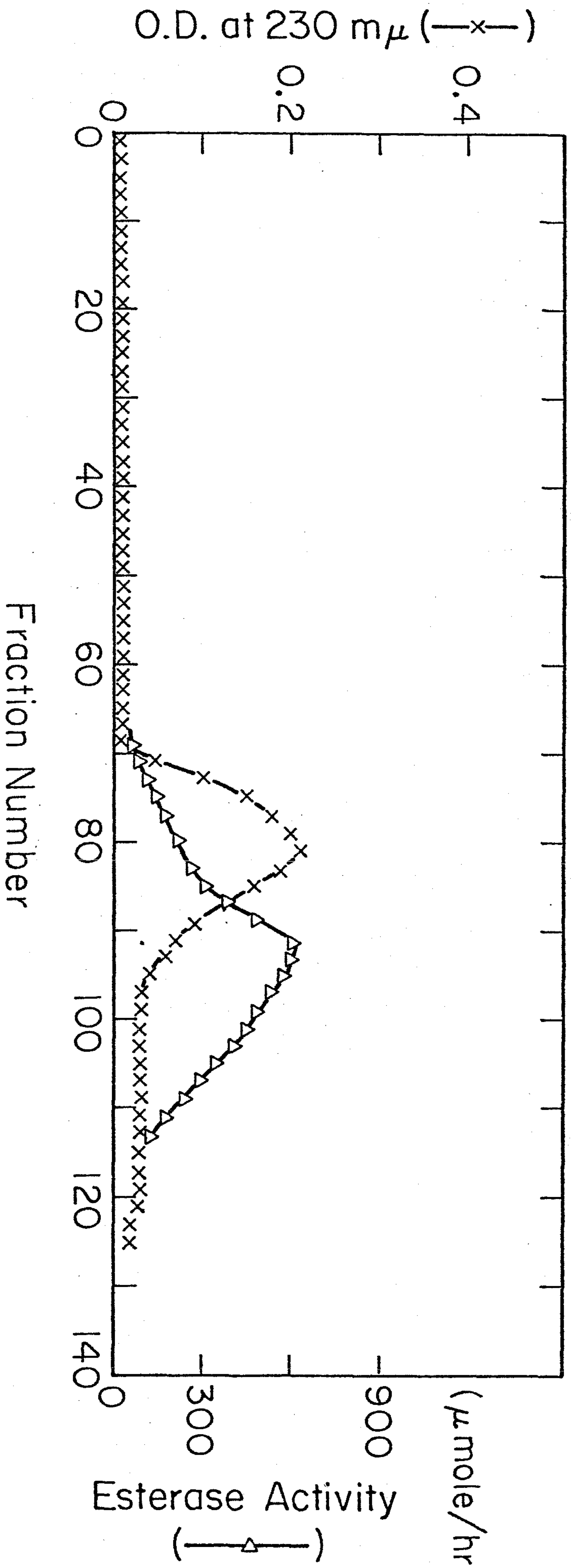

O.D. at 230 mμ (—×—)
0
0.2
0.4
Fraction Number
0
20
40
60
80
100
120
140
Esterase Activity (—△—)
(μ mole/hr)
0
300
900

FIGURE 4

Chromatography of esterase activity on SE Sephadex C-50. Enzyme (9×10^4 μmol/h, 64 μg) in 0.3 M NaCl - 10 mM Tris (pH 8) buffer was applied to the column (0.9 x 25 cm). Elution was with a linear sodium chloride gradient from 0.3 M to 1 M in 10 mM Tris (pH 8). 3 ml fractions were collected; the flow rate was adjusted to 20 ml/h; all procedures were carried out at 4°.

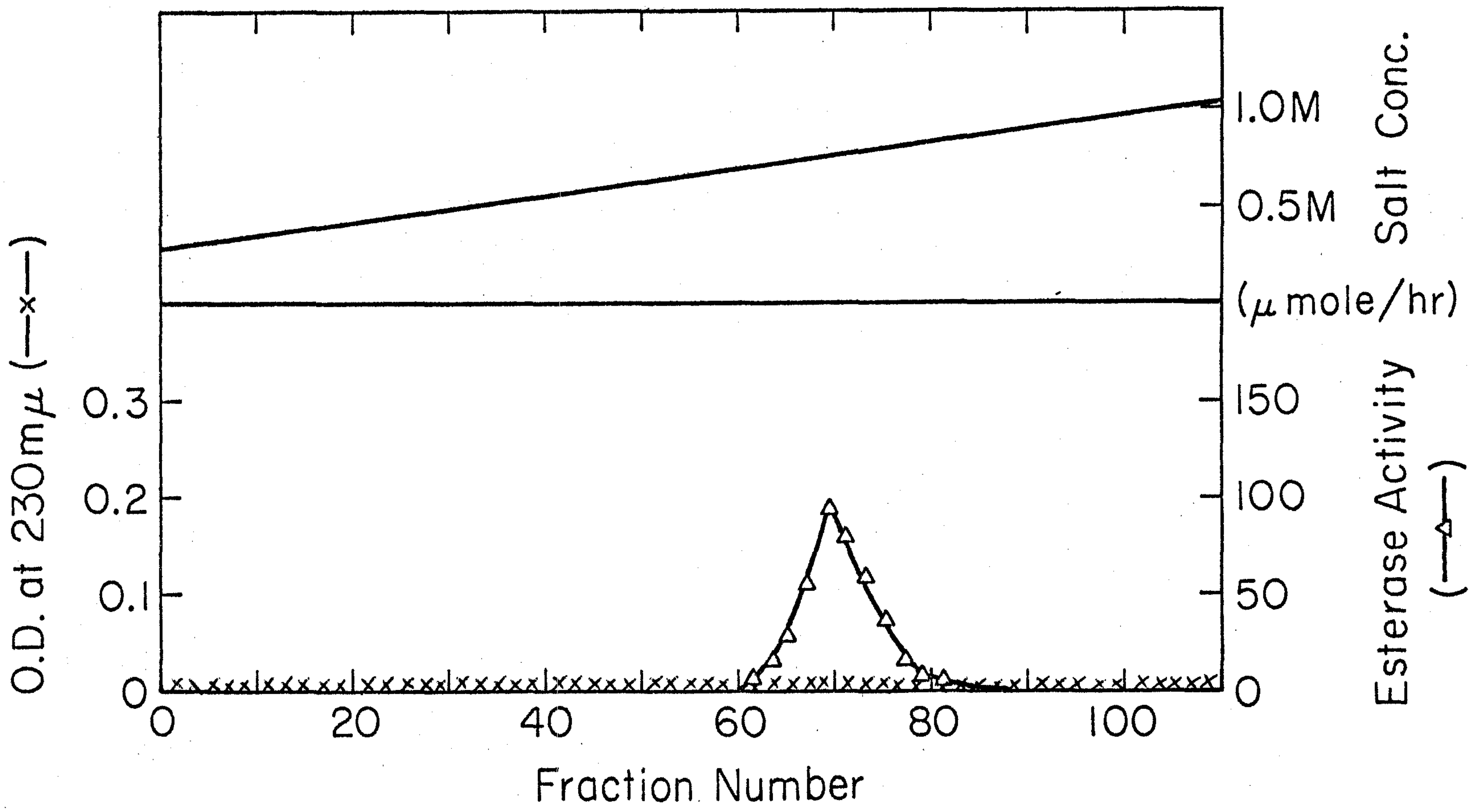

O.D. at 230mμ (—x—)
0.3
0.2
0.1
0
0
20
40
60
80
100
Fraction Number
1.0M
0.5M
Salt Conc.
(μ mole/hr)
150
100
50
0
Esterase Activity (—△—)

FIGURE 5

Estimation of the molecular weight of esterase by SDS polyacrylamide gel electrophoresis. The mobilities of different standard proteins are plotted against the log of the molecular weight. The intercept for esterase indicates a molecular weight of about 15,000.

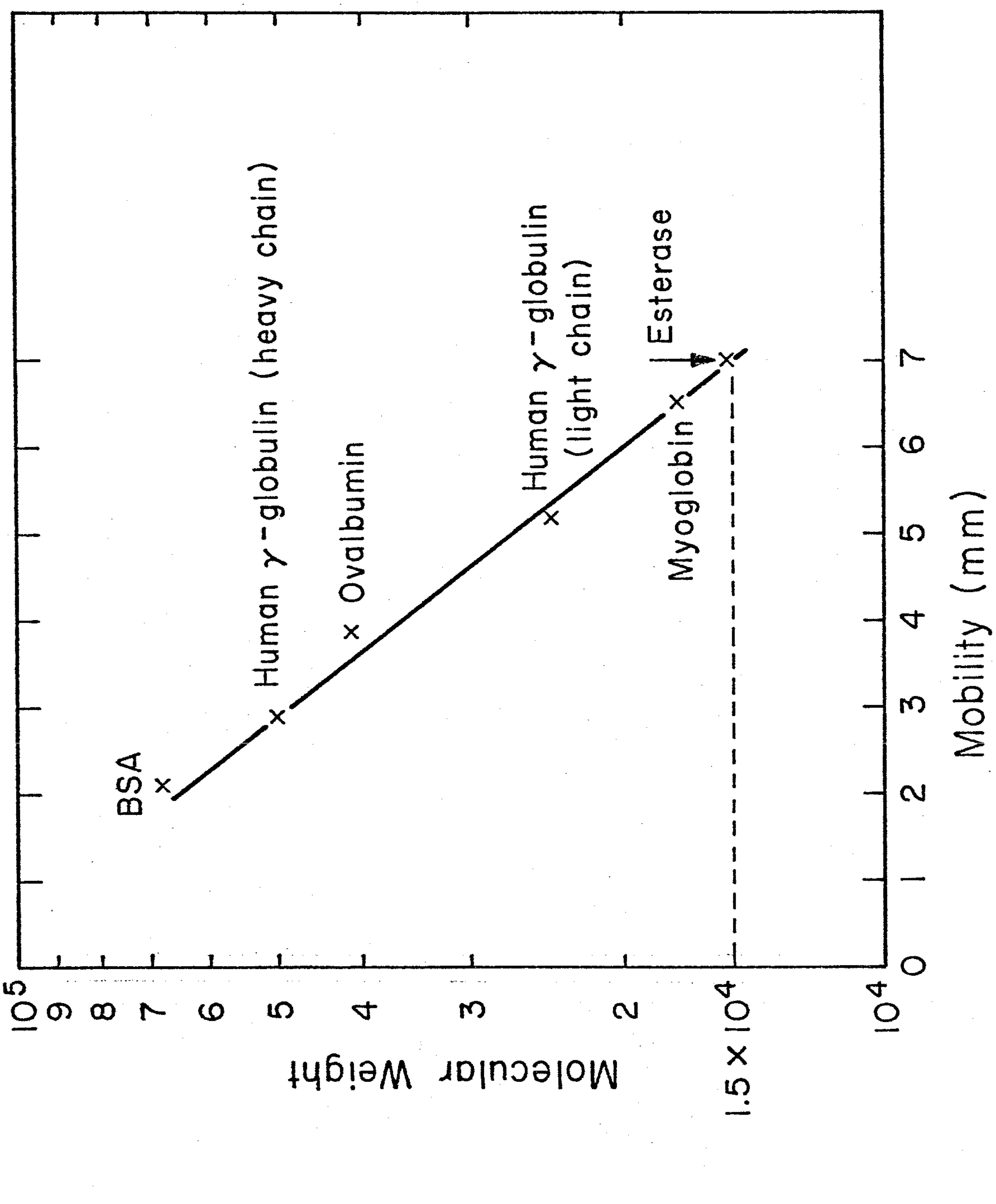
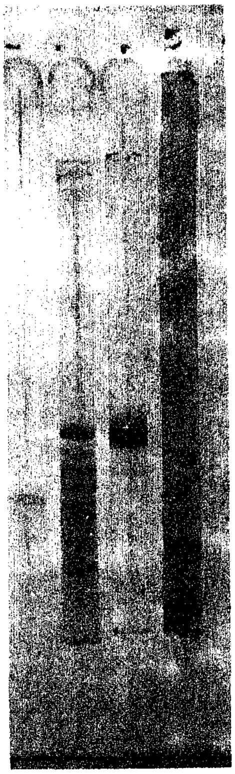
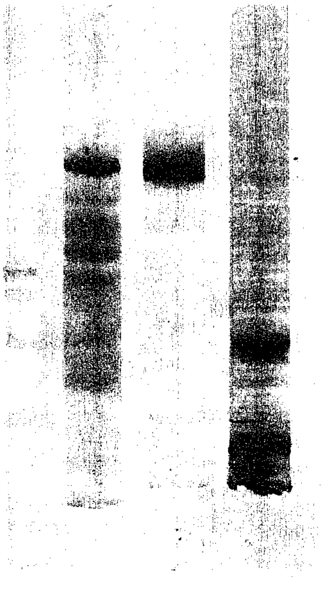

BSA
Human γ-globulin (heavy chain)
Ovalbumin
Human γ-globulin (light chain)
Myoglobin
Esterase
Molecular Weight
10^5
9
8
7
6
5
4
3
2
1.5×10^4
10^4
Mobility (mm)
0
1
2
3
4
5
6
7

FIGURE 6

Disc electrophoresis of esterase on different polyacrylamide gel systems. Gels 1 and 2 were the urea gel system; gel 3 was the SDS gel system. Gel 1 was the protein pooled from fraction #100 to #110 of Sephadex G-200. Gels 2 and 3 were the purified enzyme from SE Sephadex C-50 column.



1 2 3



1 2 3

activity is essentially lost below pH 5.0, and decreases above pH 11, presumably due to denaturation (Fig. 7).

The effects of various inhibitors of esterase activity are shown in Table III. The serine hydroxyl group reagents DFP and PMSF are potent inhibitors; sodium bisulfite is also effective but needs higher concentrations.

Effect of Substrate Concentration. Fig. 8 shows hydrolysis rate of TAME as a function of substrate concentration. Inhibition of the reaction rate by increasing substrate concentration (above 5 mM was observed). When the experimental points obtained are plotted according to the method of Lineweaver and Burk (Fig. 9), the Michaelis constant for hydrolysis was 0.16 mM.

FIGURE 7

Effect of incubation pH on the esterase activity. Enzyme (800 µmol/h, 0.3 µg protein) was incubated under different pH conditions. Enzyme activity assay was performed as given in Material and Methods.

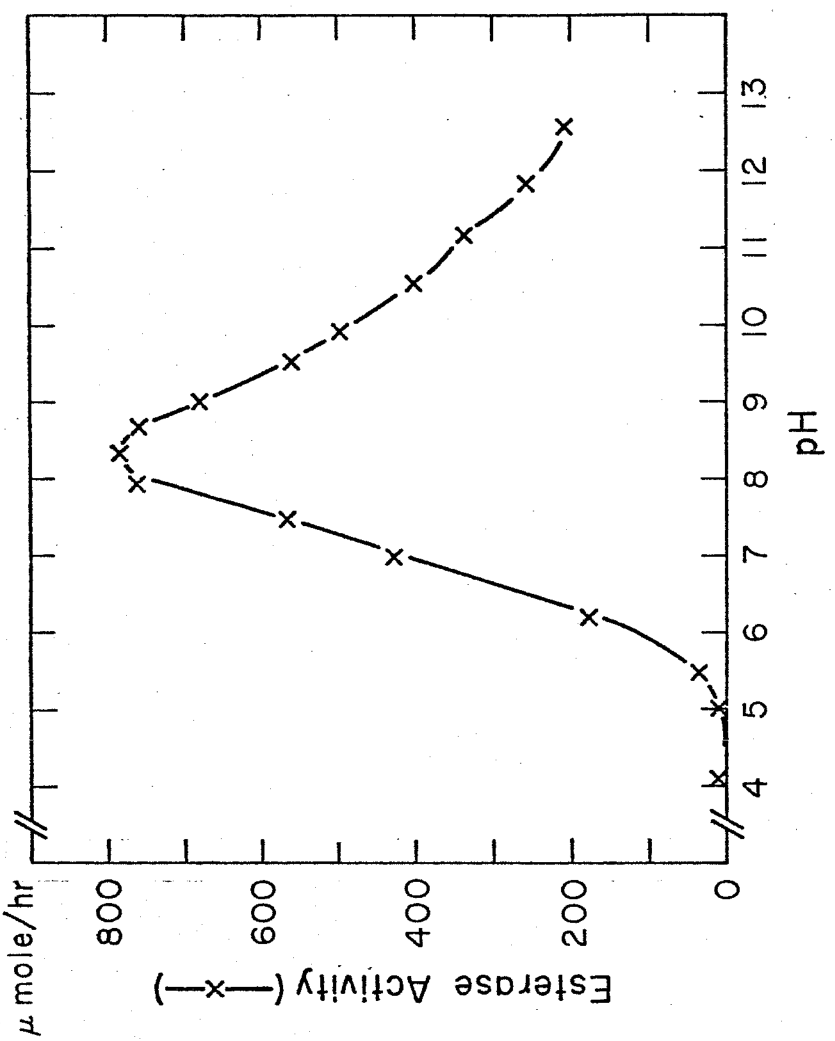

μ mole/hr
Esterase Activity (—x—)
0
200
400
600
800
pH
4
5
6
7
8
9
10
11
12
13

Table III

Effect of Inhibitors on Esterase Activity

Additions	Concentration (mM)	Percent of Initial Activity
None	-	100
DFP	0.1	0
	0.01	50
	0.001	100
PMSF	1	0
	0.1	67.5
	0.01	85.0
	0.001	100.0
$NaHSO_3$	10	0
	1	90
	0.1	100

FIGURE 8

Effect of substrate concentration on esterase activity.

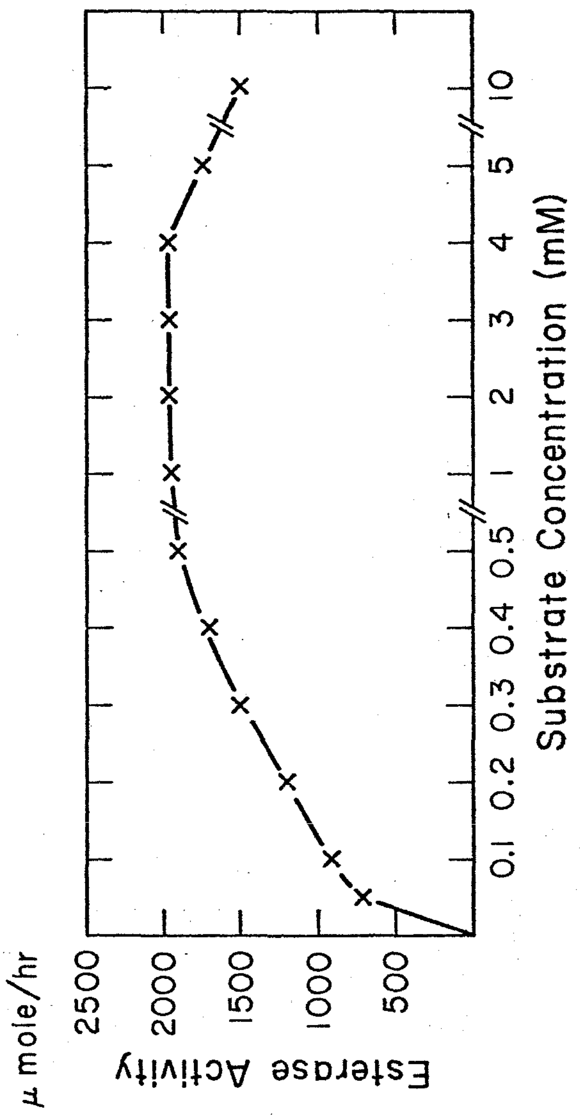
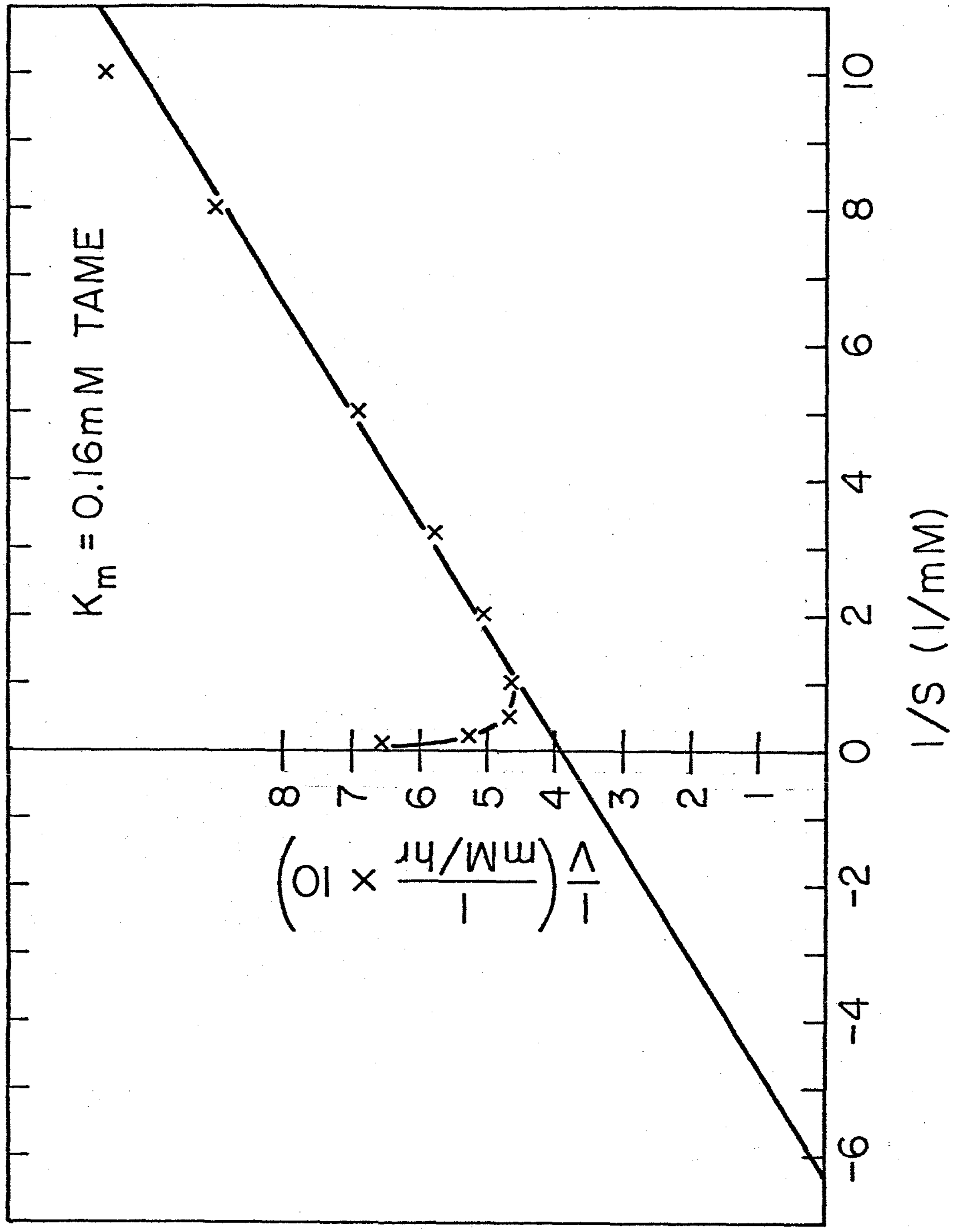

µ mole/hr
Esterase Activity
2500
2000
1500
1000
500
0.1
0.2
0.3
0.4
0.5
1
2
3
4
5
10
Substrate Concentration (mM)

FIGURE 9

Lineweaver-Burk plot for the hydrolysis of TAME by the purified esterase.


K_m = 0.16mM TAME
$\frac{1}{v}\left(\frac{1}{mM/hr} \times 10\right)$
1
2
3
4
5
6
7
8
1/S (1/mM)
-6
-4
-2
0
2
4
6
8
10

DISCUSSION

Esterase, in the stricter sense of the word, catalyzes the hydrolysis of a large number of carboxylic esters. Aldrige (1953) proposed a frequently used classification of esterases based on their behavior toward organophosphorus compounds. Accordingly, A-esterases (EC 3. 1. 1. 2) are not inhibited by organophosphorus compounds but hydrolyze them as substrates. They also split aromatic esters such as phenyl acetate and, therefore, have been designated as arylesterases. In contrast, B-esterases (EC 3. 1. 1. 1) are inhibited by organophorphorus. These enzymes have been formerly known as "ali-esterases" or, because of their wide specificity, as unspecific esterases. In pig kidney a third type of esterase has been described (Bergmann et al., 1957). This enzyme is neither inhibited by organophosphorus compounds nor does it hydrolyze them and therefore, has been named C-esterase. Since the esterase here purified is inhibited by DFP and PMSF, it may be tentatively classified as a B-esterase. However, none of the present classifications of esterases is completely satisfactory and unambiguous.

In analogy to the mechanism of ester hydrolysis by endopeptidases as studied in detail in many laboratories, the participation of a histidine residue at the active site of the esterases is suggested by the pH profile.

However, direct experimental evidence for this is still lacking.

At present the metabolic function of most carboxylesterases is still obscure. Almost all substrates used are foreign compounds which do not occur normally in intermediary metabolism. Some of them are of course of pharmacological significance. Unspecific esterases may play a role in detoxication systems. So far the physiological substrates for most of the esterases are still unknown.

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CHAPTER IV

MAPPING CHROMOSOMAL RNA GENES BY ELECTRON MICROSCOPY OF HYBRIDS OF FERRITIN-LABELED CHROMOSOMAL RNA AND DNA

INTRODUCTION

One difficulty in the study of eukaryotic gene control is the complexity of such genomes compared to those of prokaryotic cells. The genome size of E. coli. is 4.5 10^6 nucleotide pairs, i. e., about 5,000 genes coding for average sized polypetides (M. W. = 3×10^4); in dictyostelium discoideum, 5×10^7 nucleotide pairs or about 50,000 genes; in man, 8×10^9 nucleotide pairs, 8 million genes and mind boggling since it appears highly unlikely that any cell need employ 8 million different polypeptides during its life cycle. Major portions of the DNA of higher organisms have been thought by one speculator or another to consist of reserve genes, repetitive genes, regulatory genes or evolutionary garbage. However, a reasonable explanation has not yet been advanced for such large genome sizes. Most of the known biosynthetic pathways are already represented in unicellular organisms. It seems unlikely that the 1,800 fold increase in genome size from bacteria to mammals can be attributed to a 1,800 fold increase in the number of structural genes. Quite possibly, the principal differences between bacteria and mammals presumably lie in the degree of integrated cellular activities and degree of cell differentiation, and thus in a vastly increased complexity of regulation rather than a vastly increased number of structural genes.

A second problem has to do with the repetition of DNA sequences. Examination of prokaryotic DNA reveals that, apart from r-RNA cistrons, it is composed of sequences, each of which is repeated a single time in the genome (the unique sequences). In contrast, eukaryotic DNA consists of three classes of sequences: highly repetitious, middle repetitious and unique. In most higher organisms, highly repetitious DNA makes up 8 - 12% of total nuclear DNA. This fraction of the genome is of low complexity, relatively homogeneous, present in very high frequencies (10^5 to 10^6 copies per haploid genome), reassociates to form rather well base paired duplexes and has, in some cases, an unusual base composition.

Purified highly repetitive DNA, when denatured, reannealed and mounted for EM study, yields a high proportion of circles (Henning *et al*., 1970). The repetitive regions are therefore in tandem arrangements (Rae, 1970; Jones, 1970; Henning and Walker, 1970) and are known to be clustered in the centromeric areas of chromosomes (Jones, 1970; Pardue and Gall, 1970; Botchan et al., 1971; Saunders *et al*., 1972). It is debatable whether highly repetitive sequences function as a template for RNA synthesis in vivo. (Flamm *et al*., 1969; Hennig and Walker, 1970). They might serve a role in chromosomal "housekeeping" (Walker, 1968; Kram *et al*., 1972; Jones, 1970).

Moderately repetitive sequences make up to 10 - 30% of the total nuclear DNA of higher organisms. Repetition frequencies for this class of DNA range from tens to thousands of copies per haploid genome. This class of DNA sequences is highly heterogeneous, and forms imperfect duplexes upon renaturation; the majority are interspersed throughout the genome but some are clustered in certain area of chromosomes (e. g. 5s-RNA, r-RNA, t-RNA, histone genes) (Pardue and Gall, 1974).

In the last ten years a variety of observations been made, bearing on the intertwined issues of DNA sequence organization and the mechanism of gene regulation in higher organisms. Of course such genomes are so large (up to 10^{11} nucleotide pairs) that knowledge of these actual nucleotide sequences might not be very useful. However, it now appears that the genomes of higher organisms are organized in recognizable patterns. Early studies (Waring and Britten, 1966) indicated that in mammalian DNA many repetitive sequences were interspersed throughout the genome. It was observed that most high molecular weight DNA formed partial duplexes as a result of repetitive sequence interactions. Networks were formed indicating that many fragments of 10,000 nucleotides in length contained more than one repetitive sequence element. When the size of the input DNA was reduced, a smaller amount of DNA formed partial duplexes at repetitive reaction conditions. These results suggested that repetitive and unique sequences were interspersed. Experiments on calf DNA (Britten and Smith, 1970), measuring the reassociation of long labeled fragments with a great excess of short unlabeled fragments, showed that three-fourths of the 4,000 nucleotide labeled fragments contained repetitive sequences and that many of these were interspersed with unique sequences. Recent experiments on sea urchin and frog showed that much of the repetitive DNA is interspersed among unique sequences and that the modal length of the interspersed repeated sequence elements is about 300 nucleotides (Davidson and Britten, 1973). One middle repetitive sequence is followed by a unique sequence of 700 to 1,100 nucleotides in length. This fine level interspersion occurs in at least 35 to 40 % of total genomal DNA. More than 20% of these genomes is in the long period interspersion pattern where the length of the unique sequence is on the average at least 4,000 nucleotides (Davidson and Britten, 1973). Recently Bonner and his associates (1974) observed that about 56 mass % of the rat genome is organized as follows: one middle repetitive sequence approximately 300 ± 200 base pairs in length is followed immediately in about 60% of cases by a second repetitive segment of the same length, to make a doublet middle repetitive segment about 400 to 600 base pairs in length. (In the remaining 40% of the cases only one repetitive segment is observed). This is then followed by a unique sequence of 2,000 to 16,000 nucleotides in length. About 23 mass % of the rat genome is arranged similarly but with unique sequences of 500 to 2,000 base pairs.

The experimental observations reviewed above supply relatively precise information on the patterns of interspersion of repetitive and unique sequences. The similarity of the results for an echinoderm, an amphibian and a mammal suggests that interspersion of repetitive and unique sequences appear to be a general, if not universal, property of higher organism DNA. These pattern arrangements are very likely to be of functional significance and are consistent with a model proposed by Britten and Davidson (1969) for gene regulation in eukaryotes.

The model proposes that each structural gene in a given gene battery is preceded by a certain repetitive sequence. The sequences serve as recognition and binding sites for sequence-specific regulator molecules, which are termed "activators". Since either RNA or protein could serve this function, Britten and Davidson (1969) considered both alternatives as possible. In either case, genomic sequences coding for the activator must exist, and these they termed "integrator genes". These integrator genes are physically associated with "sensor genes", which respond to external effectors (e. g., hormones) in such a way that transcription becomes induced in the adjacent integrator genes. Thus simple external signals can trigger a vast response from a very large number of non-contiguous genes; their function implies the existence of multipoint switches in the regulatory system. The model requires the existence of repetitive sequence elements and predicts that specific locations of particular repetitive sequences are of crucial functional significance.

At the very heart of this model is the possibility that RNA could serve as an activator. Such RNA molecules would have to be: 1) confined to the nucleus 2) bound to chromatin 3) repetitive gene transcripts 4) highly heterogeneous.

Bekhor, Kung and Bonner (1969) and also Huang and Huang (1969) presented evidence suggesting that sequence-specific reconstitution of chromosomal proteins to DNA requires chromatin-bound RNA, termed chromosomel RNA, which hybridizes to the middle repetitive sequences of homologous DNA (Holmes, 1973). This class of RNA is heterogeneous, organ specific, confined principally to the nucleus and of general occurrence (Bonner and Widholm, 1967 , Benjamin, et al., (1966), Huang, 1967 , Dahmus and McConnell, 1969 , Shih, 1969 , Mayfield and Bonner, 1971). If the chromosomal RNA is an activator as proposed by Britten and Davidson (1969), it becomes important to ask the question, with which classes of DNA does chromosomal RNA interact, and how are they arranged in the genome. Since the size of chromosomal RNA is small (about 16,000 daltons), when it hybridizes with DNA the hybrids would be too short to be visualized directly by electron microscopy. Therefore, we have covalently coupled chromosomal RNA to ferritin, an electron dense marker, to allow the mapping of these arrangements in genome. Preliminary results of such a study are presented below.

MATERIALS

Horse ferritin was purchased from Polyscience. Bromoacetyl bromide was purchased from Aldrich Chem. Co, Inc. Mercaptosuccinic dihydrazide was a generous gift from Dr. E. R. Atkinson of Arthur D. Little, Inc. Cesium chloride was purchased from Rare Earth Division, American Potash & Chemical Corp. DEAE Sephadex A-25, and Sephadex G-25 were from Pharmacia Fine Chemicals Inc. Pronase, Grade B, was from Calbiochem.

METHODS

Growth of Rat Ascites Cells. The Novikoff ascites tumor line used in the following investigation was maintained by a serial transplantation in male albino Sprague-Dawley rats purchased from Berkeley Pacific lab. Transfer was carried out every 6th day and ascites cells were harvested at the same time.

Preparation of c-RNA from Rat Ascites Cells. C-RNA was prepared from rat ascites cells according to the method of Dahmus and McConnell (1969) with a slight modification. Unless otherwise noted, all operations were carried out at 4°. Ascites cells were diluted with an equal volume of TNKM buffer (50 mM Tris, pH 6.7 - 0.13 M NaCl - 25 mM KCl - 2.5 mM $MgCl_2$) and centrifuged at 700 g for 10 min. The pellet was washed several times in three volumes of deionized water and centrifuged at 700 g until no contamination by erythrocytes was apparent. The purified ascites cells were lysed with 1% (v/v) of Triton - X 100 in saline-EDTA (75 mM NaCl - 24 mM EDTA, pH 8). The crude nuclear pellet was collected by centrifugation at 3,000 x g and washed once with saline-EDTA. Nuclei were lysed using a Potter-Elvehjem homogenizer in 10 mM Tris (pH 8) and then sedimented at 10,000 g for 15 min. This step was repeated twice. The resulting pellet, referred to as crude chromatin, was used as the starting material for c-RNA preparation.

The crude chromatin was suspended in an equal volume of 10 mM Tris (pH 8) and diluted with two volumes of 6 M CsCl in 10 mM Tris (pH 8) to give a final CsCl concentration of 4 M. The resulting solution was extremely viscous and was homogenized in a Waring blender at 20 volts for 30 sec. The solution was stirred overnight and centrifuged at 82,000 g for 15 hours . The chromosomal proteins and c-RNA formed skins at top of the centrifuge tubes. The skins were removed with a spatula and washed three times with 70% ethanol. The chromosomal proteins were then digested with 1 mg/ml of pronase (preincubated for 90 min at 37°) in 10 mM Tris (pH 8) for 4 - 6 hours at 37°. SDS was added to a final concentration of 1% followed by the addition of an equal volume of water saturated phenol containing 0.1% 8 - hydroxyquinoline. After shaking for 30 min at room temperature, the phases were separated by centrifugation at 1,000 g for 10 min and the aqueous phase was collected. Nucleic acids were precipitated by the addition of one - tenth volume of 20% potassium acetate and two volumes of 95% ethanol. After 2 hours at -20°, the precipitate was recovered by centrifugation; washed once with 70% ethanol and dissolved in several ml of 0.2 M NaCl - 7 M urea - 10 mM Tris (pH 8). The resulting solution was applied to a A - 25 DEAE sephadex column (0.9 x 25 cm) preequilibrated with 0.2 M NaCl - 7 M urea - 10 mM Tris (pH 8) and eluted with a linear gradient of

NaCl from 0.2 M to 1.0 M in the presence of 7 M urea-10 mM Tris (pH 8). C-RNA was recovered by precipitation with two volumes of 95% ethanol in the presence of 2% potassium acetate for 2 hours at -20°.

In Vitro Labeling of c-RNA with ^3H - dimethyl Sulfate. C-RNA was labeled in vitro by the procedure of Mayfield and Bonner (1972). One mg of c-RNA was dissolved in 0.1 ml of 0.1 M potassium phosphate buffer (pH 7.5). The solution was incubated overnight with 1 mCi of ^3H - dimethyl sulfate (specific activity 400 mCi/mmole) at 4°. The incubation mixture was passed through a Sephadex G-25 column (1.5 x 60 cm) equilibrated and eluted with 0.1 M potassium phosphate buffer. The labeled c-RNA was recovered from the column eluant by centrifugation after precipitation with 95% ethanol at -20° overnight. The specific activity of labeled c-RNA was 10,000 cpm/μg.

Modification of c-RNA. ^3H - labeled c-RNA was mixed with unlabeled c-RNA to yield a specific activity of 2,000 cpm/μg. Two mg of this mixture was dissolved in 1 ml of 0.1 M sodium acetate buffer (pH 4.6) and was oxidized by addition of 1 ml of freshly prepared 0.05 M of $NaIO_4$ in the dark for 1 hour at room temperature. Five μl of ethylene glycol were added to reduce the excess $NaIO_4$. The oxidized c-RNA was dialyzed against 0.1 M sodium acetate buffer (pH 4.6) in the dark at 4° overnight. To the resulting oxidized c-RNA solution, 2 μmoles of mercaptosuccinic dihydrazide (from a freshly prepared 0.01 M solution) were added. After reaction in the dark for 10 hours at room temperature, the solution was dialyzed against 0.1 M phosphate buffer (pH 8). Dithiothreitol was added to a final concentration of 5 mM. All operations beyond this point were carried out under an argon atmosphere. The dithiothreitol treatment for one hour assured that all the mercapto groups of the coupled mercapto-succinic dihydrazide were in the reduced form. Dithiothreitol was removed by dialysis against 0.01 M phosphate buffer (pH 7.5).

Purification and Modification of Ferritin. Ferritin was modified by the method of Wu and Davidson (1973) but with slight modification. Commercial 6 times recrystallized horse spleen ferritin was dissolved in 2 % ammonium sulfate (pH 5.8) at a concentration of 10 mg/ml. Three volumes of the ferritin solution were gently mixed with one volume of 20% $CdSO_4$ and the mixture allowed to stand overnight at 4°. The crystallized ferritin was pelleted by centrifugation at 39,000 g for 20 min at 4° and redissolved in 2% ammonium sulfate to give a final concentration of 10 mg/ml. Insoluble material was removed by centrifugation as above. The recrystallization procedure was repeated 2 more times. The resulting ferritin solution was dialyzed against 10 mM potassium phosphate buffer (pH 7.8) and was adjusted to a concentration of 5 to 8 mg/ml with 0.5 M potassium phosphate buffer (pH 7.8). To 5 ml of ferritin solution (8 mg/ml in 0.5 M sodium phosphate buffer, pH 7.8) was added 25 μl of bromoacetic bromide in 10 equal portions with constant stirring at 4° for 30 min. The pH was maintained between 7 and 8 by the addition of 0.1 M NaOH. After acetylation, 2 ml of 4 M sodium acetate buffer (pH 4.5) were added to the reaction mixture and stirred for ten minutes at 4°. The resulting solution was dialyzed extensively against 10 mM phosphate buffer (pH 6.5) at 4°. Denatured ferritin was removed by centrifugation at 1,000 g for 15 min. The supernatant was collected and centrifuged at 132,000 g for 2 hours to pellet the acetylated ferritin. The modified ferritin was dissolved in 1 ml of 10 mM phosphate buffer (pH 7.8).

Ferritin-C-RNA Coupling. One mg modified c-RNA, dissolved in 0.5 ml of 0.1 M NaCl - 1 mM EDTA - 10 mM phosphate buffer (pH 8) was mixed with 30 mg acetylated ferritin in 0.5 ml of 0.3 M borate buffer (pH 9). The reaction mixture was incubated under an argon atmosphere in the dark for 14 days at 4°.

For separation of unreacted c-RNA from the conjugated ferritin-c-RNA and free ferritin, 0.1 ml of the reaction mixture was layered on top of a 5 ml of sucrose gradient (15 to 25%) in 0.1 M NaCl - 10 mM phosphate buffer (pH 7.5) and centrifuged at 132,700 g for 7 hours at 10°. The gradient was fractionated and counted. The fractions containing ferritin-c-RNA conjugates were combined, dialyzed against 0.1 M NaCl and pelleted by centrifugation at 132,700 g for 2.5 hours. The coupling efficiency between ferrin and c-RNA was about 5%.

Hybridization of Ferritin-c-RNA to DNA. The hybridization mixture contained 40% formamide - 0.1 M tricine buffer (pH 7.2) - 1 mM trisodium EDTA - 0.3 M NaCl - 5 μg of unsheared denatured DNA, 2.5 μg or 25 μg ferritin-c-RNA complex (RNA to DNA ratio 0.5 : 1 and 5 : 1 respectively).

The native DNA was first denatured by addition of 1 N NaOH. After incubation at room temperature for 10 min, the solution was neutralized with an equal amount of 2 M NaH_2PO_4. The denatured DNA solution was mixed with ferritin-c-RNA complex in phosphate buffer and formamide. *E. coli* DNA, instead of rat ascites DNA was used as the control.

Hybridization was carried out by incubation at 37° for 15 and 24 hours (40% formamide, approx. 0.18 M Na^+). After incubation, the solution was diluted with an equal volume of 10 mM Tris - 1 mM trisodium EDTA (pH 8). An equal volume of freshly distilled phenol, saturated with 0.1 M sodium borate (pH 8) was gently added and mixed thoroughly. This mixture was centrifuged at 1,000 g for 10 min. The resulting aqueous phase was collected and dialyzed extensively against 10 mM Tris - 1 mM trisodium-EDTA, (pH 8) at 4°. The phenol extraction step removed traces of free ferritin and ferritin-c-RNA complexes which had not hybridized to the DNA, thus reducing the background in electron micrographs.

Electron Microscopy. The procedure used for preparation of specimens for electron microscope is described by Davis et al., (1971). The hyperphase contained 10 μl of DNA (5 μg/ml); 8 l Tris (1 M, pH 8.5), 10 μl of trisodium EDTA (0.1 M, pH 8.5), 5 μl of cytochrome c (1 mg/ml) and 60 μl formamide (68%). The hypophase contained 10% formamide - 10 mM Tris (pH 8.5). Uranyl acetate (5 x 10^{-5}M in 90% ethanol) was used for staining. The grids were in all cases rotary - shadowed with Pt - Pd (80 - 20) and examined using a Phillips 300 electron microscope.

RESULTS AND DISCUSSIONS

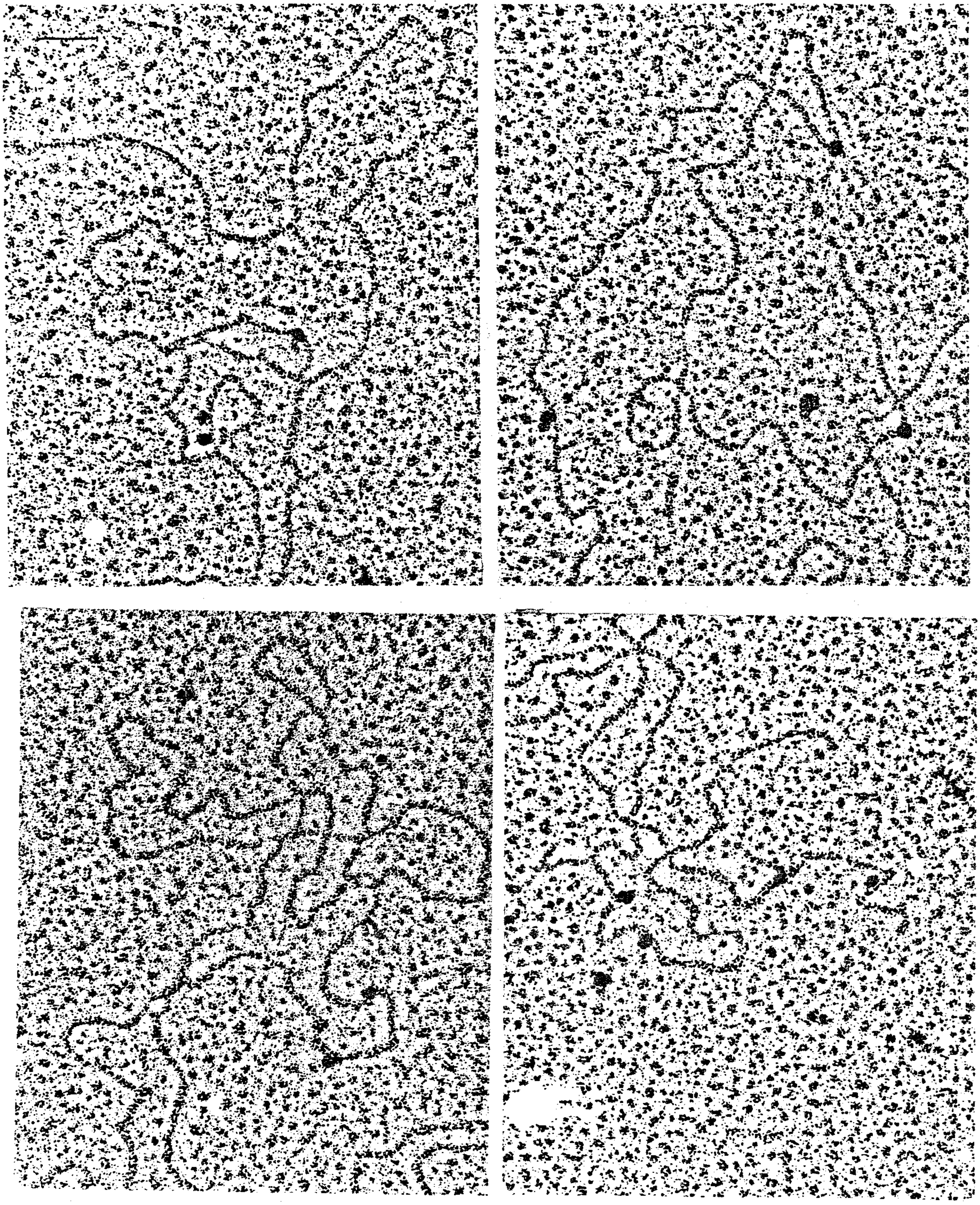
Distribution of Ferritin-C-RNA complexes in the DNA. Ferritin tagged c-RNA molecules were hybridized to ascites nuclear DNA in an effort to map the arrangement of these sequences in the genome. The ferritin acts as an electron dense marker so that the positions at which c-RNA hybridize to DNA can be visualized directly by electron microscope. Typical results of this experiment are shown in the micrographs of Fig. 1. By measuring the distances between ferritin molecules, a histogram of the interspersion was constructed (Fig. 2). These results reveal that for those DNA strands which contain ferritin molecules, two levels of interspersion exist; one of 100 - 300 nucleotides and another of 500 - 1,500 nucleotides in length. Since the interferritin distance is also the inter RNA distance and since c-RNA specifically hybridizes to moderately repetitious DNA sequences (Holmes, 1973), this observation is in accord with other studies on the arrangement of moderately repetitious DNA sequence in the rat genome (Bonner et al., 1974). However the amount of c-RNA used for this hybridization experiment was not enough to saturate all the sites available in DNA. Further experiment using saturating amount of c-RNA for hybridization is necessary. We have, however, shown that the sites to which repetitive sequence of gene expression binds may be mapped by electron microscope.

Acknowledgements

This work was carried out in collaboration with Dr. J-R Wu. We wish to thank professor Bonner for continuing guidance.

FIGURE 1

Electron micrographs of ferritin-c-RNA hybrids.



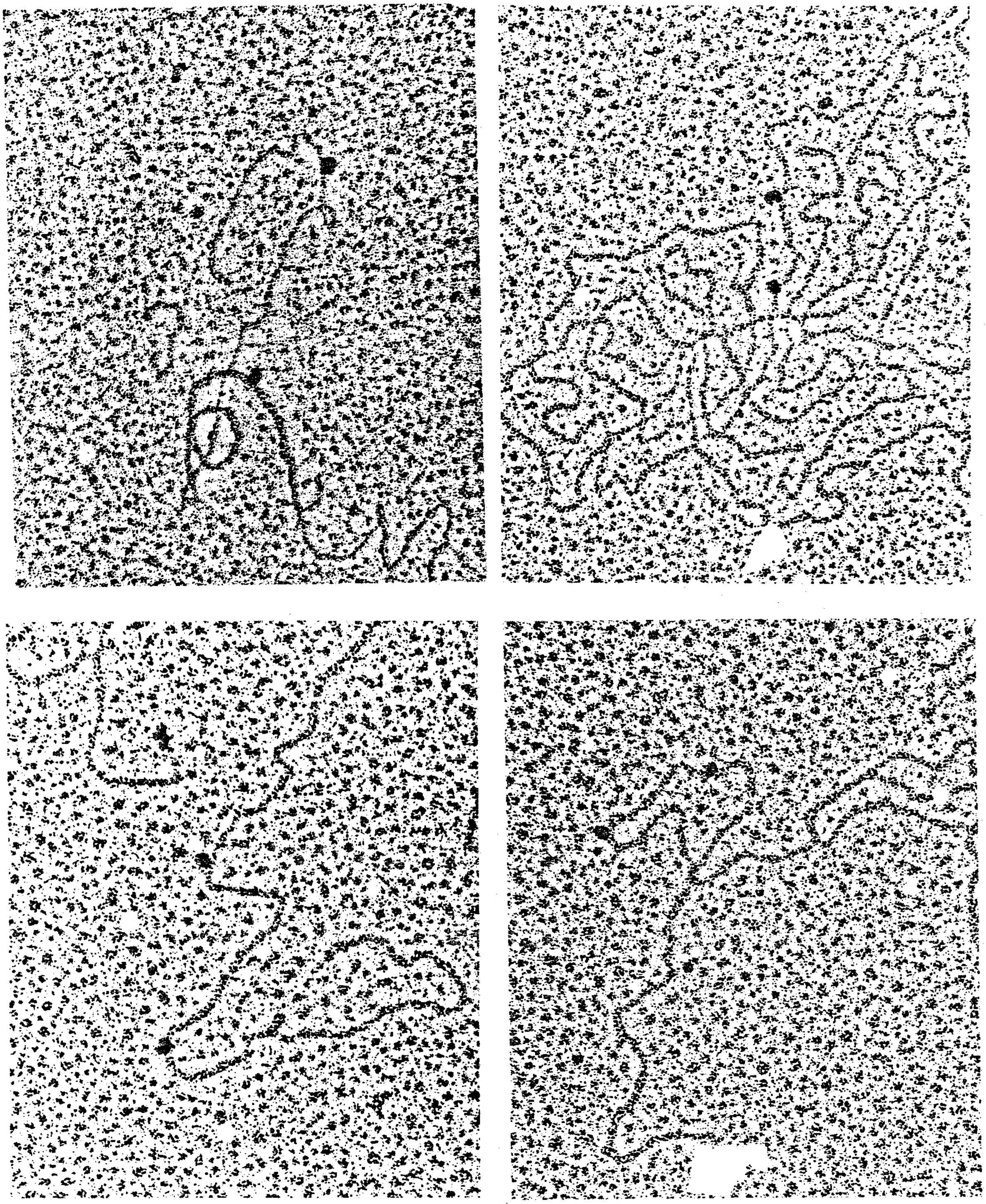
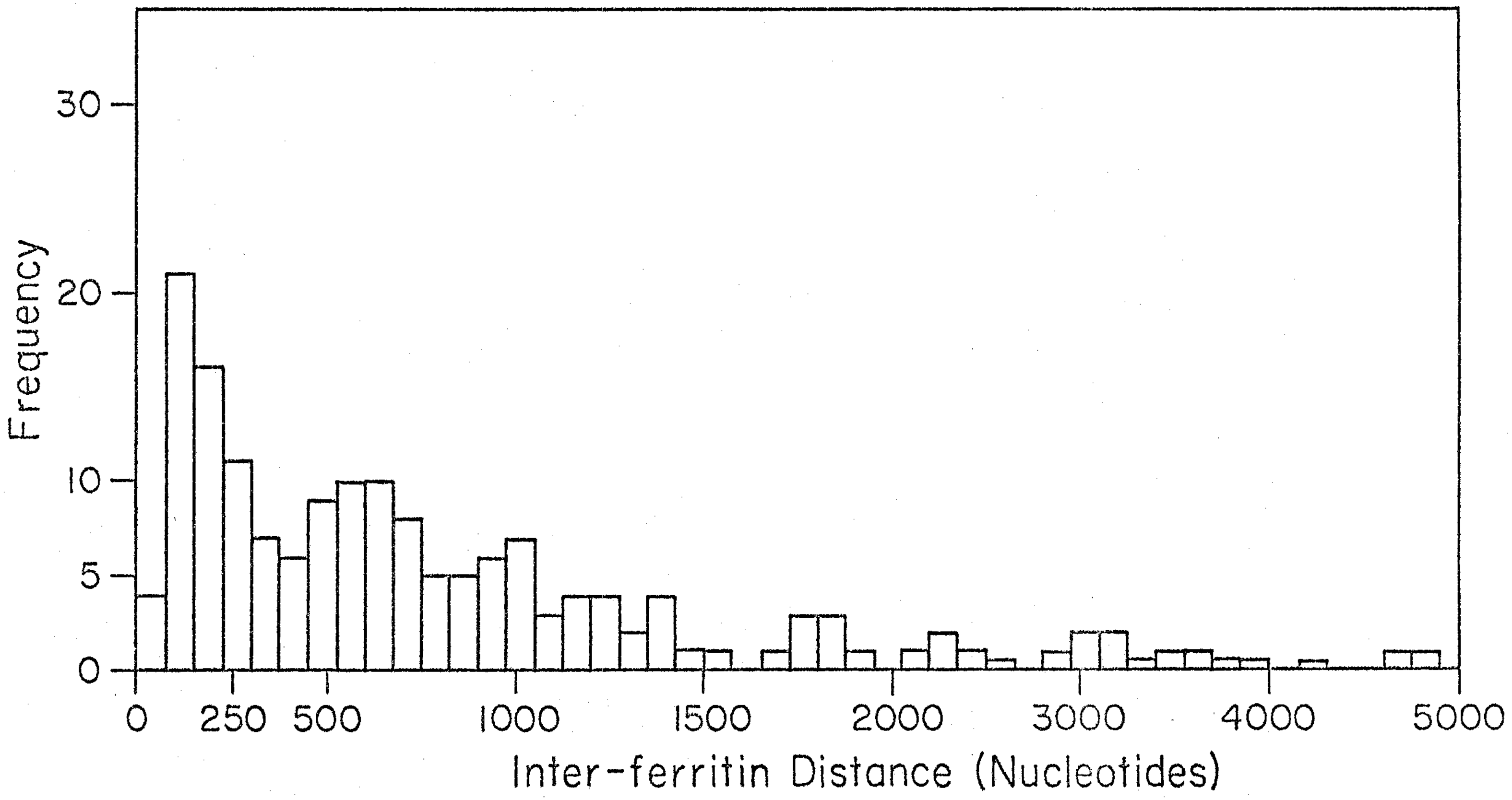


FIGURE 2

Frequency distributions of length between ferritin-c-RNA molecules.


30
20
10
5
0
Frequency
0
250
500
1000
1500
2000
3000
4000
5000
Inter-ferritin Distance (Nucleotides)

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