AN ELECTROPHORETIC METHOD FOR THE ISOLATION OF ISOHISTONES FROM THE EMBRYO OF THE SEA URCHIN PARECHINUS ANGULOSUS

by

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CONTENTS

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CHAPTER 1

INTRODUCTION

1.1 Outline of Project

It is the aim of this project to isolate sea urchin embryo histone variants by preparative polyacrylamide gel electrophoresis and to prove the identity of the isolated proteins by amino acid composition and partial sequencing. This serves two purposes, namely, the unequivocal identification of chromosomal proteins characterized only by their electrophoretic mobility as histone variants, and secondly the creation of a micropreparative electrophoretic method. These are the prerequisites for later investigations into the specific roles of histones in the process of differentiation.

Histones were isolated from sea urchin embryos using Johns' selective extraction procedure (Johns, 1964). Optimal electrophoretic resolution for the histone variants within the different classes H2A, H2B, H3 and H4 was established on analytical urea gradient polyacrylamide slab gels at constant Triton X-100 concentration. The thus identified conditions were subsequently used for preparative polyacrylamide slab gels. Standard analytical gel electrophoresis equipment was used for such gels. The recovery procedure established allowed staining of the gel with Amido Black to locate the protein fractions. The gel areas containing the separated histone fractions were excised and the dye-protein complex was extracted from the homogenized gel. The dye was subsequently separated from the proteins by anion exchange column chromatography. Finally histones were recovered by acetone precipitation, and their identity established by amino acid composition and partial sequencing.

1.2 Histone Variants

Histones are the major protein components of chromatin. The histones of most cells consist of five distinct protein types, namely histone H1, H2B, H2A, H3 and H4. Chromatin is made up out of subunits, the nucleosomes, which consist of a pair each of the main histones H2A, H2B, H3 and H4 associated with a superhelical stretch of DNA of about 200 base pairs (Kornberg, 1977 and Felsenfeld, 1978). H1 is not part of the nucleosome, but is attached to it in a not yet clearly defined way. Although histones have many properties that make them suitable for genetic control (Allfrey, 1971), the failure of conventional methods to demonstrate extensive histone diversity or tissue specific variability has in the past discouraged serious consideration of a regulatory role for these proteins. However, improvement in the development of techniques showed that histone variants do exist, both as far as different organisms are concerned, thereby indicating complexity at different levels of evolution, and as a result of genetic activity of cells during the life cycle of one organism at any particular evolutionary level (von Holt et al., 1979).

It appears now firmly established that the electrophoretic heterogeneity of histones is the result of the presence of variants synthesized at different stages of development (Hill et al., 1971, Cohen et al., 1975, Arceci et al., 1976, Newrock et al., 1978, Brandt et al., 1979). In addition each of the variants may undergo cell cycle dependant post-translational modifications. In view of this it is conceivable that the multiple reiteration of histone genes (Kedes and Birnstiel, 1971) may be the expression of closely related histone gene batteries which are switched on at different stages of differentiation. Cohen et al. (1975) supplied electrophoretic evidence for such a view, showing programmed

appearance of pulse labelled histones in developing sea urchins.

Newrock et al. (1978) describe the presence of stage specific transcription of histone mRNA. Kunkel and Weinberg (1978) have identified two distinct populations of histone gene transcripts, showing up as qualitative changes in the histones synthesized at the mesenchyme blastula and gastrula stages. Brandt et al. (1979) have structurally identified by partial sequence analysis stage specific histones at early embryonic stages in the sea urchin. So far not much can be said whether and how histones variants influence chromatin structure. Compton et al. (1976) reported that there is no general correlation between the size of DNA chromatin repeat length and the stage in the cell cycle or rate of cell division. On the other hand, Noll (1976) suggests that changes in amino acid composition of H1 can effect the micrococcal nuclease digestion pattern of chromatin. As far as sea urchin embryo chromatin is concerned, a DNA repeat length of 222 base pairs has been reported which appears to remain constant throughout development, although the rate and extent of solubilization decreases during micrococcal digestion as development proceeds (Keichline and Wasserman, 1977).

1.3 Histone Variants Isolation

The presence of small amounts of histone variants in chromatin is overshadowed by the presence of five standard histones. This makes their isolation by conventional methods such as exclusion chromatography and ion exchange chromatography difficult, because of similarity in size and charge. Histones purified by such methods and analysed by polyacrylamide gel electrophoresis (Panyim and Chalkley, 1969, Laemmli , 1970) are considered homogeneous and invariable. The two electrophoretic methods applied are capable of differentiating between molecular sizes (SDS PAGE,

Laemmli, 1970) and size:charge ratio (Panyim and Chalkley, 1969). The addition of Triton X-100, a non-ionic detergent, (Franklin and Zweidler, 1975) to acetic-acid urea polyacrylamide gels added a new dimension to histone analysis. Triton X-100 is thought to bind specifically to helical regions and will thus change the size:charge ratio (Zweidler, 1976) resulting in a differential reduction in the electrophoretic mobility of proteins depending on the extent of helicity. Often a single amino acid change leads to difference in Triton X-100 binding e.g. the oxidation of methionine to the sulfoxide results in a different mobility (Zweidler, 1978). Triton X-100 reduces electrophoretic mobility in the absence of urea but results in poorer resolution. Urea, by interfering with the helical structure of the protein, antagonizes Triton X-100 binding to the different histones. The degree of interference depends on the particular amino acid sequence. Therefore different urea concentrations will affect the Triton binding of the individual histones to different degrees which in turn results in optimal resolution of the different variant classes. The effect of urea on Triton X-100 binding has been demonstrated by Zweidler, (1978) using a urea gradient in polyacrylamide slab gel at constant Triton X-100 concentration.

How can such a sensitive analytical tool for histone variant characterization be adapted to a preparative purification technique? Two methods are generally used for the preparative recovery of proteins from polyacrylamide gels.

1) Separation of proteins on cylindrical or slab gels, visualization, excision of protein bands and recovery by, (a) extraction with various solvents (Houston, 1971, Gibson and Gray, 1979, Wada and Snell, 1972, Djondjurov and Holtzer, 1979, Kerckaert, 1978, Martinage, 1976), or (b) electrophoretically (Mardian and

Isenberg, 1978, Braatz and McIntire, 1977, Hanaoka et al., 1979).

2) Continous electrophoretic elution from a separating gel (Stephens, 1975, Koziarz and Köhler, 1978).

CHAPTER 2

PREPARATIVE POLYACRYLAMIDE GEL ELECTROPHORESIS(PAGE)

2.1. Introduction

Polyacrylamide gel electrophoresis has become a powerful tool for the fractionation of proteins,and its methodology is applicable to molecules virtually without size limit and lends itself to the fractionation on the basis of molecular size, net charge and hydrophobicity over wide ranges of pH, ionic strength and temperature and in the presence of a wide variety of detergents. Extensive reviews and monographies have been published in this respect (Panyim and Chalkley, 1969, Laemmli, 1970, Hardison and Chalkley, 1976, Hamana and Iwai, 1976, Chrambach et al., 1976). It is applicable to the analysis at the microgram to nanogram level. Because of the fractionation power and the versatility of the method, attempts have been made to adapt the system to a preparative scale. Up to date, preparative PAGE is a preparative tool in the microgram range, and improvements will depend on future development of simple and reliable preparative PAGE apparatus. Different apparatus and techniques will probably become necessary for either g or mg preparations.

The general strategy to be followed for the fractionation of protein molecules by PAGE is as follows:

The determination of stacking conditions (Ornstein, 1965) for proteins under selective conditions of pH, ionic strength and temperature. Stacking improves the resolution of the protein sample to be separated by PAGE and is presented pictorially in Fig. 1 page 7 (Williams and Reisfeld, 1964). Stacking is achieved whenever an electric field is applied to a system of ionic species differing in their electrophoretic mobilities, contained

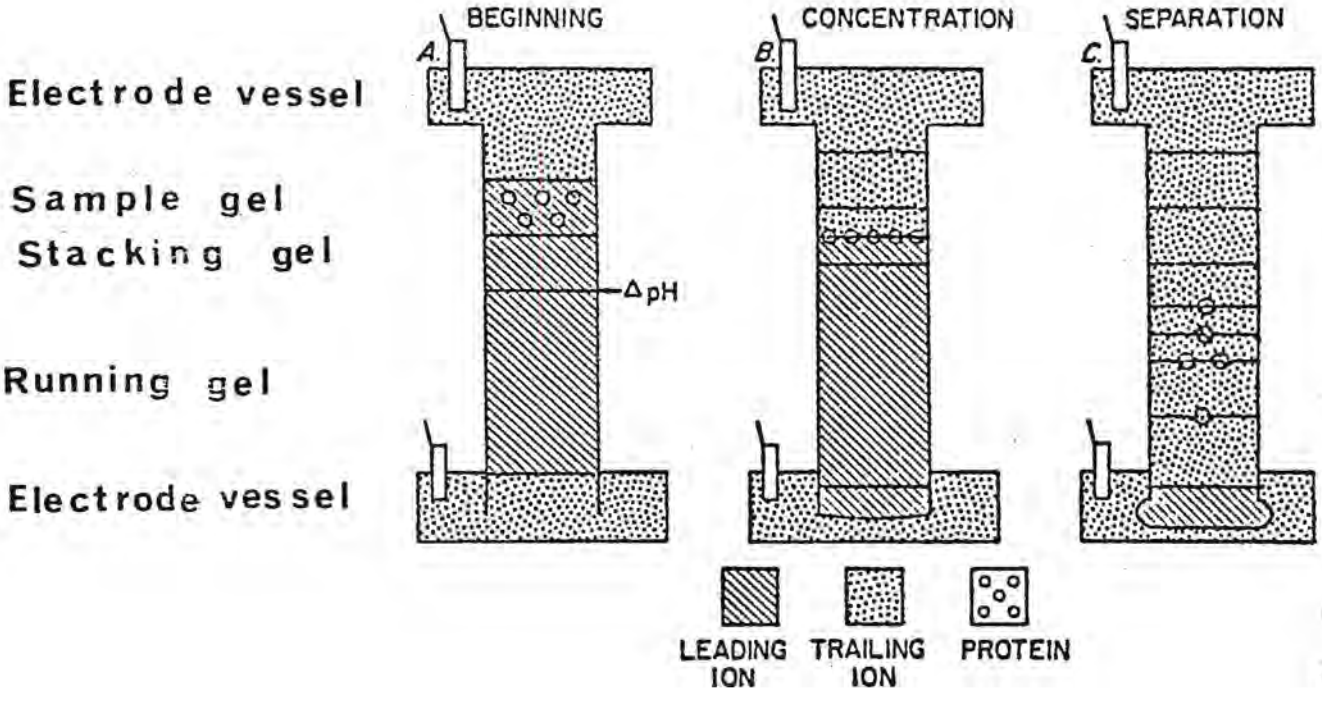


Fig. 1 Steady state stacking
(Williams and Reisfeld, 1964)

in separate phases (sample gel, stacking gel and running gel), and separated by a stationary boundary. The high mobility or leading ion species pull away from the slower or trailing constituent, causing an increase in the voltage gradient behind the leading constituent. The slower moving species will be accelerated until all the trailing and leading constituents separated by a moving boundary will move with the same speed (i.e. iso-tachophoretically) (Chrambach, 1980). Optimal stacking conditions can be quantitated (Jovin, 1973), and Jovins' theory has been adopted in the form of an easily applied computer program and sample output (Rodbard and Chrambach, 1971,1974). The determination of the optimal gel concentration for the separation of the protein of interest involves PAGE at different gel concentrations (total acrylamide and cross linker concentration). The electrophoretic mobility is logarithmically dependant on the gel concentration. From the Ferguson Plot Kr, the retardation constant can be calculated. The latter is related to the molecular weight and molecular radius and to the net charge. The Ferguson Plot can be used as a guide for the design of further analytical and preparative methods to achieve separation due to differences in size, in charge or both. Rodbard and Chrambach (1971, 1974) designed a series of computer programmes designated as the PAGE-PACK, which facilitates and automates the determination of the optimal operative pH. The data from multiple zone electrophoresis (MZE) with a large number of buffer systems (Jovin, 1973) give optimal stacking conditions. The fractionation conditions as derived from the Ferguson plot and based on the Rf values from 3-7 different gel concentrations are included. *

* PAGE-PACK (Rodbard and Chrambach, 1971, 1974)

Program name	Program function
1. FERGUSON	Calculates and plots Ferguson plot: $\log(R_f) = \log(Y_0) - K_R T$
2. MOLWT	Calculates and plots molecular weight (MW) calibration curves, obtains $\bar{R}$ and MW for unknowns, calculates and plots $\bar{R} = a + b\sqrt{K_R}$ $\mathrm{MW} = a + bK_R$ $\log(\mathrm{MW}) = a + bR_f$ $\mathrm{MW} = a + b\log(R_f)$ $\log(\mathrm{MW}) = a + b(V_e/V_0)$ $\bar{R} = a + b\sqrt{-\log(K_{av})}$
3. CHARGE	Calculates free electrophoretic mobility M_0 and valence V
4. T-OPT	Calculates T_{max} and T_{opt}
5. IDENT	Tests for identity by an F test and K_R–Y_0 ellipse overlap criteria
6. SIGMOID	Estimates MW for SDS-PAGE or gel filtration using a sigmoidal relation between log (MW) and R_f, V_e/V_0, V_e, or K_{av} K_{av} or $R_f = \dfrac{a}{1 + (\mathrm{MW}/c)^b}$ V_e/V_0 or $V_e = \dfrac{a - d}{1 + (\mathrm{MW}/c)^b} + d$ (for V_e/V_0, d should be unity)

Generally there are two methods for preparative PAGE in use:

1) Simultaneous Zone Elution Method

Electrophoresis is carried out for a fixed time. The fractionation of the proteins is executed at a gel concentration already optimized in the analytical gel system. The proteins are eluted from gel slices and concentrated (Houston, 1971, Gibson and Gracy, 1979, Wada and Snell, 1972, Mardian and Isenberg, 1978, Braatz and McIntire, 1977, Hanaoka et al., 1979).

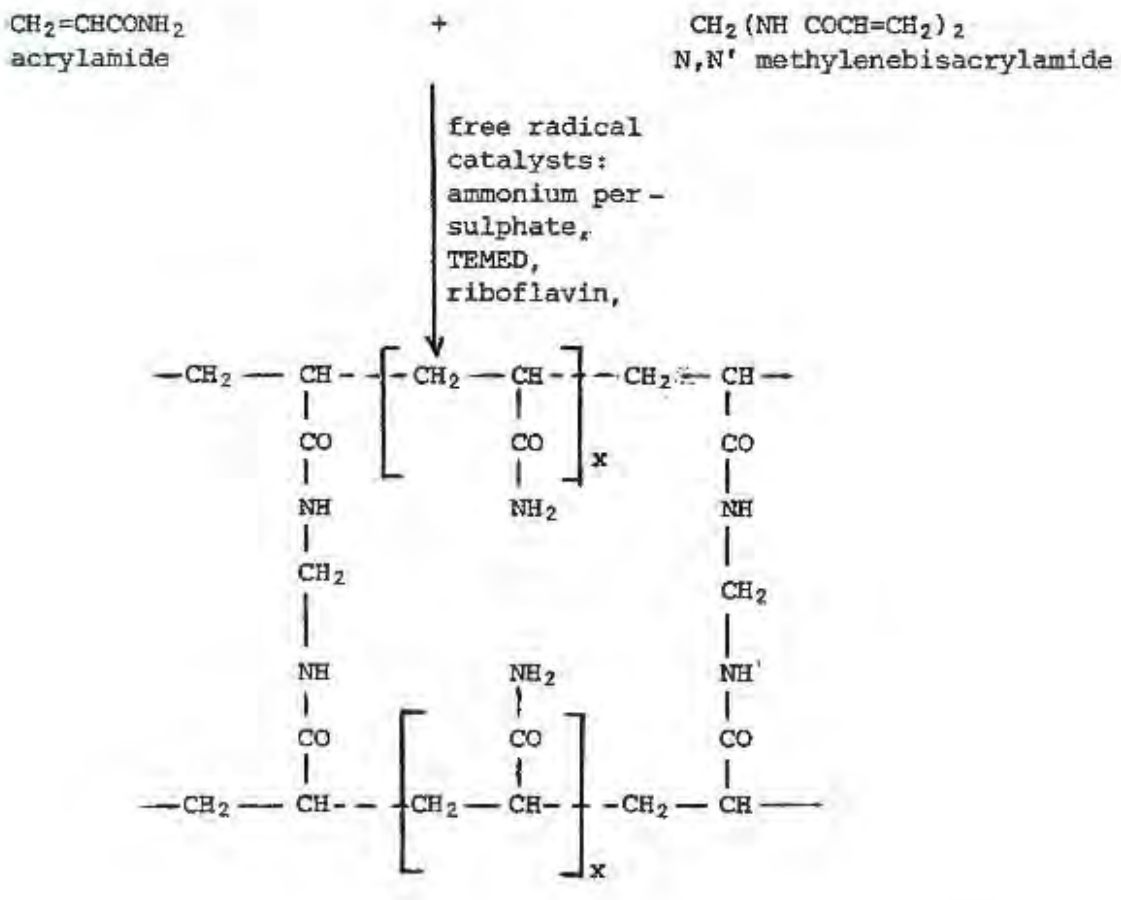
2) Continuous Zone Elution Method

Electrophoresis is carried out at a fixed path length, and the optimal gel length and gel concentration have to be established to obtain the maximal resolution per unit time (Stephens, 1975; Koziarz and Köhler, 1978). The method still needs a convenient system for the continuous analysis of the eluate, followed by the eluate concentration.

2.2 Factors Affecting the Performance of Preparative PAGE

2.2.1 Control of Polymerization

a) Polymerization reaction



b) Polymerization reagents

The acrylamide monomer and the cross linker should be of the purest grade in order to form a gel of a reproducible pore size. Often acrylamide monomer preparation contains acrylic and inorganic ions making recrystallization necessary (Shuster, 1971). The acrylamide stock solution should be kept cool and in the dark as on incorrect storage polymers may form spontaneously.

c) The Control of Pore Size

The effective pore size is an inverse function of the total monomer (% T), which is defined as the sum of the concentration of acrylamide and crosslinker (Johnson, 1979). Therefore reproducibility of pore size is dependent on the quantitative conversion of monomer to polymer, which depends on the concentration of the catalyst, the possible presence of inhibitors of polymerization and the redox conditions in the gel.

d) The Initiators

In selecting a catalyst andan accelerator system, it is desirable for the preparation of gels to keep the concentration of extraneous ions at a minimum. The free radical catalyst-accelerator used is ammonium per sulfate together with TEMED,or riboflavin together with TEMED (Davis, 1964). TEMED is selected because of its high efficiency, that is its capacity to accelerate polymerization in low concentrations. Triethanolamine was less efficient. Ammonium persulfate is used as a free radical generator. It is a strong oxidant, therefore pre-electrophoresis is necessary to remove excesses of persulfate in order to prevent oxidation of the protein sample during its passage through the gel (Brewer, 1967). Riboflavin is a photo-chemical initiator. It has the advantage that polymerization time can be better controlled as it is initiated by exposure to light, and very low concentrations of riboflavin are effective whereas in using ammonium persulfate, the polymerization time is set by the concentration of ammonium persulfate in the polymerization mixture. The initiator concentration is inversely proportional to average chain length. Therefore one has to compromise between maximum initiator concentration to obtain a high degree of

conversion and minimum concentration to give maximum average chain length (Chrambach et al., 1976).

e) The Inhibitors

Polymerization is inhibited by compounds which act as free radical traps e.g. atmospheric oxygen. Therefore degasing of the polymerization mixture is essential. Inhibition is an important consideration in choosing the apparatus. Glass plates (borosilicate) are chosen to form the gel shell rather than Perspex as, due to the polarity of the former, they have a better gel adherence. This is of particular importance in preparative gel preparations because of the larger gel mass. In the present work Teflon spacers have been used rather than rubber spacers as the latter were found to be inhibitory.

f) The Redox Condition in the Gel

The redox state in the gel is affected by the free radical concentration. The latter does not directly affect the pore size, but the peroxide which is formed as an intermediate in the polymerization reaction initiated with persulfate or riboflavin leaves the gel in a highly oxidized state. This in turn may affect the protein conformation depending on the number of reactive reducing groups present in a protein. The result will be a change in the electrophoretic mobility of that protein. Mercaptoethanol, to prevent peroxide formation, cannot be added to the gel as during polymerization it inhibits the reaction,and even if used at low concentrations, it would become fully oxidized by conditions of vinyl polymerization. While pre-electrophoresis to remove oxi-

dizing species can partially overcome this problem, the procedure complicates the method, because it will reliably remove only charged oxidizing molecules whereas uncharged molecules may or may not remain in the slab depending on the time available for diffusion. This results in a slab condition which is difficult to define. Thiodiglycol alleviates the oxidation problem (Zweidler, 1976) where pre-electrophoresis is not desirable.

2.3 Suitability of Slab Gels for Preparative PAGE - General Observations

It was considered desirable to design preparative PAGE on the basis of analytical PAGE procedures already used in the laboratory and in particular, complicated cooling procedures should be avoided. This meant that analytical gel conditions could be used and merely required adaption to a preparative scale by increasing the surface area for sample application and the gel length for improved separation. This limited the gel width to 10 cm, with the gel length and the thickness being variable.

When the surface area is increased, a higher current has to be applied to maintain a constant voltage gradient this results in increased heat generation. Therefore the current must be selected to minimize heating, particularly in "Hot Spots" where higher protein concentrations in the gel produce a higher resistance. Temperature variations in the gel cause uneven migration zones. When gels with a surface area of 1,5 cm^2 in the sample slot and a thickness of 0,17 cm were electrophoresed at a low current (10 mA constant current) overnight, the gel temperature could be adequately controlled with a fan.

Gels with a greater surface area will have to have an internal cooling

system if they are to give reproducible results in a comparable time.

When the whole gel width was used for sample application, the separated protein zones showed a marked increase in mobility at the sides, resulting in overlap between the bands (Fig. 2a pp. 16). It was thought that uneven entry of the sample into the gel caused by slight inhibition of polymerization at the edges of the gel surface was the main cause. However, the effect could not be prevented by careful overlaying of the gel with water during the polymerization procedure. Therefore the sample well was changed by designing a well comb which left 0,5 cm of unused surface area at either side of the sample (Fig. 2b pp. 16). This, however, did not affect the phenomenon (Fig. 2b pp. 16). To test whether an ionic imbalance caused by excessive high protein concentrations at the edges was the underlying reason, a comb was designed which formed a gel surface, as depicted in Fig. 2c pp.16, that allowed the sample to be applied to the centre well and application buffer to the two side wells. Now the protein fractions separated moved in straight bands and the edge effect disappeared. This procedure for producing straight protein zones was successfully used throughout the project. A simpler comb design achieves the same result. This comb provides equal distances of the sample well from the edges as in the previous one. However, no extra buffer wells are included. The gel width for sample application within the comb is 7,5 cm. Fig. 2d pp. 16 shows that with this design, straight bands are also obtained. It thus appears that polymerization for some reason does not proceed to the same degree and uniformity at the edges of the gel as at the centre. One explanation may be that the temperature of polymerization is higher in the centre of the gel, thereby proceeding faster than at the sides. Possibly the riboflavin concentration could be lowered to delay the speed of polymerization.

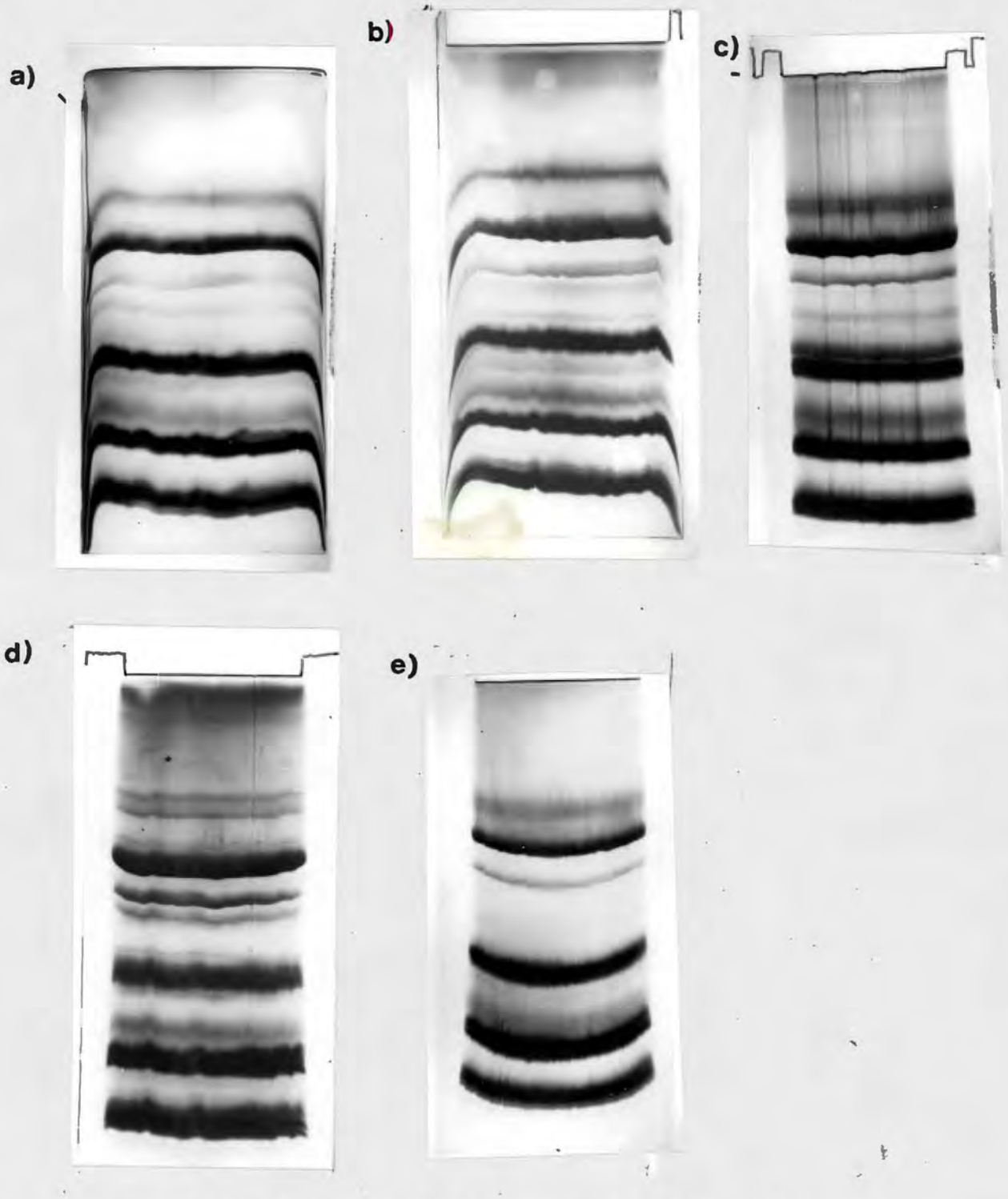


Figure **2** *a-d) Effect of sample slot design on straightness of separated protein bands.*

e) Effect of overheating during electrophoresis on straightness of separated protein bands.

The optimal gel length and thickness were established in order to achieve maximum separation in a convenient time without distortion of separated bands due to inadequate heat dissipation, and to provide maximum surface area in the sample well in order to optimize the loading capacity. Fig. 3 pp. 18 compares (a) 10 and (b) 20 cm long gel. The separation of the protein zones improves with the length of migration. The 20 cm long gel was electrophoresed overnight for 16-17 hours which is convenient. The current applied was 10 mA which was low enough so that heat generated could be adequately dissipated by a fan placed in front of the gel, resulting in even migration zones. Increasing the electrophoresis time beyond 17 hours did not improve separation as the zones were becoming diffused. This may be attributed to change in gel conditions at the bottom of the gel due to diffusion of urea and other constituents from the gel into the lower tray. Increasing the loading capacity by increasing the gel thickness from 0,15 to 0,3 cm and keeping the gel length at 20 cm did not achievethe desired separation in a set time at low current. Separation at low current is desirable because a fan could be used as a cooling device. Electrophoresis in the cold room does not overcome the problem as it is the temperature difference within the gel, created by localized protein concentrations differences that causes uneven boundary migration. These localized "hot spots" cannot be cooled sufficiently by simple convection. In Fig. 2e pp.16, a current of 40 mA was used resulting in a curvature in the protein zone as a result of local overheating, the effect being more pronounced at a higher protein concentration. The amount of protein that can be applied and successfully separated on a gel is affected by the adsorption of the protein to the gel. At 0,1 mg/cm^2 the resolution is high but the recovery is only 30%, whereas when all the adsorption sites are saturated and 3 mg/cm^2

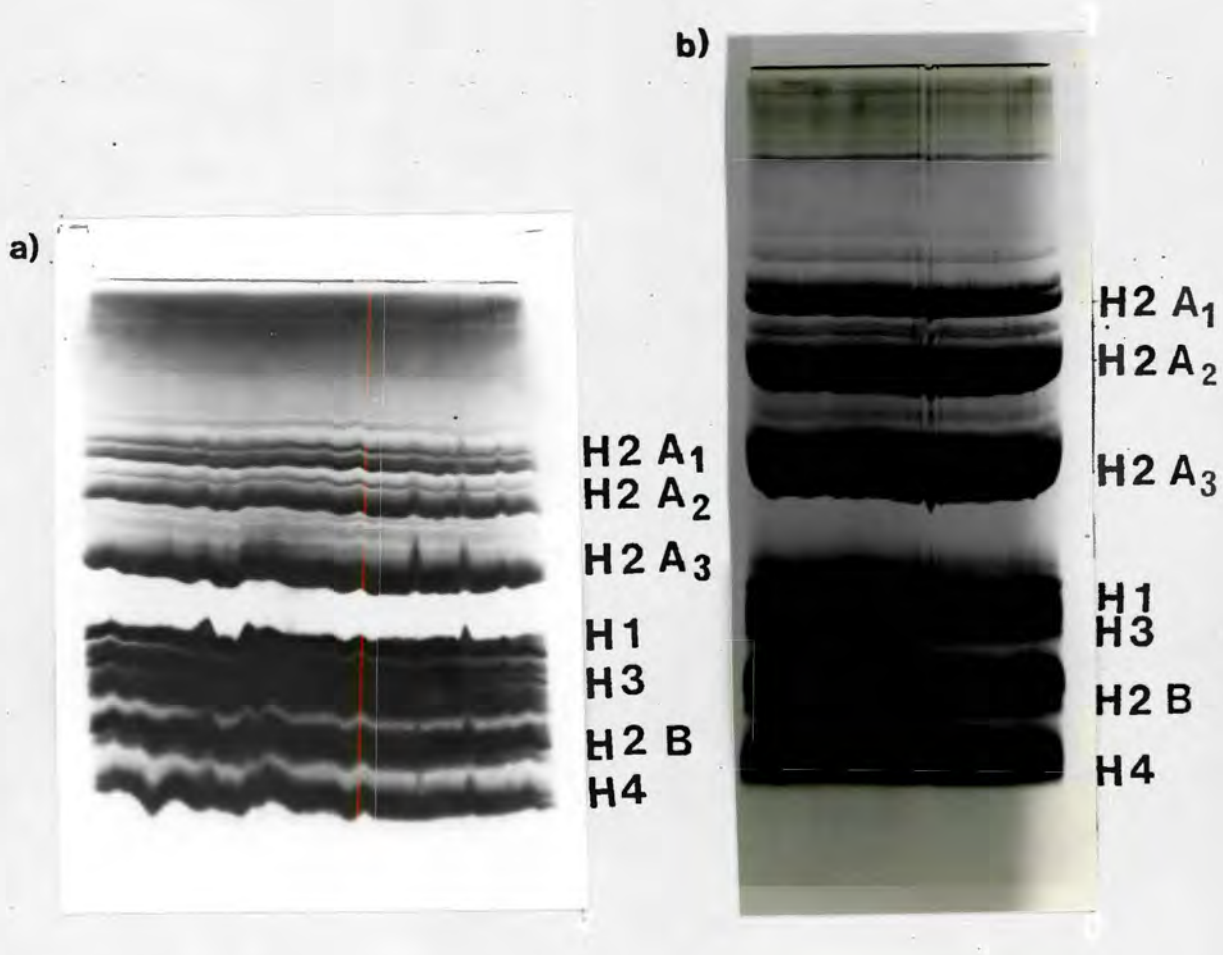


Figure **3** *Effect of increase of gel length on separation*

8,7 M urea 6 mM Triton-PAGE of Sea urchin embryo histone mixture after 5% perchloric acid extraction, which removes most of the H1.

a) 10 cm long slab gel

b) 20 cm long slab gel

are applied, the recovery is 90% (Kapadia and Chrambach, 1972). Jovin et al., (1964) recommended that 1-2 mg of protein of interest can be applied per cm^2 gel surface if the fractions are separated by one band-width from its nearest contaminant. In other words, the better the separation between the contaminants and the protein of interest the higher the loading capacity. As a result of the various observations the following conditions were chosen:

Slab dimensions: 0,17 x 7,5 x 20 cm, the available surface area for sample application is 1,27 cm^2.

Polymerization of the gel: with riboflavin as a catalyst, this makes pre-electrophoresis unnecessary, thiodiglycol (Zweidler, 1978) is included in polymerization mixture, as a sink for oxidizing species thus protecting methionine.

Electric field: 10 mA constant current for 16-17 hours.

Sample size: 10 mg of protein mixture containing up to 5 proteins of interest. 4-6 preparative slab gels can be electrophoresed conveniently overnight.

Staining solutions: If freshly prepared Amido Black solution is used, staining of gel for 5 minutes is sufficient. Destaining should be for at least 6 hours with several changes of destainer to allow for diffusion of urea and detergent from the gel.

CHAPTER 3

3. Recovery of Proteins from Polyacrylamide Slab Gels

3.1 Introduction

There are two types of methods used for the recovery of proteins from gels.

I) Batchwise elution of protein fractions following electrophoretic separation.

II) Continuous elution from a separating gel.

I) The batchwise elution can be achieved in various ways:

a) electrophoretically (Mardian and Isenberg, 1978, Braatz and McIntire, 1977, Hanaoka et al., 1979),

b) after solubilization of the gel matrix (Tas et al., 1979),

c) with suitable solvents (Houston, 1971, Gibson and Gracy, 1979, Wada and Snell, 1971, Djondjurov and Holtzer, 1979).

The advantages of batchwise elution are:

a) The optimal concentration established for analytical PAGE can be adopted for preparative purposes.

b) The analytical laboratory equipment is easily adaptable.

c) The separated protein zones can be easily located by staining. Some of the methods used allow staining of the entire gel(Djondjurov and Holtzer, 1979), whereas in others, stained marker strips have to be used (Martinage et al., 1975).

d) The recovery of the proteins from the elution buffer is simple.

The disadvantages are:

a) Inefficient heat dissipation excludes the use of thick slabs resulting in the need to process a larger number of thin slabs to recover proteins. But this does not present a major obstacle as the system is easily set up so that six preparative slabs can be run conveniently in parallel.

b) The number of steps involved are numerous. Five days are needed to isolate a freeze dried purified protein.

c) The removal of gel contaminants like acrylic acid or remnants of the extraction solvents e.g. SDS can cause problems.

II) Continuous elution from a separating gel (Stephens, 1975, Koziarz and Köhler, 1978) followed by analysis and concentration of the eluate. In the various modifications of these methods, many parameters are difficult to control. As a result there is no suitable preparative apparatus permitting proper control of heat dissipation, buffer flow and uniformity of the electrical field. In addition, suitable gel matrices have to be developed to allow the elution of the slowest moving component to be eluted in a reasonable time.

At the present stage of the development of the methodologies, with both types of methods, protein is only recovered in the µg region. It was therefore decided to investigate the less complex methods grouped under I.

3.2 Batchwise Elution of Protein Fractions

3.2.1 Electrophoretic Elution

The method developed for electrophoretic elution was based on a design described by Mardian and Isenberg (1978). The method involves locating protein zones in unstained gels with the aid of stained marker strips. The thus identified zones are then accurately excised. The gel regions containing the proteins are chopped up into convenient sizes and transferred to the electrophoretic chamber where the proteins are eluted electrophoretically. Subsequently the sample volume can be concentrated through freeze drying.

The following procedure was adopted to test the effectiveness of the gel elution chamber (Fig. 4 pp.23). 40 mg whole Calf Thymus histone was applied to a small acid-urea slab gel and electrophoresis was carried out for 2-3 hours to effect electrophoretic transfer of the protein into the gel, no separation was attempted. The gel was cut into little pieces and placed into the gel elutor. The elution solution (5% acetic acid, 2% thiodiclycol) was added, and the elution chamber assembled (Fig. 4 pp.23). The elution was carried out at 10 mA, 200 V for 48 hours. At hourly intervals the tap between the elution chamber and the collecting chamber was closed and the current switched off, a 2 ml sample was withdrawn to record the OD_{260} and the sample was returned..Fig. 5 pp.24 shows the increase in absorbance due to histone elution. After 48 hours the content of the collection chamber was desalted on a Sephadex G-25 column in 0,01 M HCl. The sample was subsequently freeze dried.

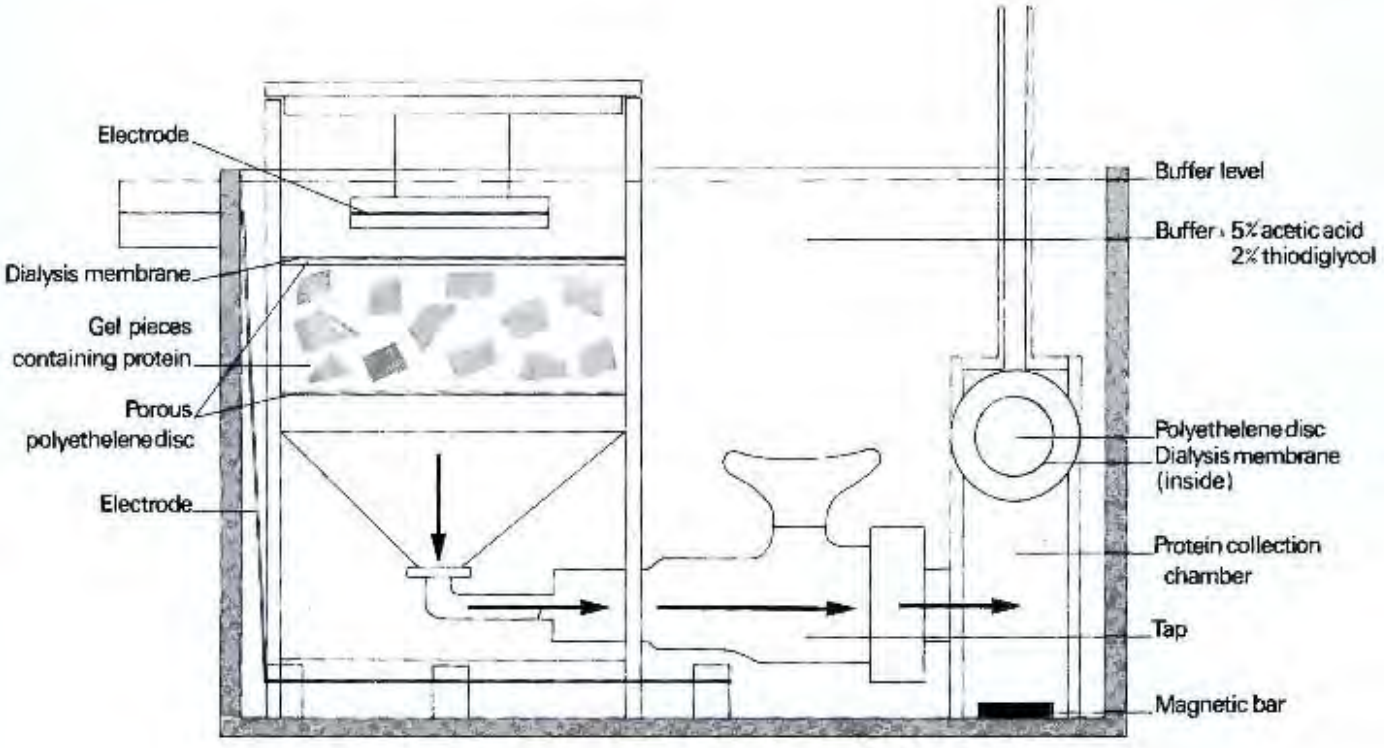


Figure 4 Electrophoretic Gel Elutor

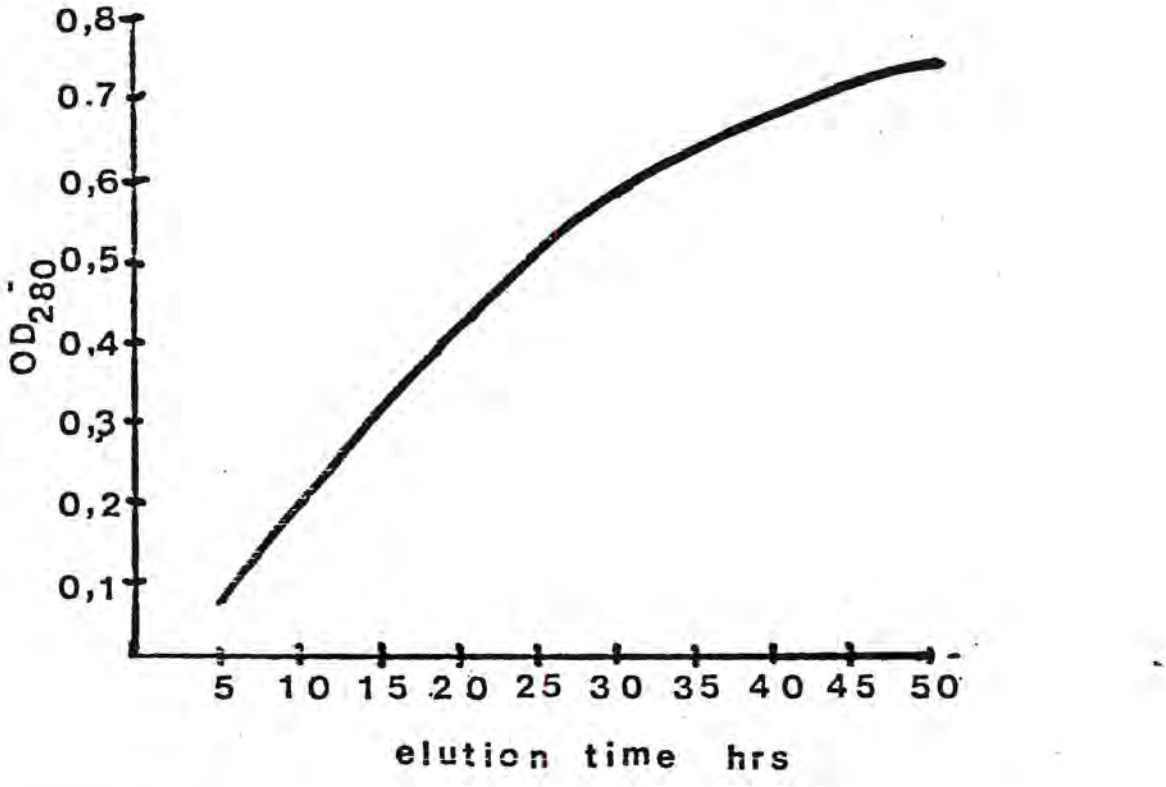


Figure 5 *Absorbance monitoring of histone elution*

The solution in the collecting chamber was partially removed and this absorbance was measured at the times shown.

Elution time (hrs)	OD_{280}	Temperature °C = 21,5 at 0-time
1	-	23
3	-	25
5	0,078	25
6	0,102	25
7	0,134	25
22	0,433	25
25	0,507	25
28	0,580	25
30	0,580	24
48	0,755	25

After 48 hours the protein transfer from the gel approaches completion. The recovery in several experiments was between 60-70% (by weight). In similar experiments using a Triton polyacrylamide slab gel, the recovery was also 70%. From these results it appears that electrophoretic gel elution is a suitable method. However, the preparative isolation of histones from gels requires the staining of the gel, to allow for precise location of the separated protein fractions. The recovery, when using partially destained gel slabs, dropped to 10%. Djondjuov and Holtzer (1979) have described a technique for the removal of Coomassie from protein samples recovered from gels, based on the dissociation of the protein-dye complex in trichloroacetic acid (TCA). The method was applied to histones. 10 mg whole Calf Thymus histone was applied to a small Triton slab gel and electrophoresis was carried out for 2-3 hours to effect electrophoretic transfer of the protein into the gel, no separation was attempted. The gel was stained for 15 minutes in 0,25% Coomassie in 50% methanol, 10% acetic acid, followed by destaining in 25% ethanol, 10% acetic acid until the bands were clearly visible. The gel was homogenized with an Ultra-Turax in 10% trichloroacetic acid (TCA) in 30% ethanol, to affect the dye protein dissociation. The homogenate was filtered through Whatman filter paper No.1. Electrophoresis in the TCA solution, because of the strong electrolyte properties of the latter is not possible. Therefore the gel homogenate was equilibrated in 70% acetic acid and 2% thiodiglycol prior to electrophoretic elution in the gel elution chamber for 48 hours. The collection chamber content was desalted and freeze dried. The recovery was 20%.

Djondjuov and Holtzer (1979) used a Coomassie stained SDS gel. The poor recovery in our experiment from Triton gels may be due to stronger

hydrophobic binding of Coomassie to proteins in a Triton system than in a SDS system. The observed poor destaining of Triton gels in TCA-ethanol indicates such a stronger dye protein interaction.

The electrophoretic elution of proteins as described was successful when proteins were eluted from unstained gels. But the use of stained guide strips was found to be inadequate as it does not allow faithful alignment of stained and unstained gel zones containing the protein and their subsequent precise excision. The method was therefore abandoned.

3.2.2 Chemical Extraction

3.2.2.1 Solubilization of Cross-Linked Gels

Solubilization of cross-linked gel is possible with 30% H_2O_2 (Young and Fulhorst, 1965) but these conditions are hardly suitable in protein chemical work. Tas et al. (1979) have reported N,N-diallyltartardiamide (DATD) as a cross-linker and found that such gels could be dissolved in 10 mM HIO_4. However, similar reservations apply. Even if the cross-linker can be cleaved, no satisfactory method has been devised to remove linear polymer of polyacrylamide from the protein. The complete removal of the former is however of greatest importance when samples are subjected to amino acid analysis. Polyacrylamide gel and products derived from it during the solubilization contribute to the amino acid composition of the hydrolysate (Brown and Howard, 1980).

3.2.2.2 Extraction of Histones with 0,1% SDS or 70% Acetic Acid

The extraction of proteins from gels with 0,1% SDS has been described by Sommer (1978). Pastink has proposed 70% acetic acid as a suitable solvent (1979). The separation of the dye-detergent-protein complexes by ion pair extraction has been described by Henderson (1979). The suitability of the combination of these methods has been investigated.

10 mg whole Calf Thymus histones were applied to a small Triton slab gel and electrophoresis was carried out at 16 mA constant current for about 2 hours to effect electrophoretic transfer of the protein into the gel, no separation was attempted. The gel was stained in 0,25% Coomassie in 50% methanol, 10% acetic acid for 15 minutes, followed by destaining in 25% ethanol, 10% acetic acid until bands were clearly visible. The gel was then cut in half; the one half was homogenized with an Ultra-Turax in 0,1% SDS, whereas the other half was homogenized in 70% acetic acid. Both samples were processed individually through the following steps. The homogenate was shaken overnight at 4°C and then filtered through Whatman filter paper No. 1. The filtrate was dialyzed and freeze dried. The freeze dried sample was dissolved in 1-2 ml distilled water and precipitated with 10 volumes of acetone:triethylamine:acetic acid (85:5:5). The precipitate was collected by centrifugation in a bench top centrifuge. The precipitate was washed twice with acetone, and then redissolved in water and freeze dried. The respective recoveries by weight using 0,1% SDS and 70% acetic acid were 135% and 141%. This indicates heavy contamination of the sample with either SDS or gel components. This becomes evident on comparing the analytical gels of the recovered proteins (Fig. 6 pp.30). About 3-4 times the amount of the recovered histone fractions had to be applied to result in comparable staining

intensity.

3.2.2.3 Extraction with 70% Acetic Acid or 70% Formic Acid

Acetic acid (Pastink, 1979) as well as formic acid (Wada and Snell, 1972) are good protein solvents and have both been used to extract proteins from gels. The separation of the complex formed between Amido Black and protein is cleaved by both acids and the components of the complex can be separated on an ion exchanger (Wada and Snell, 1972). The suitability of this process has been investigated.

10 mg Calf Thymus histones were applied to a small Triton polyacrylamide slab gel and electrophoresis was carried out for 2-3 hours to effect electrophoretic transfer of the proteins into the gel, no separation was attempted. The gel was stained for 5 minutes in 0,1% Amido Black and destained in 7% acetic acid until bands were clearly visible. The gel was then cut in half, the one half was homogenized with an Ultra-Turax in 70% acetic acid, whereas the other half was homogenized in 70% formic acid. Both samples were then processed individually through the following steps. The homogenate was shaken overnight at 4°C and then filtered through Whatman filter paper No.1. The filtrate containing the Amido Black-histone complex was passed over a Dowex 1 x 8 column equilibrated with 40% formic acid. The Amido Black is bound by the resin whereas the protein is eluted from the column. The eluant was dialyzed and freeze dried. The freeze dried sample was dissolved in 1-2 ml 0,2 M H_2SO_4 and precipitated with 6 volumes acetone, the precipitate was collected by centrifugation and washed twice with acetone. The precipitate was then dissolved in water and freeze dried. In several experiments the recovery using 70% acetic acid was between 55-60% and formic

acid, between 70-80%. Both procedures yield fluffy white powders. Fig.7 pp. 30 shows recovered samples on an analytical SDS gel. The higher recovery from the formic acid extract becomes evident on comparing the different lanes to the Calf Thymus control.

It was investigated whether the ion exchange separation of the dye histone complex is only applicable to Amido Black stained protein or whether it also applies to the Coomassie-histone complex. When Coomassie stained Triton gels were processed according to the protocol on page 28 the Coomassie-histone complex was not dissociated but eluted from the column. This suggests different binding forces between the two dyes. Fig. 8 pp.31 gives the structure of Amido Black and Coomassie. Amido Black appears to bind to proteins mainly via ionic bonds between the sulphonic acid groups and the amino groups of the protein (Vickerstaff, 1950), which are easily dissociated in the acid and exchanged on the anion exchange column. Coomassie on the other hand, with its more extended structure, appears to offer more hydrophobic binding sites in addition to the ionic sites, making dissociation under the above conditions impossible.

From the results obtained, it was decided to use 70% formic acid for extraction of histones from Amido Black stained Triton gels and subsequently separate the Amido Black-histone complex on an anion exchange column (for detailed description of the procedure see 8.1.1.7).

Figure 6 Gel electrophoresis of recovered histones from SDS and acetic acid extracts of Coomassie stained Triton gels

1 and 7	acid extracted Calf Thymus C.T. histones						10 µg	(control)
2	C.T. histones	recovered	with	0,1%	SDS		5 µg	
3	"	"	"	"	"	"	10 µg	
4	"	"	"	"	"	"	20 µg	
5	"	"	"	"	"	"	40 µg	
6	"	"	"	"	"	"	100 µg	
8	C.T. histones	recovered	with	70%	acetic	acid	5 µg	
9	"	"	"	"	"	"	"	10 µg
10	"	"	"	"	"	"	"	20 µg
11	"	"	"	"	"	"	"	40 µg
12	"	"	"	"	"	"	"	100 µg

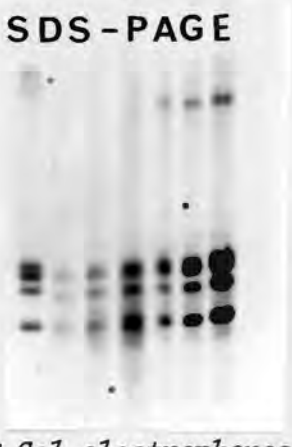
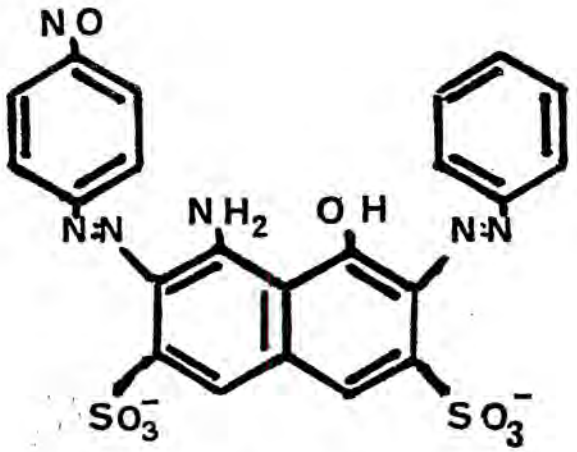


Figure 7 Gel electrophoresis of recovered histones from acetic acid and formic acid extracts of Amido Black stained Triton gels

1	acid extracted Calf Thymus histones						10 µg	
2	C.T. histones	recovered	with	70%	acetic	acid	5 µg	
3	"	"	"	"	"	"	10 µg	
4	"	"	"	"	"	"	20 µg	
5	"	"	"	70%	formic	acid	5 µg	
6	"	"	"	"	"	"	10 µg	
7	"	"	"	"	"	"	20 µg	



Amido Black

Coomassie brilliant blue

Figure 8 *Structure of Amido Black* and Coomassie brilliant blue***

* Houston 1971

** Fazekas de St. Groth et al. 1963

CHAPTER 4

THE EFFECT OF TRITON X-100 ON POLYACRYLAMIDE GEL ELECTROPHORESIS

4.1 Introduction

The electrophoretic resolution of proteins has been significantly improved over that achieved with the acid urea system of Panyim and Chalkley (1969) by the incorporation of the nonionic detergent Triton X-100 in the separation gel. The presence of Triton X-100 has made it possible to resolve analytically, the histone variants differing only by substitution of a single neutral amino acid (Franklin and Zweidler, 1977, Zweidler, 1978). Fig. 9 pp.33 compares the 2 gel systems, and whereas a) the acid urea system resolves 6 bands, b) the Triton gel is able to distinguish between 11 bands in the same protein sample.

Maximum Triton binding is dependent on the proton and urea concentration. It has been suggested that the pH dependence is the result of minimal unfolding of the protein at pH 7 (Zweidler, 1976). The effect of urea will be considered under 4.2. pp. 35. It has been well established that the oxidation state of methionine, if that amino acid is situated in a critical area, can have a profound influence on the Triton binding with resulting alterations of electrophoretic mobility. To prevent the oxidation, thiodiglycol is added to the gel polymerization mixture, as it is an efficient oxygen scavenger (Zweidler, 1978). An excess of thiodiglycol can, however, inhibit the polymerization. To demonstrate the effect of methionine oxidation on Triton binding, a mixture of Sea Urchin embryo histones was oxidized with performic acid (Hirs, 1967) and analysed on a urea gradient gel at constant Triton concentration (Fig. 10 pp. 34). The oxidation of methionine to its sulfoxide results in reduced Triton

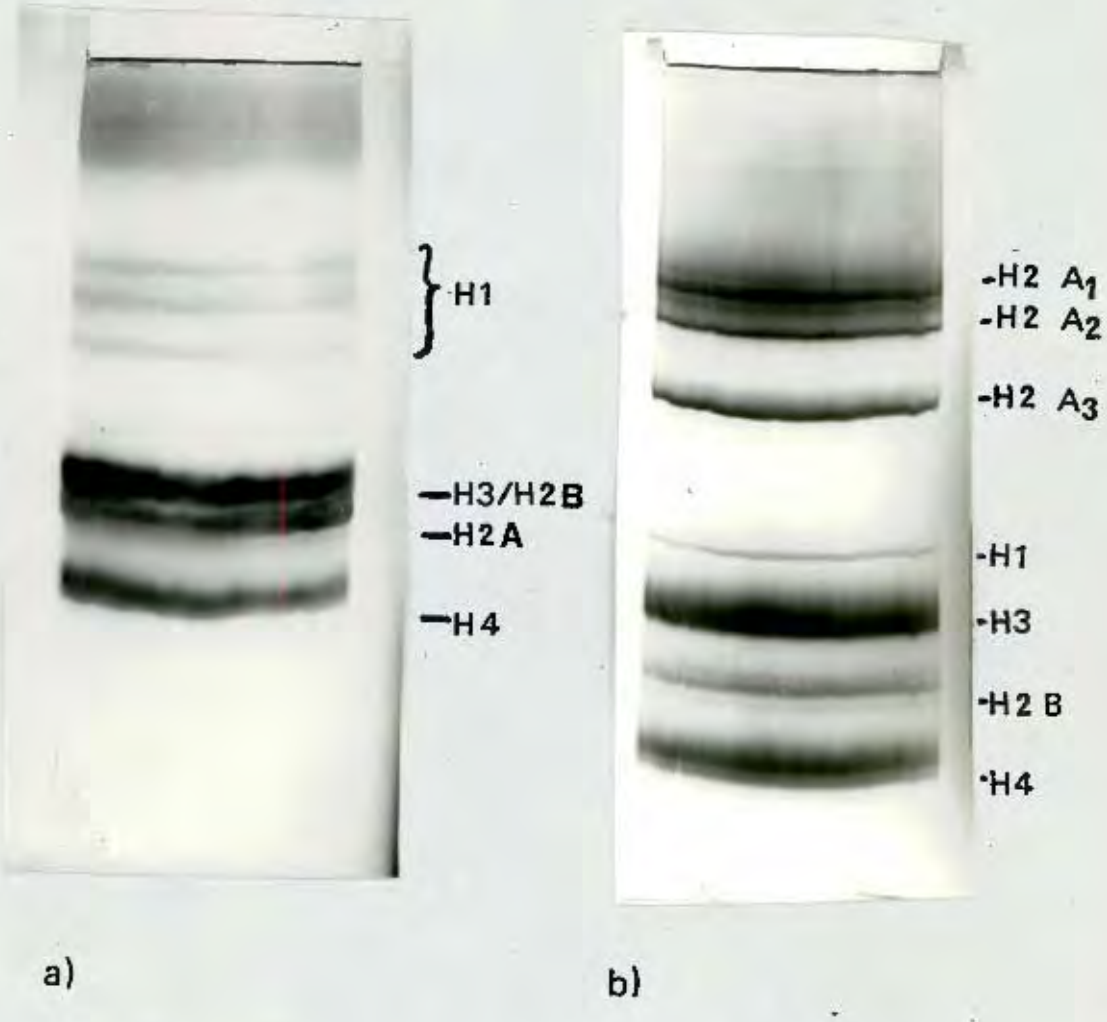


Figure **9** *Preparative polyacrylamide gel electrophoresis (PAGE)*

a) Acid-urea (8,7 M) PAGE and

b) 8,7 M urea 6 mM Triton X-100 PAGE of Sea urchin embryo enriched histone H2A fraction (Johns 1964).

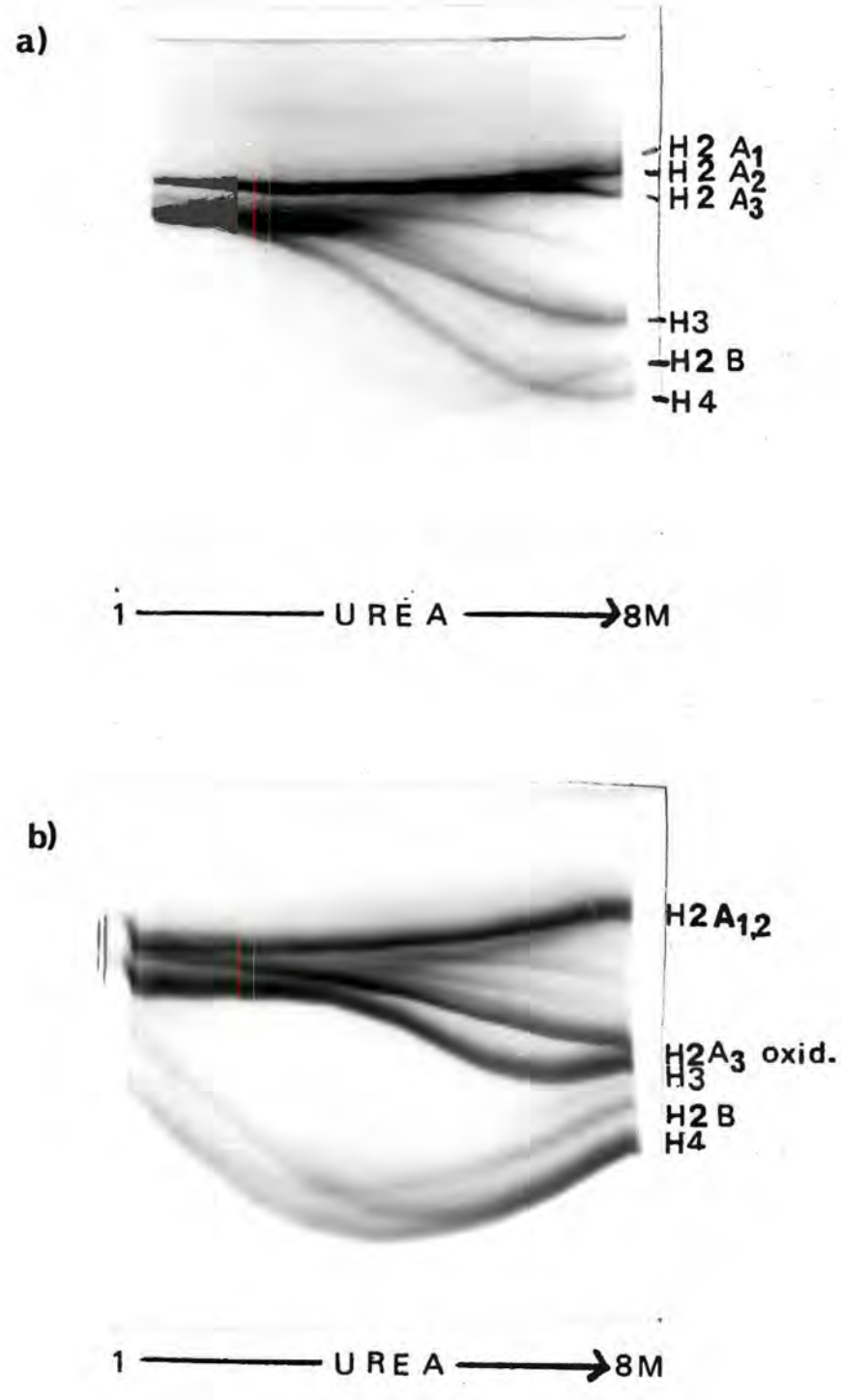


Figure **10** *Effect of performic acid oxidation on Triton binding*

A Johns enriched H2A Sea Urchin histone mixture was oxidized with performic acid and electrophoresed on a 0-9 M urea gradient slab gel at constant 6 mM Triton X-100 concentration.

a) unoxidized histone mixture

b) oxidized histone mixture

binding which is observed on the gel as an increase in the electrophoretic mobility. The histone most markedly affected is $H2A_{(3)}$, which now exhibits a mobility close to that of histone H3. Of the other histones only H2B and H4 are affected, thus indicating that the remainder of the other histones do not possess a methionine residue or that the methionine is situated in a non critical area of the molecule.

4.2 Mode of Triton Binding

Triton X-100 is thought to bind specifically to helical regions in the protein molecule, thereby altering the size:charge ratio, thereby affecting in a different fashion, the electrophoretic mobility depending on the extent of helicity in the protein (Zweidler, 1976, Hamann and Iwai, 1976). Hamann and Iwai calculated the helical content according to Lewis and Bradbury (1974) and the total hydrophobicity for Calf Thymus, and showed that Triton binding can be better correlated with the helical content rather than with the total hydrophobicity. The helical regions are probably partially identical with the hydrophobic centre of the molecule. If only the helicity determines the Triton binding, then the strong Triton effect even at 8 M urea on histone H2A, which has a larger hydrophobic centre, would indicate a high stability of those helical regions even at high urea concentration. Similarly the cysteine (Calf Thymus) serine (Chicken Erythrocyte) substitution in histone H3 (Brandt et al., 1974) has a large effect on Triton affinity resulting in an increase in the electrophoretic mobility of C.T. H3 (Fig. 11 pp. 36). This is consistant with the generally observed correlation between detergent affinity and helix promoting ability (Hamann and Iwai, 1976). Cysteine and serine are similar in configuration, but belong to different categories of helix promoting ability (Scheraga, 1970).

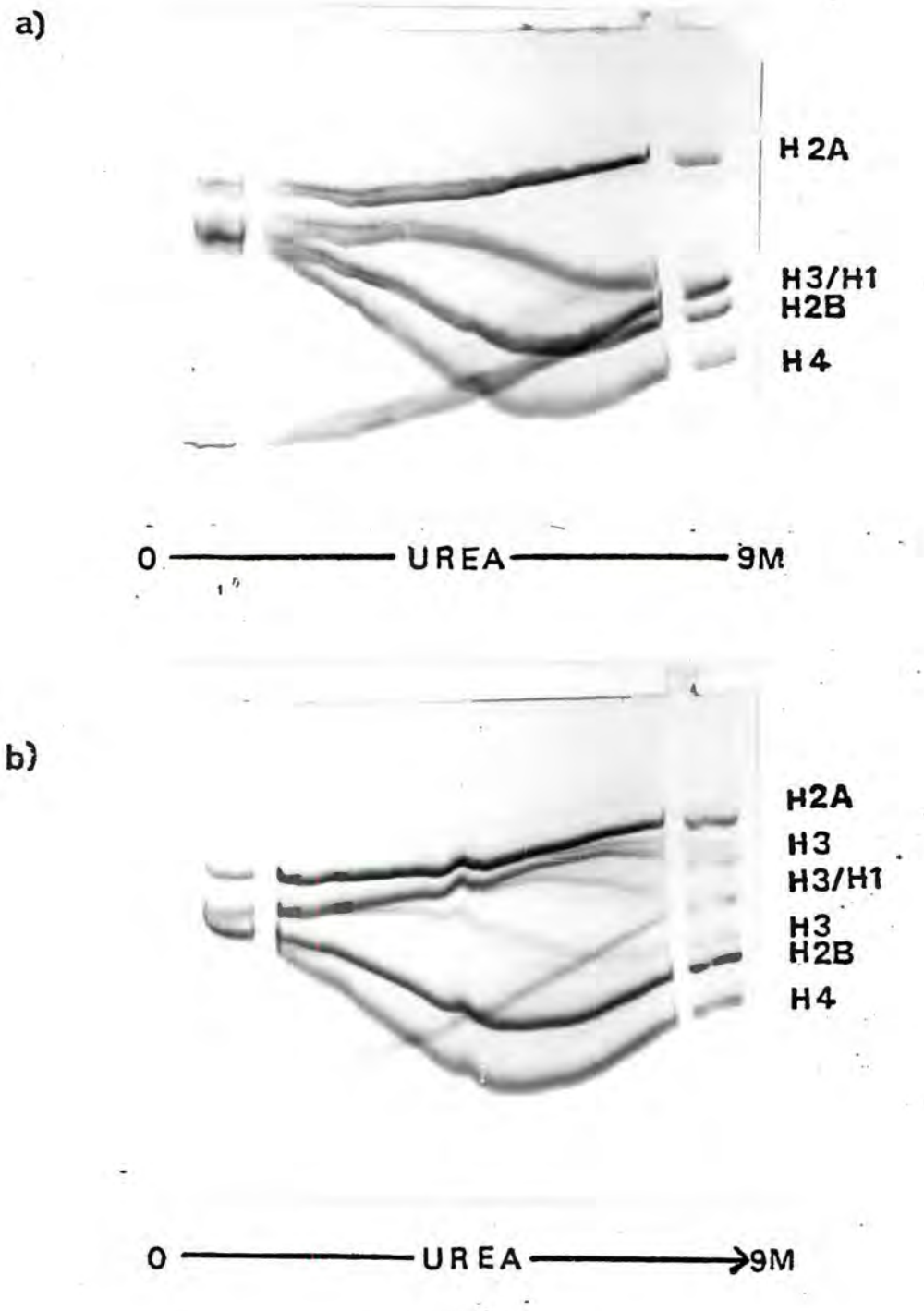


Figure 11 *Dependence of Triton binding on amino acid (A.A.) sequence of histone H3.*

0-9 M urea gradient 6 mM Trition slab gel electrophoresis of
a) Chicken erythrocyte histones
b) Calf Thymus histones

Note the decrease in electrophoretic mobility of Calf Thymus H3 co mpared to Chicken erythrocyte H3 due to the Cys⟷Ser substitution at position 96.

So far, only the destabilizing effect of urea on the helical regions in the protein molecule has been considered which results in a decrease in the Triton affinity. There is however another factor, namely, the effect of urea on Triton micelle formation. Triton is thought to bind to high affinity sites (helical regions) in the protein molecule preferably in the micellar form of the detergent (Makino, S. et al., 1973, Hoffman and Dowben, 1978). The low critical micellar concentration (CMC) of 0,24 mM (Helenius and Simons, 1972), makes it difficult to maintain a sufficiently high monomeric concentration to effect cooperative binding. Urea was found to have a marked effect on the CMC of Triton even at low concentration of the former (Gratzer and Beaven, 1969).

At high urea concentration much more Triton is needed to bind to the protein molecule, and only those proteins with a stable helical region in high urea will still be able to bind Triton. This effect is clearly demonstrated in the gradient slabs in Fig. 12 a-c pp. 38. In the three Triton gradients at different urea concentrations, the increasing CMC becomes obvious in the abrupt decrease in mobility. The CMC of Triton changes from 2,3 mM at 4 M urea, to 3,2 mM at 6 M urea and 6 mM at 8,7 M urea. Histone H2A stands out predominantly in all 3 gels. Because of its stable helical region at 8,7 M urea it binds the most Triton, although a much higher Triton concentration is needed to achieve maximum separation. At 8,7 M urea, (Fig. 12c pp. 38) none of the other histones are stable enough to bind Triton even at high concentration of the detergent. At 4 M urea all the other histones bind Triton except histone H1 which can serve as a reference point. The H1 is the fastest moving fraction in the 4 M urea gel after the CMC of Triton has been

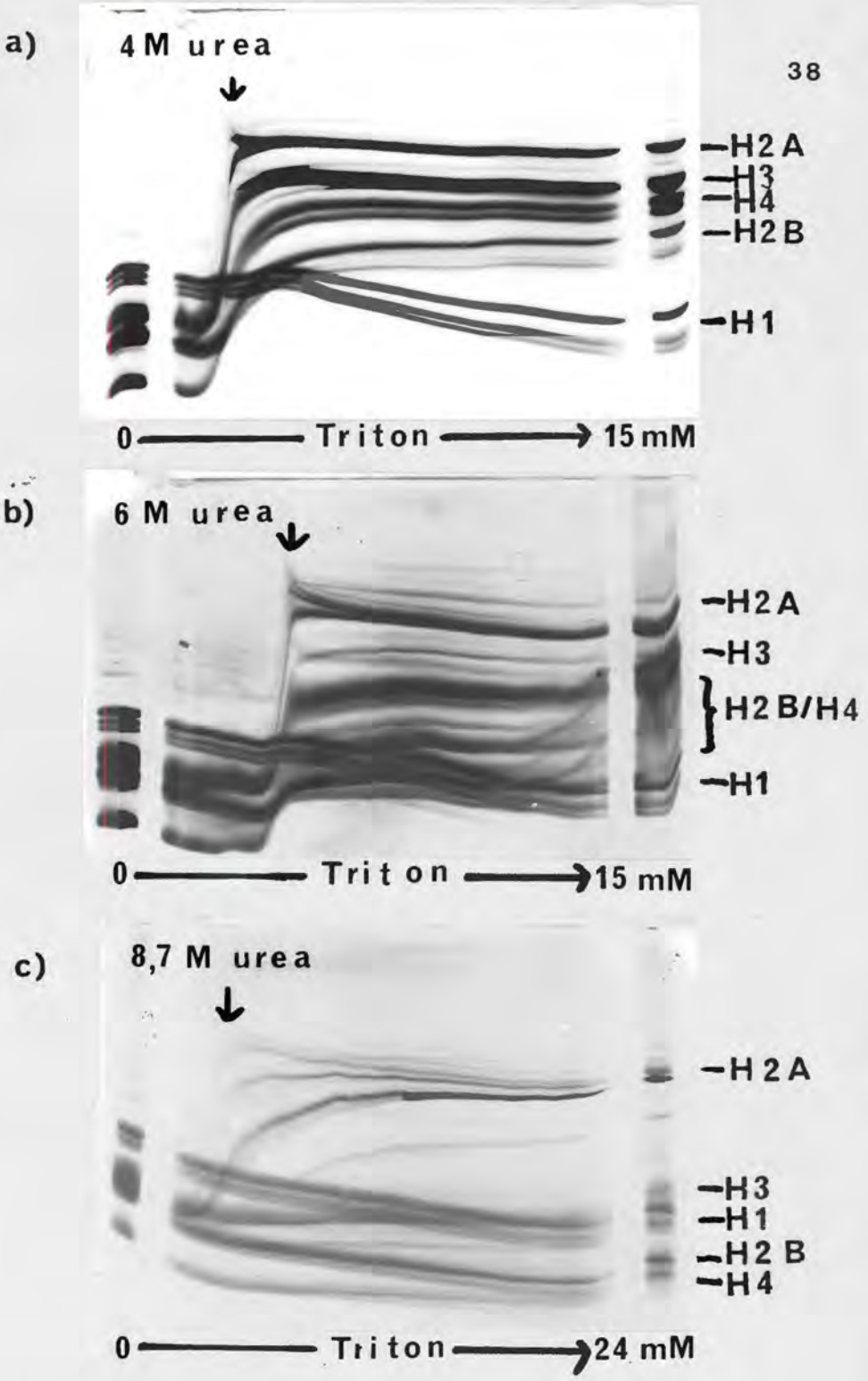


Figure **12** *Triton gradient gel electrophoresis of total Sea Urchin embryo histones at different urea concentrations.*

a) 0-15 mM Triton X-100 4 M urea
b) 0-15 mM Triton X-100 6 M urea
c) 0-24 mM Triton X-100 8,7 M urea

The change in mobility of histones H2A, H2B, H3 and H4 is due to the amount of Triton bound by them - H1 does not bind Triton and thus is not retarded in its mobility. It can serve as a reference. The change in CMC of Triton with increasing urea concentration (a-c) is evident in the sudden change of mobility, indicated by ↓ At 4 M urea the CMC equals 2,3 mM Triton, at 6 M urea 3,2 mM Triton and at 8,7 M urea 5,8 mM respectively.

exceeded. H3 and H4 have all markedly reduced mobility but are well resolved. This pattern changes drastically at 6 M urea (Fig. 12 b pp.38), when all histones except H1 can still bind Triton but due to urea interfering more and more with hydrogen bonding the amount is much less. Histones H4 and H2B are retarded to a lesser degree and are not clearly resolved from each other as well as from H1. At 8,7 M urea H2B and H4 do not bind Triton any longer and move with a faster mobility than H1, exhibiting a chracteristic acid urea gel pattern, except for H2A which can still bind Triton. The degree of interference of urea on Triton binding appears to depend on the primary structure. Therefore different urea concentration will effect Triton binding to individual histones to different degrees. Information gained from such gradient slabs will allow one to set up specific conditions for the optimal resolution of the different histone variant classes for analytical or preparative purposes.

4.3 Establishment of Triton X-100 and Urea Concentrations for Preparative Histone Variant Isolation on Gradient Polyacrylamide Gels

The optimum Triton and urea concentration for separation of Sea Urchin embryo histone variants was established on analytical gradient gels. A total Sea Urchin embryo histone mixture was electrophoresed on a 0-24 mM Triton 8,7 M urea slab gel Fig. 12c pp. 38. The Triton gradient at the high urea concentration was chosen as it resolved H2A into three variants whereas the other histones, though not markedly affected by Triton, were well resolved as homogenous classes. The separation achieved was at least one bandwidth (Fig. 9b pp. 33). This allowed the sample load per preparative gel to be increased by at least a factor of 5 when compared to separation in acid urea gel (Fig. 9 pp. 33).

Optimal resolution for the histone variants in the different classes H2A, H2B, H3 and H4 was established on a 0-9 M urea gradient gel at constant 6 mM Triton concentration (Fig. 13a pp.41). 6 mM Triton is high enough to maintain a critical CMC at all urea concentrations. Thus the difference in mobility observed for the various histones (Fig. 13a pp.41) is solely due to the effect of urea on the helical regions and not due to limiting Triton micellar concentration (Fig. 13b pp.41). In the latter the Triton concentration is 2,3 mM which as can be seen in Fig.12a pp.41, is high enough for all histones to bind Triton at low urea concentrations. But as soon as the urea concentration is increased, the CMC of Triton becomes limiting, and at about 7 M urea all Triton micelles are destroyed and even histone H2A, which is stable at high urea concentrations, is not retarded any longer and moves with a mobility observed on a standard Panyim and Chalkley acid-urea gel.
In the urea gradient gel, (Fig. 13a pp.41), it becomes obvious that urea affects Triton binding particularly for H2B and H4, less for H3 and H2A and hardly at all for H1. The decrease in mobility for H1 as well as for the other histones, especially in the high urea concentration, probably reflects the effect of the viscocity of urea on molecular sieve properties of the gel. This is supported by gel (Fig. 14 pp. 42) which has an inverse (10-15%) acrylamide gradient superimposed on the urea gradient so as to produce comparable intrinsic mobilities of most proteins in O M and 8 M urea (Creighton, 1978). Though the compensation of the two effects is not complete, the retardation of all histones in the high urea concentration is definitely reduced through the change in the gel concentration.

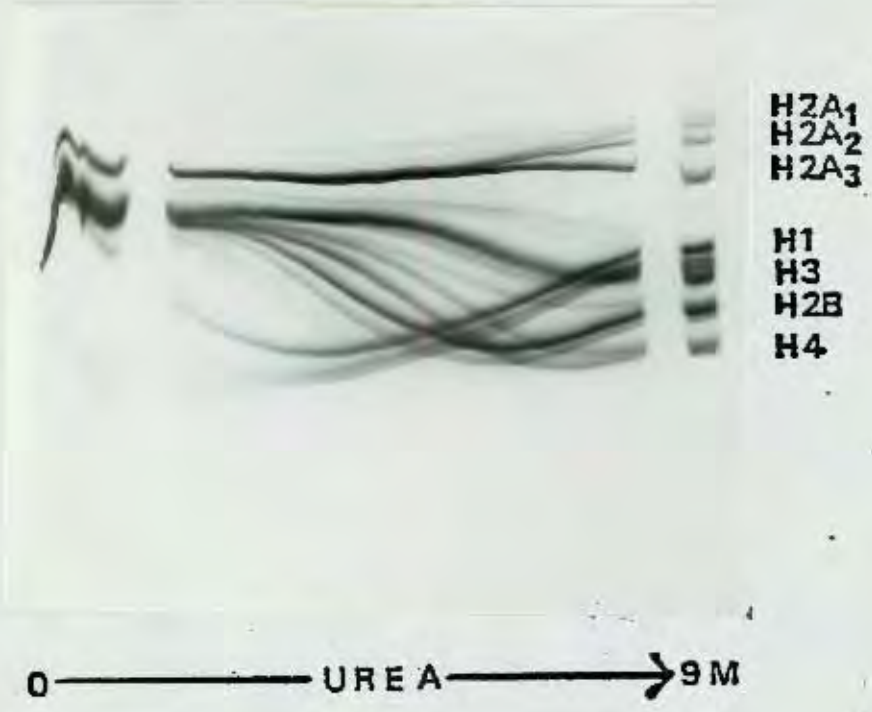
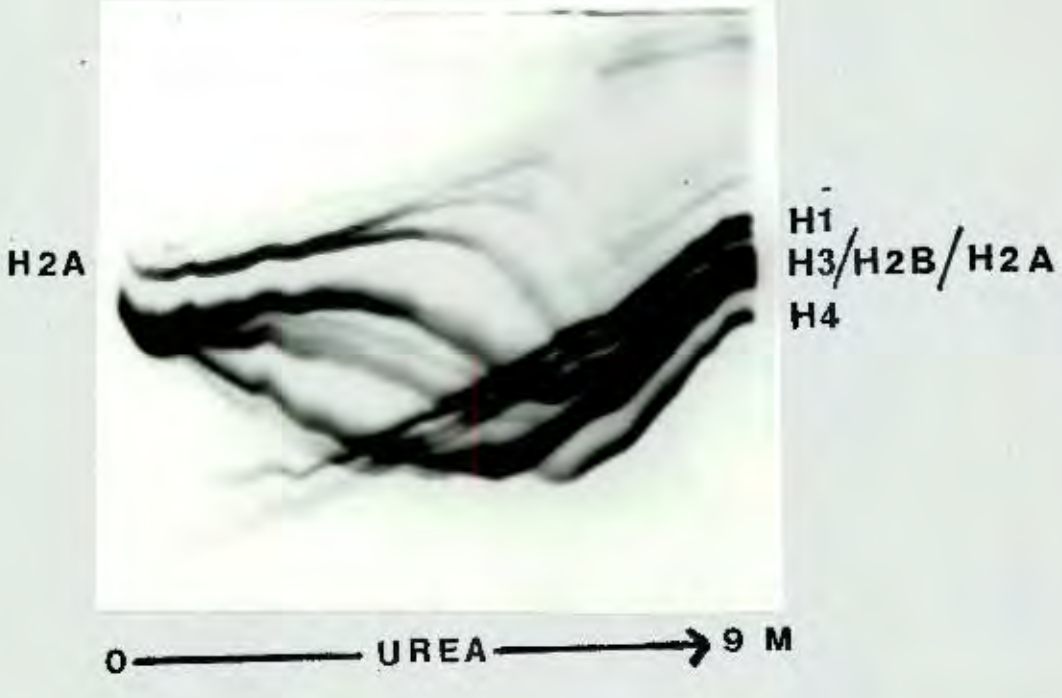


Figure 13 *Dependence of Triton X-100 binding on urea concentration*

a) Total Sea Urchin embryo histones were electrophoresed on a 0-9 M urea gradient slab gel at constant 6 mM Triton concentration.



b) Total Sea Urchin embryo histones were electrophoresed on a 0-9 M urea gradient slab gel at constant 2,3 mM Triton concentration.

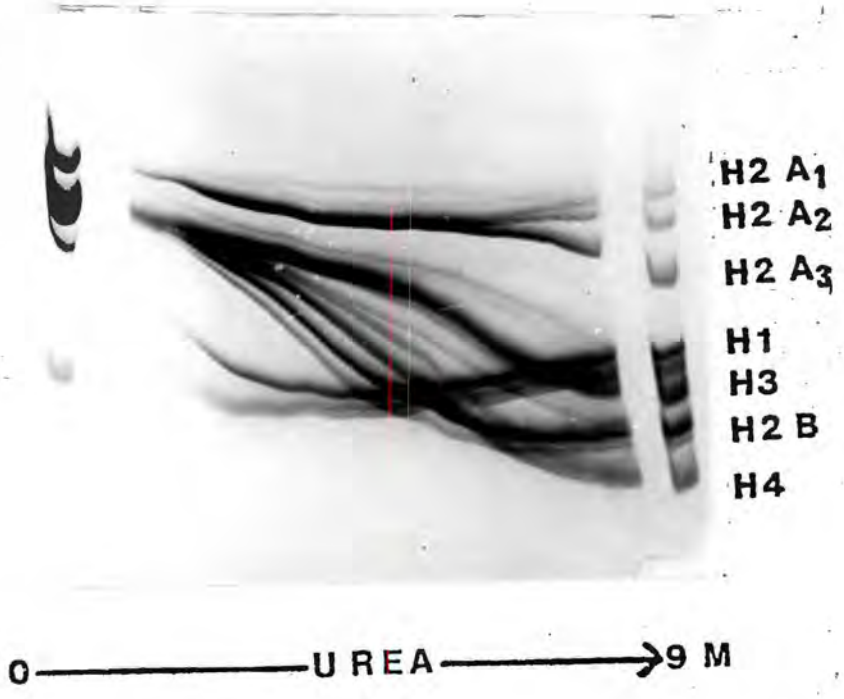


Figure 14 *Effect of Urea concentration on molecular sieve properties of polyacrylamide gel*

Total Sea Urchin embryo histones were electrophoresed on a 0-9 M urea gradient slab gel at constant 6 mM Triton concentration, with a superimposed inverse 10-15% acrylamide gradient.

The conditions for preparative isolation of Sea Urchin embryo histones in the different classes as derived from the urea and Triton gradient gels are as follows:

For H2A variants: 6 mM Triton, 8,7 M urea (9 M urea is too close to the solubility limit, leading to occasional precipitation of urea in the gel).

H2B variants : 6 mM Triton, 4 M urea;

H3 variants : 6 mM Triton, 6 M urea;

H4 variants : 6 mM Triton, 4,5 M urea.

CHAPTER 5

PREPARATIVE PILOT EXPERIMENTS

A number of pilot experiments to test the feasibility of the method was undertaken. In particular, the suitability of histone subfractions, isolated by John's method or molecular sieve chromatography, as starting material received consideration. A Sea Urchin histone mixture after 5% perchloric acid extraction, which removes most of the H1, has been fractionated on a preparative 8,7 M urea, 6 mM Triton X-100 slab gel Fig. 15a pp. 45. The H2A variants, designated 1-3 are easily excised as individual fractions, whereas the other histones are excised as groups before being further fractionated into their respective variants at a different urea concentration (see previous section). A total of 50 mg of the histone mixture has been applied to four slabs. The gels were stained and destained. The individual stained gel zones were accurately excised and corresponding fractions were combined for extraction with 70% formic acid. The recovery for individual histones were:

$H2A_{(1)}$	2,1 mg
$H2A_{(2)}$	2,2 mg
$H2A_{(3)}$	2,3 mg
H3	2,5 mg
H2B	2,8 mg
H4	2,2 mg
	14,1 mg

14,1 mg represents an overall recovery of 35%. Losses occur due to the following reasons:

The excision between separated zones has to be done in a conservative fashion to avoid overlaps. The original sample weight also contains other slow moving protein contaminants, as seen in the region above H2A.

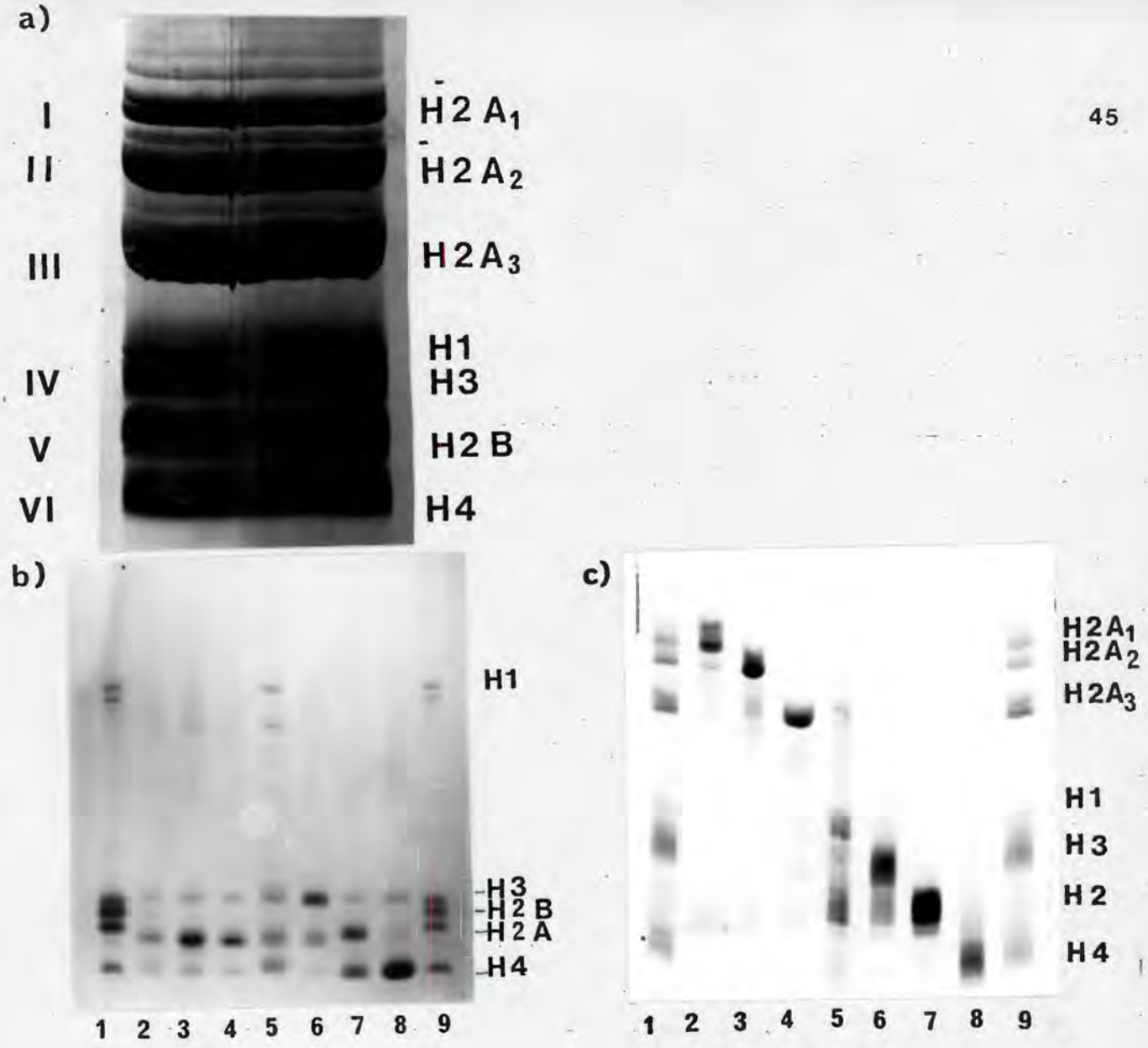


Figure **15** *Preparative Triton-PAGE of Sea Urchin embryo histones and identification of purified histone fractions by analytical PAGE.*

a) Separation of Sea Urchin embryo histone mixture after 5% perchloric acid extraction which removes most of the histone H1, by 8,7 M urea, 6 mM Triton X-100 PAGE

b) Analytical SDS-PAGE of purified histone fractions
lane 1 and 9: Calf Thymus histones
2: histone $H2A_{(1)}$
3: histone $H2A_{(2)}$
4: histone $H2A_{(3)}$
5: Sea Urchin embryo histones H2A enriched
6: histone H3
7: histone H2B
8: histone H4

c) Analytical 8,7 M urea 6mM Triton X-100 PAGE of purified histone fractions
lane 1 and 9: Sea Urchin embryo histones H2A enriched
2: histone H2A
3: histone $H2A_{(2)}$
4: histone $H2A_{(3)}$
5: sea urchin histone H2B enriched
6: histone H3
7: histone H2B
8: histone H4

The sample still contains some H1 contributing to the starting sample weight but the zone containing H1 has been discarded. Adsorption of protein to acrylamide is significant (see Fig. 16 pp.47). And finally, the extraction with formic acid with subsequent ion exchange separation of dye and protein with ensuing histone precipitation in itself has only a recovery of 70-75% (see Fig. 7 pp. 30).

The purified Sea Urchin histones H2A, H2B, H3 and H4 have been analyzed on analytical Triton (Fig. 15c pp.45) and SDS-gels (Fig. 15b pp.45), as well as on 0-9 M urea gradient gels at constant Triton concentration (Fig. 17 pp. 48). The analytical Triton gel (Fig. 15c pp.45) shows that all recovered histone fractions exhibit the original mobilities. But Figs.15 b&c pp. 45 also indicate that all fractions are still contaminated. The urea gradient gels clearly show contamination of the H2A fractions (1-3 Fig. 17 a-c pp.48) with all the other higher mobility histones, H3, H2B and H4. The contamination is thought to be the result of adsorption of histones to the acrylamide. Weber (1953) has suggested that the faster moving proteins, in matrix supported electrophoresis, migrate as zones through the matrix and are continuously depleted until they are spread along the migration path like an unrolled carpet. That such a carpet is in fact produced during electrophoretic migration of histones in acrylamide is demonstrated in the experiment in Fig. 16 pp. 47. Ten mg of Chicken erythrocyte histones were applied to a 10 cm long 8,7 M urea 6 mM Triton slab gel, electrophoresis was carried out overnight with the intention to run all the histones off the gel. The gel was stained and destained. Some of the histone H2A had not quite run off, but all the other histones were not present any longer. The entire gel slab was then extracted. The isolated protein sample was applied to an analytical SDS-gel (Fig.16pp.47). The gel clearly shows that all 5

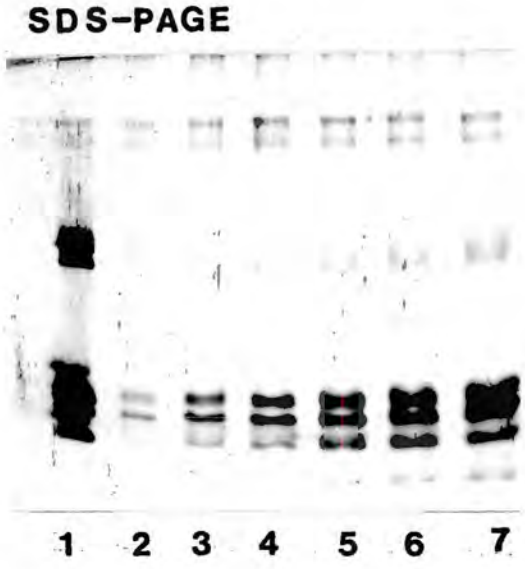


Figure 16 *Absorption of histones to the gel matrix*

SDS-PAGE of Chicken Erythrocyte (C.E.) histones
lane 1 : Whole C.E. histones control
" 2–7 : C.E. histones recovered due to absorption of histones to the gel matrix during electrophoresis

(See page 46)

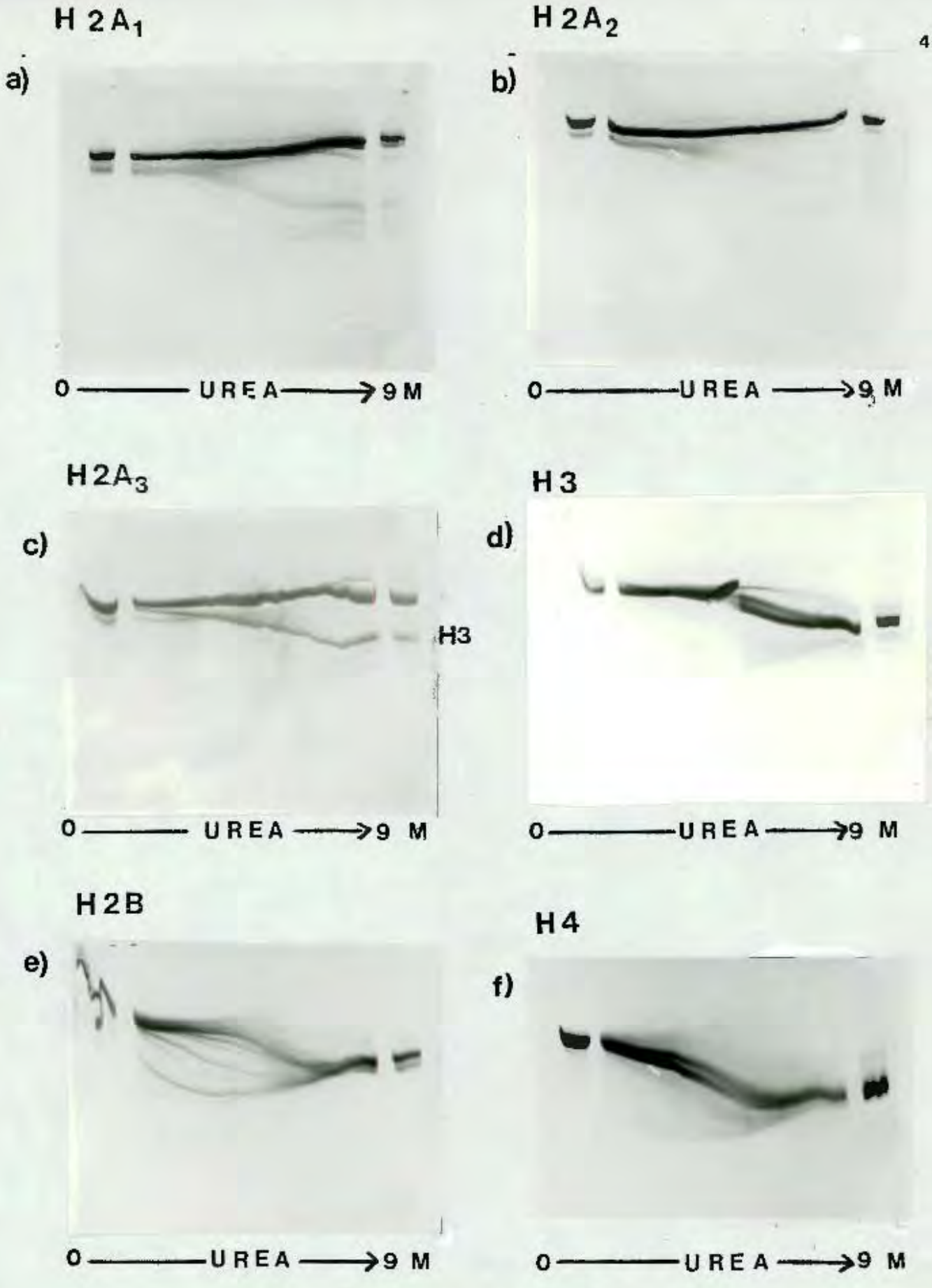


Figure 17 *Urea gradient Triton - PAGE*

Sea urchin embryo histone fractions I-VI recovered from preparative Triton gel Figure **15a** analysed on 0-9 M urea gradient 6 mM Triton X-100-PAGE

a) histone $H2A_{(1)}$

b) histone $H2A_{(2)}$

c) histone $H2A_{(3)}$

d) histone H3

e) histone H2B

f) histone H4

histones have been isolated from the gel which should only have had a small amount of histone H2A present.

The histones H3, H2B and H4 (Fig. 17 d-f pp.48) are contaminated significantly with each other, because of their close proximity in the gel. Diffusion of the zones is affected by variation in temperature, density and electrophoretic convection during electrophoresis (Morris and Morris, 1963). In addition, the high protein concentration in the zone effects the mobility. The net mobilities will decrease with increasing concentration of the ion constituents in the zone. Therefore the proteins on the edges of the concentration gradient, present in lower concentrations, will move faster than those present in the main zone itself (Morris and Morris, 1963). The boundary will therefore become diffused, and overlaps between closely separated zones such as histones H2B, H3 and H4 will result. The cross contamination in the fractions isolated was judged visually on the dye-binding to be in the order of 5%.

The amino acid analysis of recovered fractions is given in Table 1 pp. 50 The results obtained reflect the cross contamination observed in the analytical gels, as well as contamination due to contribution of the gel itself to the amino acid composition of recovered histone fractions. Brown and Howard (1980) found that the contribution, especially at low protein to gel ratios, is linearly related to the volume of the gel slices eluted. In their studies the amino acids most significantly enhanced are serine, glycine, threonine, glutamic acid, alanine and lysine. The results, however, still allow positive identification as to which histone class a particular recovered fraction belongs e.g. all fractions have a characteristic lysine:arginine ratio as well as a specific basic:acidic

TABLE 1 AMINO ACID COMPOSITION OF SEA URCHIN EMBRYO HISTONES RECOVERED BY PREPARATIVE TRITON-PAGE

Analyses are given in mole %
No corrections have been made for losses due to incomplete hydrolysis.

* Results of amino acid composition for Sea Urchin histones purified by gel filtration and ion exchange chromatography.

* H2A Brandt et al. 1979
* H2B " " " "
* H3 " " " 1974
* H4 Wouters Tyrou et al. 1976

A.A.	$H2A_{(1)}$ mole %	$H2A_{(2)}$ mole %	$H2A_{(3)}$ mole %	H2A* mole %	H3 mole %	H3* mole%	H2B mole %	H2B* mole %	H4 mole %	H4* mole%
Asp	6,6	6,7	6,2	6,7	4,2	3,8	4,9	4,9	5,3	5
Thr	4,0	3,6	4,0	4,2	6,2	6,2	7,2	6,2	6,2	6
Ser	5,3	5,1	6,1	5,6	5,3	4,2	7,2	8,4	2,8	2
Glu	8,7	8,5	7,7	8,6	8,1	11,1	7,4	8,4	6,4	6
Pro	3,8	3,9	4,1	5,1	4,9	4,6	3,4	4,4	0,9	1
Gly	12,1	11,5	11,6	11,4	7,8	5,8	9,1	7,6	17,2	17
Ala	12,4	13,6	13,3	12,0	14,5	13,3	12,1	10,1	8,9	7
Val	6,8	7,1	6,4	7,0	5,0	4,7	6,5	6,5	6,5	9
Met	0,7	0,3	1,1	0,8	1,8	1,4	1,5	1,7	1,4	1
Ile	4,3	3,9	3,4	4,0	4,6	4,8	5,3	6,0	4,6	5
Leu	10,4	11,3	10,9	10,7	9,6	8,6	6,3	5,2	8,3	8
Tyr	2,7	2,6	2,4	2,7	2,4	2,2	3,7	3,6	3,4	4
Phe	1,9	1,9	1,9	1,9	1,9	3,1	1,7	1,5	2,0	2
Lys	10,0	11,3	10,1	9,7	10,4	10,2	13,2	15,8	10,4	10
His	1,8	1,7	1,9	1,6	1,8	1,5	1,8	1,9	2,7	2
Arg	8,3	7,9	8,5	8,1	10,8	13,0	8,3	7,9	12,9	14
Lys/Arg	1,2	1,3	1,2	1,2	1,00	0,75	1,6	2,0	0,8	0,7
$\frac{Lys+His+Arg}{Asp+Glu}$	1,3	1,2	1,5	1,4	1,8	1,5	1,9	1,9	2,2	2,45

residue ratio, which distinguish the particular histone classes.

A stock of the different histone fractions was accumulated from several such preparative gels. Sufficient material was collected of the H2A variants for amino acid analysis and amino acid sequencing. Histones H2B, H3 and H4 have to be refractionated at different urea concentrations for the isolation of their variants. In view of this, it was decided to use histone enriched fractions isolated by gel filtration as starting material for their electrophoretic separation.

H2B

Fig. 18bpp.52 shows the elution profile from Bio-Gel P-60 of the histone H2B enriched Johns fraction (Fig. 18a pp.52). There is still some contamination due to histones H2A and H3 but they will not interfere as their mobility at 4 M urea is much slower and well out of the H2B region (Fig. 18c & d pp.52). The major interfering histones H1 and H4 have been removed as they would have interfered with the isolation of histone H2B variants at 4 M urea. This H2B preparation can now be used for the isolation of its variants on preparative 4 M urea 6 mM Triton slab gels, see Chapter 6.3 pp.63.

H4

Crude H4 was prepared by purifying a Johns enriched H2A fraction (Fig. 19 a pp.53) by gel filtration on Bio-Gel P-60 (Fig. 19b pp.53). Fig.19c pp.53 shows the analytical Triton gel of the recovered P-60 histone fractions. The histone H4 can now be used for the isolation of possible

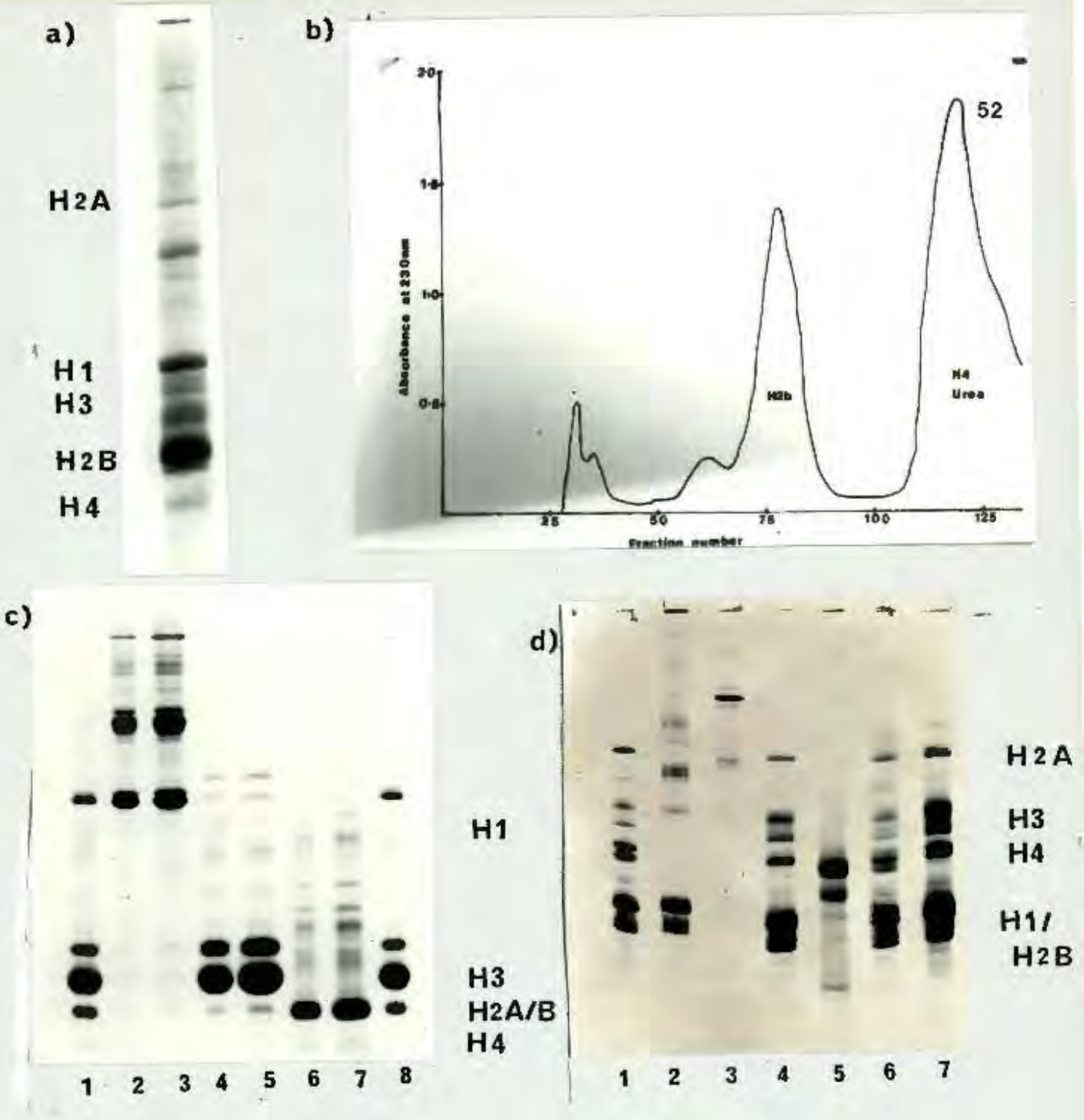


Figure 18 *Purification of Sea Urchin Embryo histone H2B*

a) Sample: enriched Johns histone H2B fraction on analytical 8,7 M urea 6 mM Triton X-100 PAGE

b) H2B enriched histone eluted from a Bio-Gel P-60 column (900 mm x 50 mm) with 0,05 M NaCl 0,02 M HCl pH 1,7

c) SDS-PAGE of histone fractions

lane 1 and 7: histone H2B enriched
2 and 3: histone H1 and aggregates
4 and 5: histone H2B, H2A and H3
6 and 7: histone H4

d) 5 M urea 6 mM Triton X-100 PAGE of histone fractions
lane 1 and 7: histone H2B enriched
2: histone H1
4 and 6: histone H2B, H3 and H2A
5: histone H4

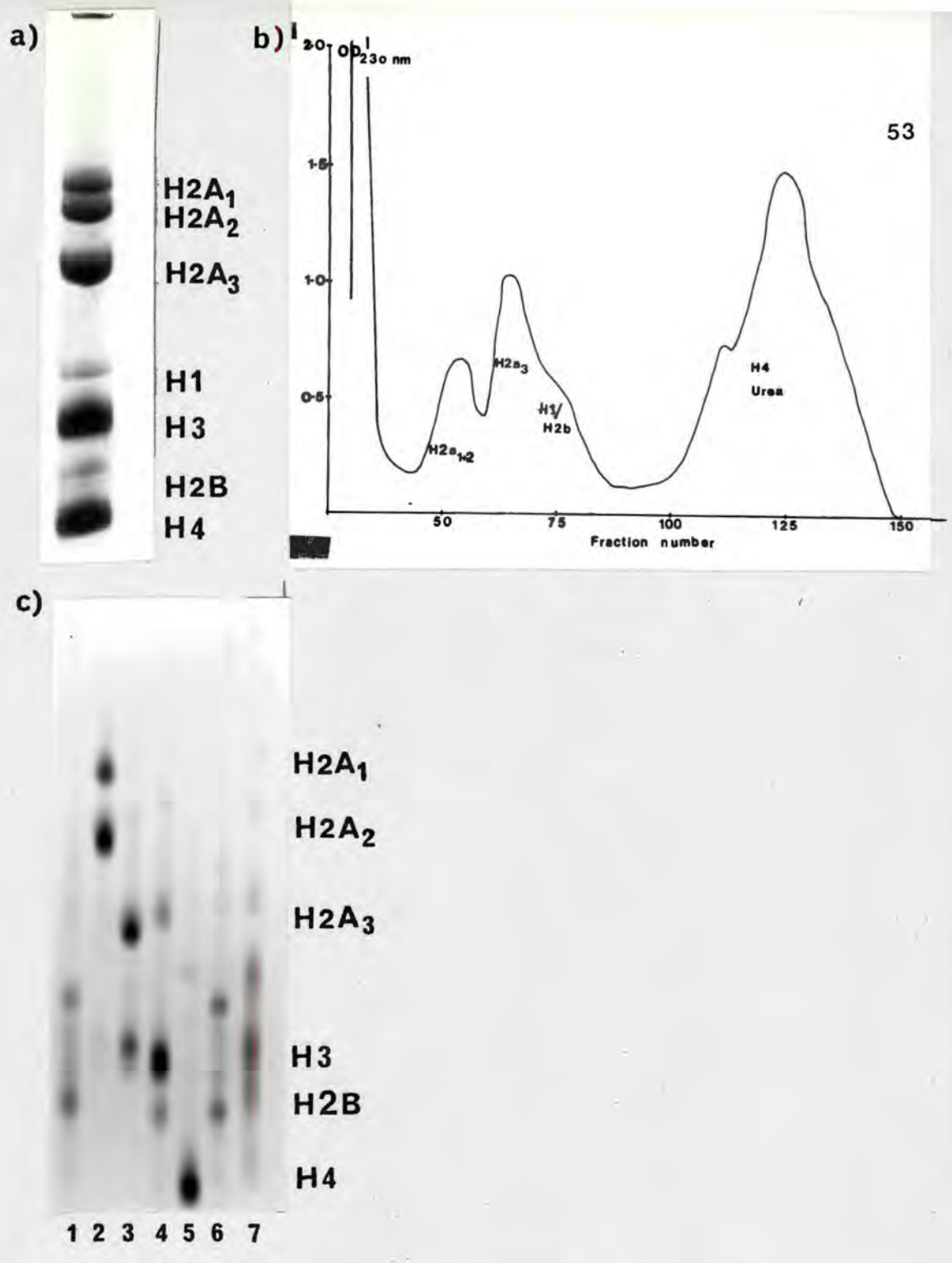


Figure **19** *Purification of Sea Urchin Embryo histone H4*

a) Sample enriched Johns histone H2A fraction on analytical 8,7 M urea 6 mM Triton X-100 PAGE

b) H2A enriched histones eluted from a Bio-Gel P-60 column (900 mm x 50 mm) with 0,05 M NaCl, 0,02 M HCl pH 1,7

c) 8,7 M urea 6 mM Triton PAGE of histone fractions
lane 1: histone H2B enriched
2: histone $H2A_{(1)}$ and $H2A_{(2)}$
3: histone $H2A_{(3)}$ and H3
4: histone H3
5: histone H4
6: whole sea urchin embryo mixture

variants on preparative 4 M urea and 6 mM Triton slab gels. In the gel depicted in Fig. 19c pp.53 , it is interesting to note that H2A variants can at least be partially separated on Bio-Gel P-60 (Fig.19c pp. 53 lanes 2 and 3). This has escaped notice earlier due to the lack of insight into Triton-binding by histones and the effect that urea exerts in Triton polyacrylamide gel electrophoresis. Such H2A fractions (Fig.19c pp.53 lanes 2 and 3) would be the ideal starting material for the isolation of variants on preparative 8,7 M urea 6 mM Triton slabs, as contamination of the histone fractions (H2B, H3 and H4), which have a faster mobility, can be avoided.

H3

As starting material for the preparative gel electrophoretic isolation of Sea Urchin histone H3 variants, a mixture of H3 has been used which had been recovered from preparative 8,7 M urea 6 mM Triton slabs (Fig. 15a pp.45), as well as fraction III from the separation of the histone enriched sample on Bio-Gel P-60, (Fig. 19c pp.53 lane 4). The latter is contaminated with H2B but this will not interfere as at 6 M urea, H2B has a faster mobility than H3.

CHAPTER 6

ISOLATION OF ELECTROPHORETICALLY HOMOGENOUS SEA URCHIN EMBRYO HISTONE VARIANTS

6.1. Introduction

The existence of histone variants is well documented in the literature (von Holt et al., 1979; Urban et al., 1980; Brandt and von Holt, 1978; Bonner et al., 1980; Cohen et al., 1975; Brandt et al., 1979; Treigyte and Gineitis, 1979; Strickland et al., 1978; Newrock et al., 1978).

It appears that the heterogeneity in histone H3 and H4 arises from minor modifications such as point mutations, e.g. cysteine-serine substitution in Calf Thymus histone H3, or deletion of one or more residues. In addition, post translational modifications through acetylation (Phillips, 1963), methylation (Murray, 1964) or phosphorylation (Kleinsmith et al., 1966) occur resulting in different electrophoretic mobility (Treigyte and Gineitis, 1979). The histones H2A and H2B show striking variabilities due to reiteration, insertion, deletion and point mutations in the major areas of the molecule. Histone H2A variability occurs mainly in the amino-terminal and carboxy-terminal end, while the hydrophobic centre is conserved (von Holt et al., 1979). In the case of Sea Urchin embryo, histone H2A made prior to the blastula stage differs in primary structure from the later H2A histones (β & δ). H2A α contains a methionine which is lacking in the later H2A histones (Newrock et al., 1978). So far it is not known whether there are primary structure differences among the later synthesized forms. The H2A variants can be resolved by electrophoresis on acid-urea gels containing Triton X-100 (Zweidler and Cohen, 1972; Franklin and Zweidler, 1977). Their micropreparative isolation from preparative Triton polyacrylamide gels should thus be possible. The precise

location of sequence differences is complex. All the known histones H2A's are blocked at their N-terminal and in addition the hydrophobic tryptic core is highly conserved. Therefore the sequence differences have to be identified via peptide mapping or the elucidation of the entire structure (Strickland, 1980).

Histones H2B have a highly variable N-terminal region and a more constant C-terminal region (89 residues) following the central methionine residue (von Holt et al., 1979). Three different H2B variants from Sea Urchin embryo can be identified on analytical Triton polyacrylamide gels. Because most of the histones H2B have a free N-terminus, the location of sequence differences may be more easily compared to histone H2A.

6.2. Sea Urchin Embryo Histone H2A Variants

Histone H2A variants have been isolated from preparative 8,7 M urea 6 mM Triton slab gels (Fig. 20a pp.57). The histone mixture to be separated results from a selective Johns extraction and is enriched in H2A, H3 and H4 (Fig. 20 a & b pp. 57) 400 mg of this histone mixture was processed on preparative gels and 30 mg of each of the three H2A variants was recovered. The H2A variants 1-3 have been analysed on analytical SDS gels (Fig. 20c pp.57), analytical 8,7 M urea 6 mM Triton gels (Fig. 20d pp.57) as well as on 0-9 M urea gradient slab gels at constant Triton concentration (Fig. 21 a-c pp. 58). From figures 20c and 20d, it is obvious that the gels are overloaded thus showing up the contaminations clearly. Contamination is due to factors discussed in Chapter 5. By visual judgement, the contaminants are present in the order of about 5%. The amino acid composition of H2A 1-3 is given in Table 2. The composition data for all three fractions are characteristic for histones H2A. In particular the lysine:arginine ratio of

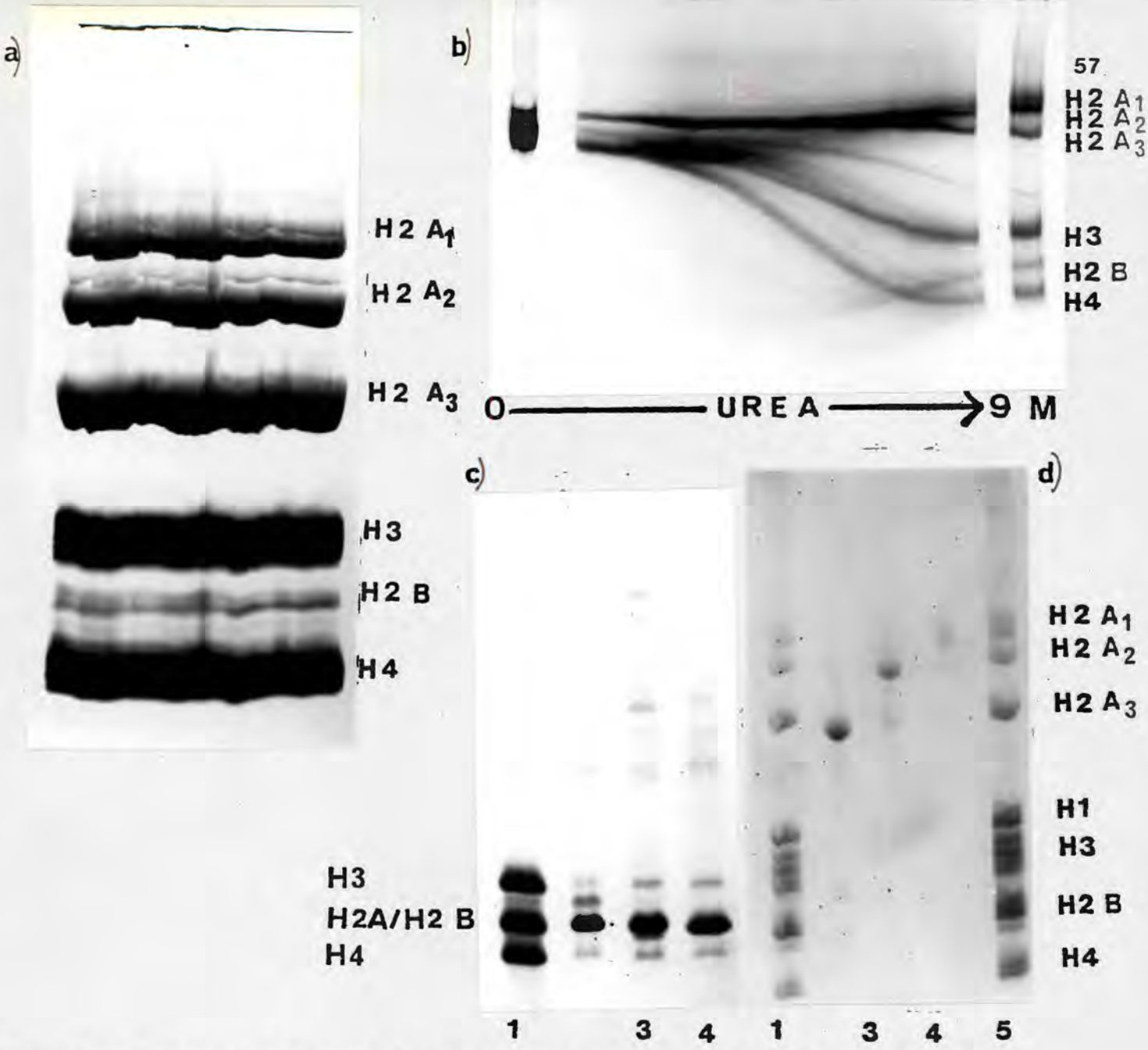


Figure 20 *Preparative Triton-PAGE of Sea Urchin embryo histones H2A enriched and identification of purified histone fractions by analytical PAGE*

a) Separation of histone H2A enriched fraction by preparative 8,7 M urea, 6 mM Triton PAGE

b) 0-9 M urea gradient 6 mM Trition-PAGE of histone H2A enriched fraction

c) Analytical SDS-PAGE of purified histone fractions
lane 1: Sea Urchin embryo H2A enriched histones
" 2: Histone $H2A_{(1)}$
" 3: Histone $H2A_{(2)}$
4: Histone $H2A_{(3)}$

d) Analytical 8,7 M urea 6 mM Triton-PAGE of purified histone fractions
lane 1: Sea Urchin embryo histones
" 2: Histone $H2A_{(1)}$
" 3: Histone $H2A_{(2)}$
4: Histone $H2A_{(3)}$

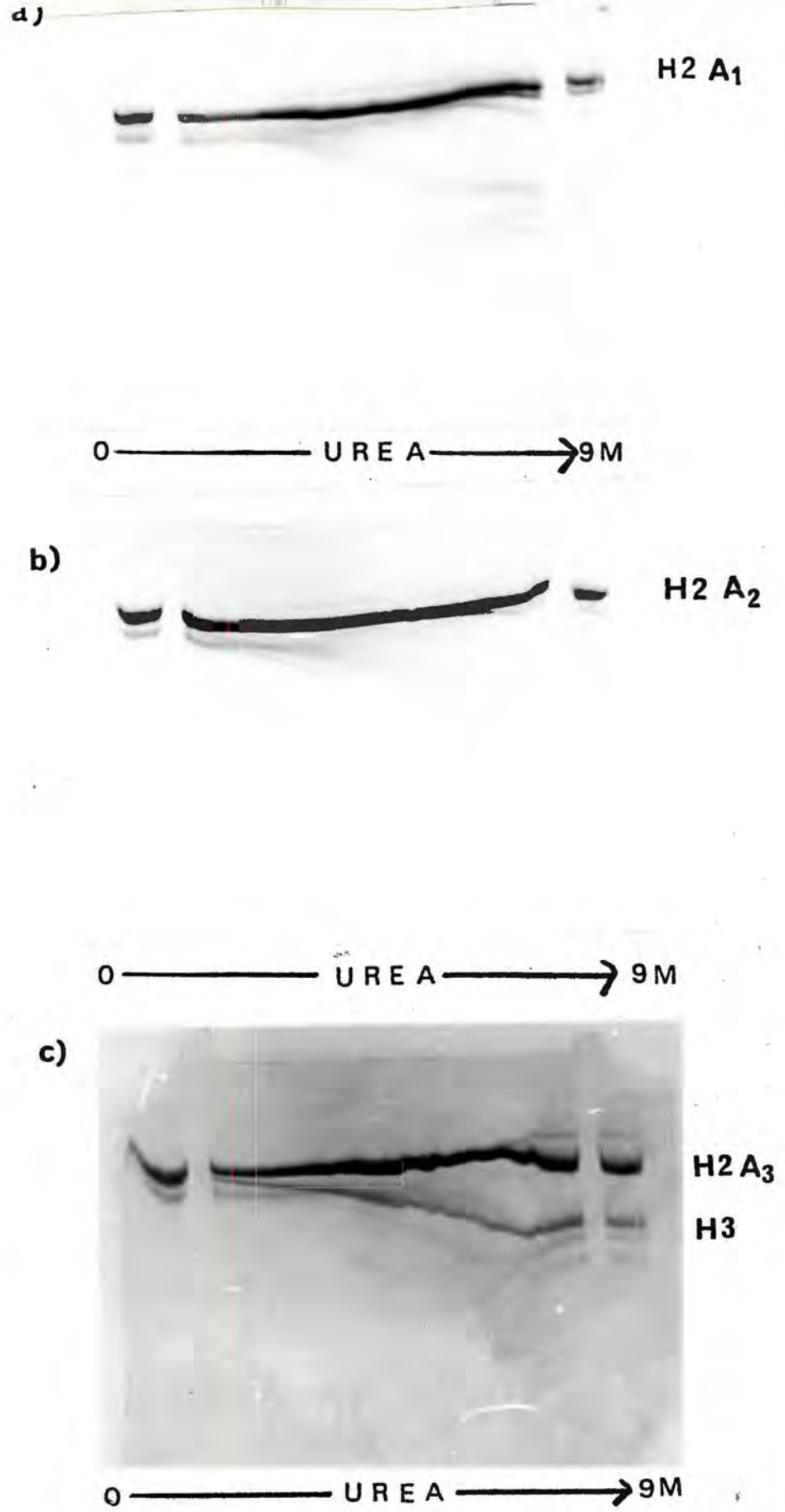


Figure **21** *Urea gradient Triton-PAGE of Sea Urchin Embryo histone H2A variants*

0-9 M urea 6 mM Triton X-100 PAGE

a) Sea Urchin Embryo histone $H2A_{(1)}$

b) " " " " $H2A_{(2)}$

c) " " " " $H2A_{(3)}$

approximately 1,2 is typical. The overall composition tallies closely with that reported for Sea Urchin H2A isolated by gel filtration (Brandt et al., 1974), see H2A* Table 2, pp.60. Both histones $H2A_{(1)}$ and $H2A_{(2)}$ exhibit a blocked N-terminus and also proved to be resistant to CNBr cleavage. Histone $H2A_{(3)}$ which is also blocked, contains at least 1 methionine, as on cleavage with CNBr in the sequencer cup, a characteristic H2A sequence was obtained. The sequence following methionine lies in the conserved hydrophobic core. It is identical (Fig. 22 pp.61) to the homologous region in Calf Thymus (C.T.) H2A (Yeoman et al., 1972). Methionine itself is substituted in C.T. H2A with leucine in position 51. Fig.11b pp.36 shows the yields of Pth-amino acid recovered per degradation cycle for CNBr 2 peptide of $H2A_{(3)}$. The presence of methionine in histone $H2A_{(3)}$ was already indicated in Fig. 10 pp. 34, where, upon treatment with performic acid, $H2A_{(3)}$ loses its high affinity for Triton due to methionine oxidation and now has an electrophoretic mobility close to histone H3. A similar effect was not observed for histone $H2A_{(1)}$ or $H2A_{(2)}$.

To locate other possible differences in primary structure of histone H2A variants, histones $H2A_{1-3}$ were subjected to trypsin digestion and the resulting peptides were separated from the tryptic core on a G-25 column. The peptides can be mapped by two dimensional chromatography and electrophoresis (Katz et al., 1959; Bennet, J., 1967). K. Patterson (member of the Department) produced the peptide maps for the H2A variants (Fig. 23 pp.62). Indications are that most peptides are in common between the variants. Peptides which exhibit a different mobility will be subjected to amino acid analysis. The tryptic cores still await sequence determination.

TABLE 2 AMINO ACID COMPOSITION OF SEA URCHIN EMBRYO HISTONE H2A VARIANTS

Analyses are given in mole %; no corrections have been made for losses due to incomplète hydrolysis.

* result of amino acid composition for Sea Urchin histone H2A purified by gel filtration and ion exchange chromatography (Brandt et al. 1979).

A.A.	$H2A_{(1)}$ mole %	$H2A_{(2)}$ mole %	$H2A_{(3)}$ mole %	$H2A^{*}$ mole %
Asp	6,5	6,9	6,5	6,7
Thr	3,7	3,9	3,7	4,2
Ser	5,3	5,4	5,8	5,6
Glu	9,0	8,8	8,5	8,6
Pro	5,7	5,1	5,7	5,1
Gly	11,3	11,4	11,2	11,4
Ala	11,9	12,4	10,9	12,0
Val	6,2	6,5	6,5	7,0
Met	-	0,5	0,8	0,8
Ile	4,4	3,9	3,9	4,0
Leu	10,0	10,5	10,2	10,7
Phe	2,4	2,3	2,4	2,7
Tyr	2,0	1,8	1,7	1,9
Lys	11,0	10,4	10,9	9,7
His	1,6	1,5	1,5	1,6
Arg	8,8	8,9	9,5	8,1
Lys/Arg	1,2	1,15	1,15	1,2
$\frac{Lys+Arg+His}{Glu+Asp}$	1,2	1,3	1,4	1,4

Sea Urchin embryo $H2A_{(3)}$: Arg-[Val-Gly-Ala-Gly-Ala-Pro-Val-
(CNBr 2 peptide) Tyr-Met-Ala-Ala-Val-Leu-Glu-Tyr-
-Leu-(Calf Tymus H2A)
Leu-Thr-Ala-Glu-Ile-Leu-Glu-Ala-
Gly-Asn-Ala-]Arg-Asp-Asn-Lys-Lys-
Thr-Arg-Ile-Ile-Pro-Arg-His

Figure 22 *Sequence of Sea Urchin Embryo Histone $H2A_{(3)}$ after CNBr cleavage.*

Position 51 shows substitution with methionine compared to leucine in Calf Thymus H2A. The [ ] sequence represents the tryptic core of histone H2A.

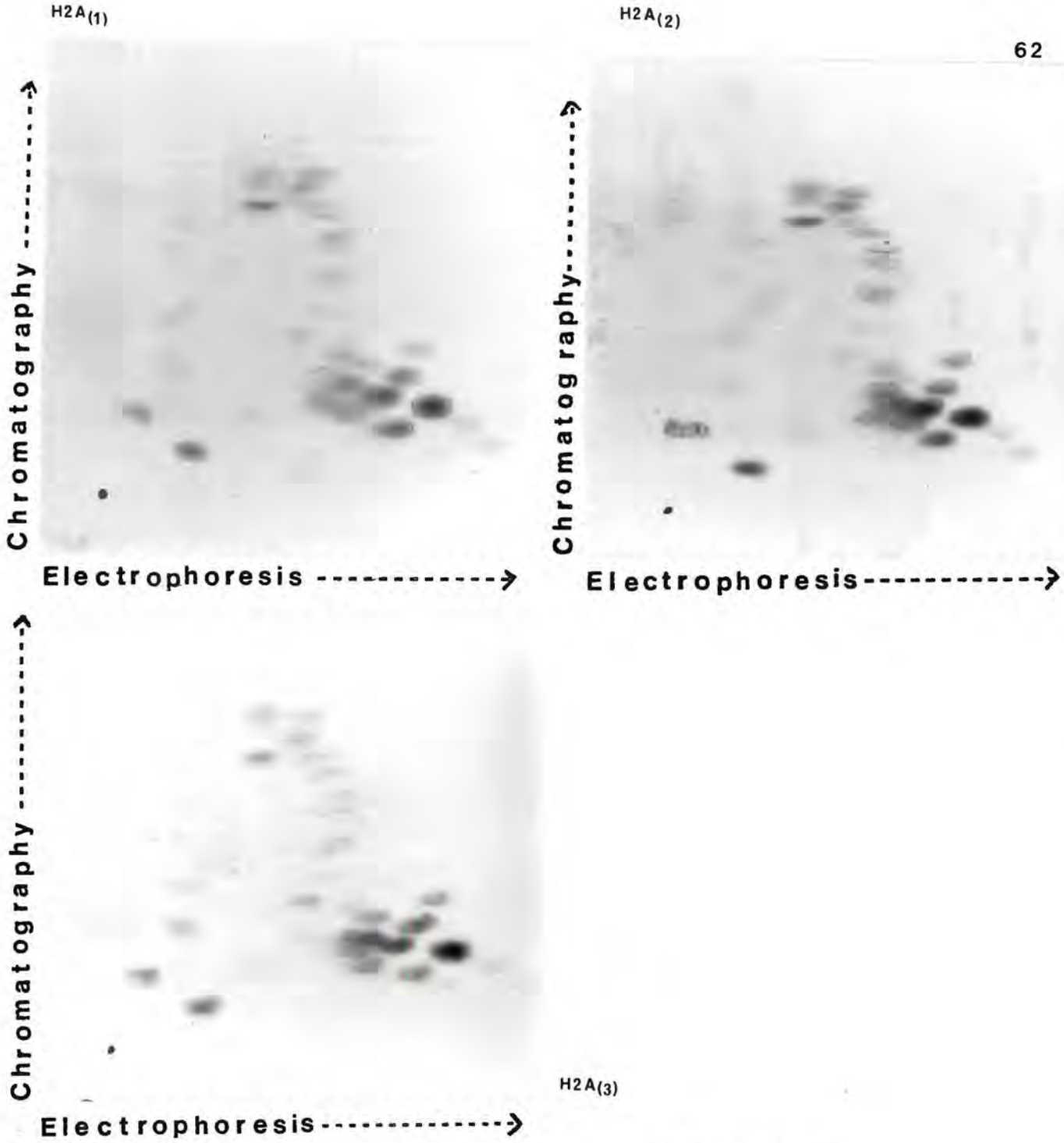


Figure 23 Peptide Map of Tryptic Digest of Histone H2A Variants

The peptides are mapped on thin layer cellulose plates. 20 nmoles have been spotted in the bottom left hand corner of the plate and electrophoresis is carried out for 2 hours at 800 volts in pyridine:acetic acid:acetone:H_2O (13:26:200:400), followed in the 2nd dimension by chromatography in pyridine:acetic acid:butanol:H_2O (40:10:55:40). The spots are located with ninhydrin. Individual peptides can be scraped off, eluted from cellulose with 0,9 M acetic acid, and on acid hydrolysis the amino acid composition can be determined.

6.3. Sea Urchin Embryo Histone H2B Variants

H2B variants have been isolated on preparative 4 M urea 6 mM Triton polyacrylamide slab gels. This optimal urea concentration has been chosen from the results reported in Fig. 13a pp.41. As starting material, a Johns enriched H2B fraction (Fig. 24b pp.64) has been used. Prior to electrophoresis, a purification step by gel filtration on Bio-Gel P-60 to remove histone H4 is necessary. At 4 M urea histone H4 travels between $H2B_{(1)}$ and $H2B_{(2)}$ (Fig.24a pp.64), resulting in contamination of the variants as well as reducing the sample load that can be applied to the gel. The partially purified H2B fraction (Fig. 24b pp.64), depleted of histone H1 and H4, still contains some histone H2A and H3. However, at 4 M urea, both contaminants have a much slower mobility. This enriched H2B fraction is shown on a urea gradient slab gel in Fig. 25 pp. 64. Here 40 mg H2B are applied to 4 preparative gels and the five fractions identified on Fig. 24b pp. 64 have been excised, and the correspoding histone fractions eluted. The recoveries per fraction are as follows:

F1 : 2,6 mg
F2 : 4,8 mg
F3 : 3,8 mg
F4 : 9,4 mg
F5 : 7,0 mg

On analytical 8,7 M urea, 6 mM Triton slab gels (Fig. 26a pp.65), analytical 4 M urea, 6 mM Triton slab gels (Fig. 26b pp.65) and analytical SDS slab gel (Fig. 26c pp.65), the three fastest moving fractions are identified as histones H2B and have been designated $H2B_{(1-3)}$. The urea gradient gels of $H2B_{(1-3)}$ show that no major contaminations are present, in spite of the close proximity of $H2B_{(2)}$ and $H2B_{(3)}$. The degree of cross-contamination can best be recognized from the urea gradient slabs (Fig. 27 a-c pp.66). The rapidly moving contaminating fractions, particularly in $H2B_{(2)}$ (Fig. 27b pp.66) and $H2B_{(3)}$ (Fig. 27c pp.66) are most probably minor H2B variants, as

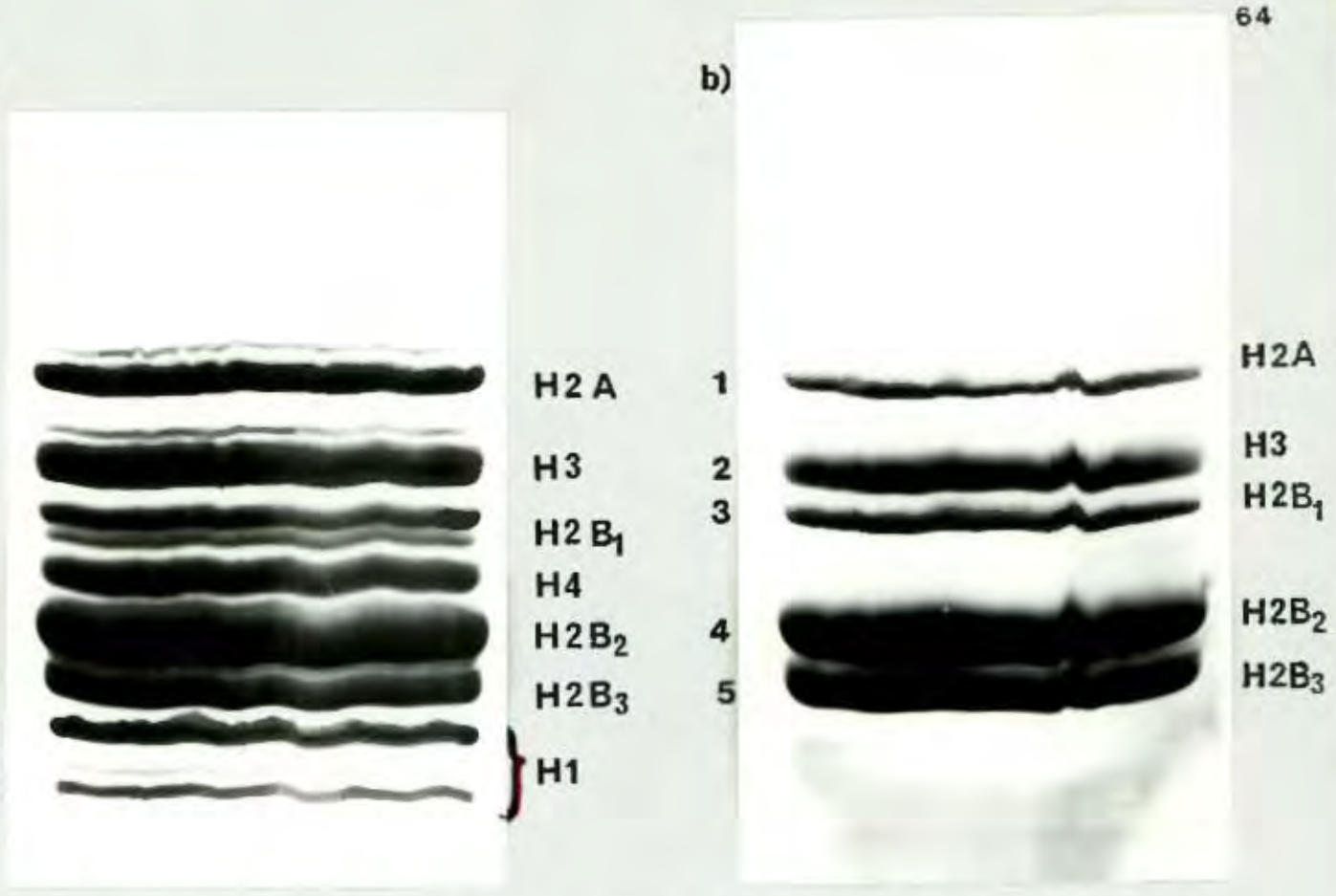


Figure **24** *Separation of Sea Urchin embryo histone H2B on a preparative 4 M urea 6 mM Triton gel*

a) H2B enriched Johns fraction
b) H2B enriched Johns fraction after further purification on Bio-Gel P-60

Figure **25** *0-9 M urea gradient 6 mM Triton-PAGE of Sea Urchin embryo H2B after purification on Bio-Gel P-60.*

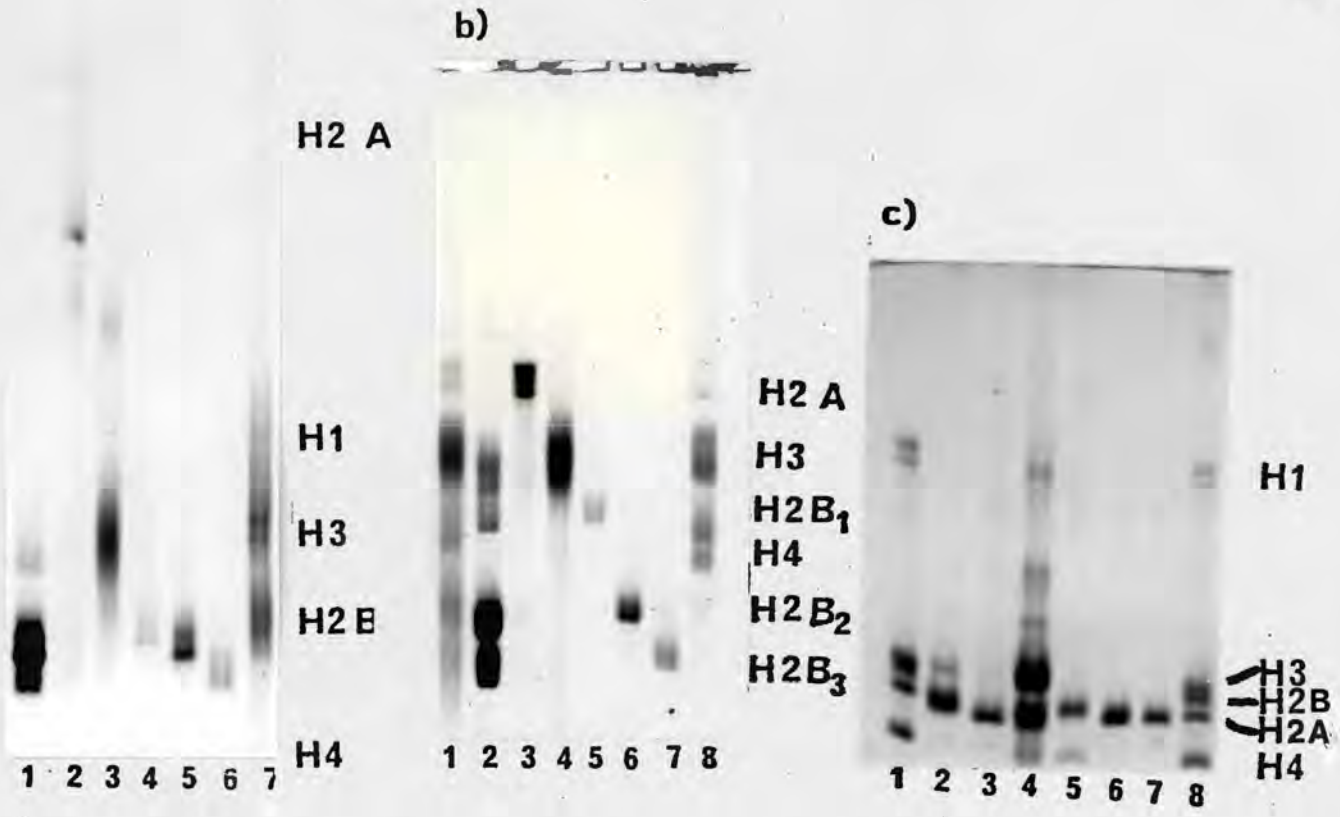


Figure **26** *Analytical PAGE of Sea Urchin Embryo histone fractions recovered from preparative Triton-PAGE (Figure* **24** *page* **64** *).*

a) 8,7 M urea 6 mM Triton-PAGE

1 control H2B as purified on P-60
2 F1 (H2A) ex preparative gel Figure
3 F2 (H2A/H3) ex " " "
4 F3 ($H2B_{(1)}$) " " " "
5 F4 ($H2B_{(2)}$) " " " "
6 F5 ($H2B_{(3)}$) " " " "
7 total Sea Urchin histones acid extract

b) 4 M urea 6 mM Triton-PAGE

1 total Sea Urchin histones acid extract
2 control H2B as purified on P-60
3 F1 (H2A) ex preparative gel Figure
4 F2 (H2A/H3) ex " " "
5 F3 ($H2B_{(1)}$) " " " "
6 F4 ($H2B_{(2)}$) " " " "
7 F5 ($H2B_{(3)}$) " " " "
8 Johns enriched histone H2A fraction

c) SDS PAGE

1 and 8 total Calf Thymus histone acid extract
2 control H2B as purified on P-60
3 F1 (H2A) ex preparative gel Figure
4 F2 (H2A/H3) ex " " "
5 F3 ($H2B_{(1)}$) " " " "
6 F4 ($H2B_{(2)}$) " " " "
7 F5 ($H2B_{(3)}$) " " " "

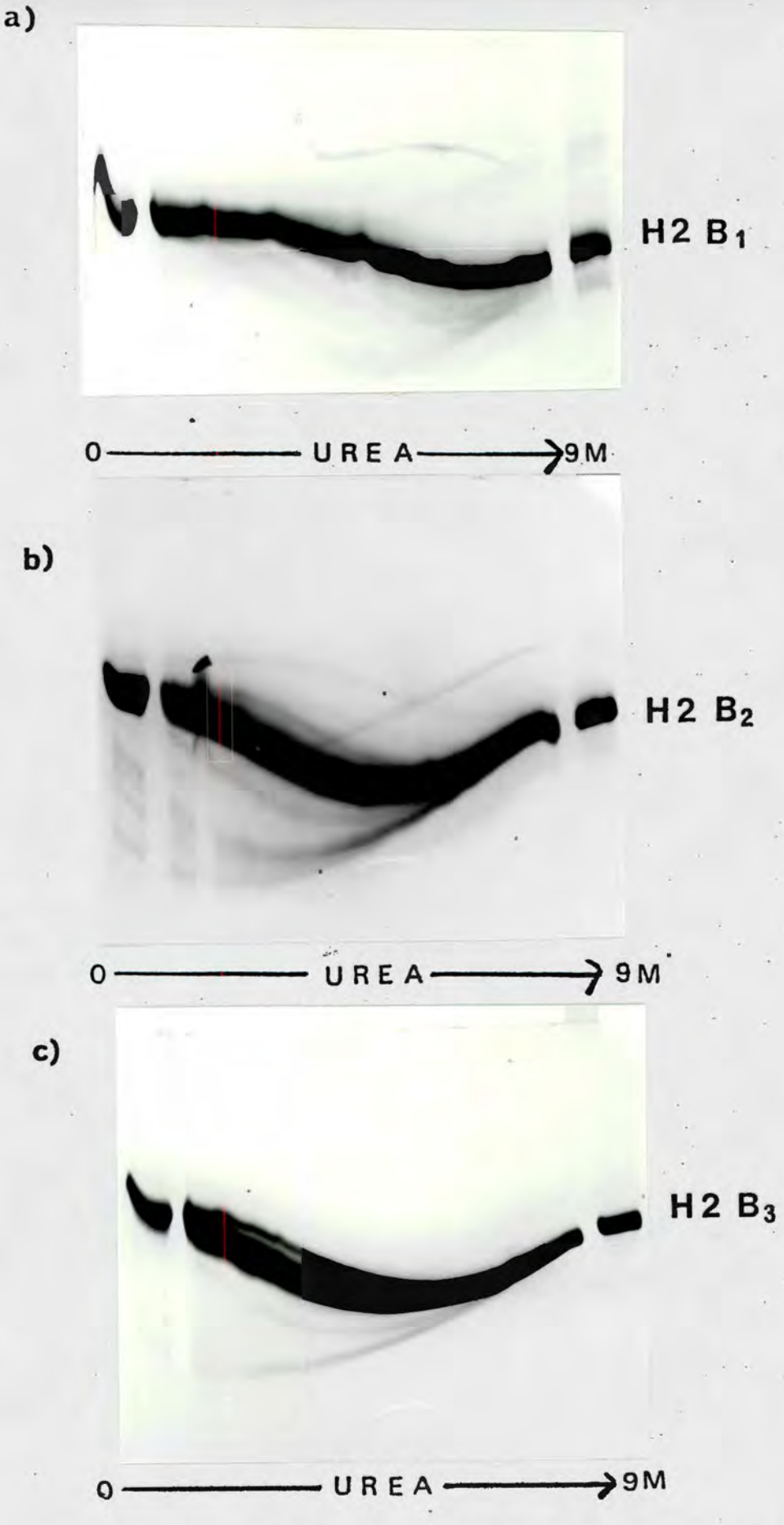


Figure **27** *Urea gradient Triton-PAGE of Sea Urchin Embryo histone H2B variants*

0-9 M urea 6 mM Triton X-100 PAGE

a) Sea Urchin Embryo histone $H2B_{(1)}$

b) " " " $H2B_{(2)}$

c) " " " $H2B_{(3)}$

from about 6 M urea onwards, they move as one band together with the three main histones H2B.

$H2B_{(1)}$ has a markedly different behaviour at the different urea concentrations compared to histones $H2B_{(2)}$ and $H2B_{(3)}$. Doubt arose whether this protein was indeed H2B. The amino acid analyses (Table 3 pp. 68), however, show characteristic histone H2B compositions for all three variants. Histone $H2B_{(1)}$ has a lower Lys/Arg ratio due to the presence of some contamination with H4 (see SDS gel Fig. 18c pp. 52). To clarify that the difference in electrophoretic mobility observed on the Triton gel reflects a genuine difference in primary structure, rather than post-translational side chain modification, histones $H2B_{(1-3)}$ have been subjected to partial sequence analysis. The yields of Pth-amino acids per degradation cycle have been plotted for each H2B variant (Fig. 36b,d,e,f pp. 106,108,110). Fig. 28a pp.69 compares the N-terminal sequences of the 3 variants. $H2B_{(1)}$ resembles the late gastrula H2B isolated by Brandt et al., 1974, by molecular sieve and ion-exchange chromatography, except that in position 6, one finds Thr instead of Val. Fig. 36b pp.106 shows that $H2B_{(1)}$ is slightly contaminated with $H2B_{(2)}$. $H2B_{(1)}$ is clearly identified as H2B type histone by homology in the region following the central methionine.

$H2B_{(2)}$ is identical to the early blastula H2B (Brandt and von Holt, 1978). It is contaminated with $H2B_{(3)}$ marked as the minor sequences in Fig. 36d pp. 108. The appearance of Lys-Ser-Ala-Ser in positions 6-9 indicates the presence of at least one other variant. Judging from the intesity of staining, $H2B_{(2)}$ is the major component of gastrula H2B (urea gradient gel, Fig. 13a pp.41). Although $H2B_{(2)}$ when isolated from preparative gel appeared fairly homogenous (Fig. 24b pp.64), sequencing showed an obvious heterogeneity within $H2B_{(2)}$ itself.

TABLE 3 AMINO ACID COMPOSITION OF SEA URCHIN EMBRYO H2B VARIANTS

Analyses are given in mole %
No corrections have been made for losses due to incomplete hydrolysis.

* Results of amino acid composition for Sea Urchin embryo histone H2B purified by gel filtration on ion-exchange chromatorgraphy (Brandt et al. 1979).

A.A.	$H2B_{(1)}$ mole %	$H2B_{(2)}$ mole %	$H2B_{(3)}$ mole %	H2B* mole %
Asp	5,0	5,0	4,8	4,9
Thr	6,5	7,0	6,4	6,2
Ser	7,1	8,7	9,6	8,4
Glu	8,0	8,6	8,0	8,4
Pro	5,4	4,7	3,8	4,4
Gly	8,2	7,5	7,8	7,6
Ala	10,9	10,4	11,2	10,1
Val	6,9	6,7	6,8	6,5
Met	1,2	1,5	1,5	1,7
Ile	5,1	6,1	5,0	6,0
Leu	5,9	5,1	5,1	5,2
Tyr	3,3	3,6	3,8	3,6
Phe	1,9	1,5	1,7	1,5
Lys	13,0	15,0	15,1	15,8
His	1,8	1,4	1,5	1,9
Arg	9,4	6,7	7,6	7,9
Lys/Arg	1,4	2,2	2,0	2,0
$\frac{Lys+His+Arg}{Asp+Glu}$	1,9	1,7	1,9	1,9

FIGURE 28

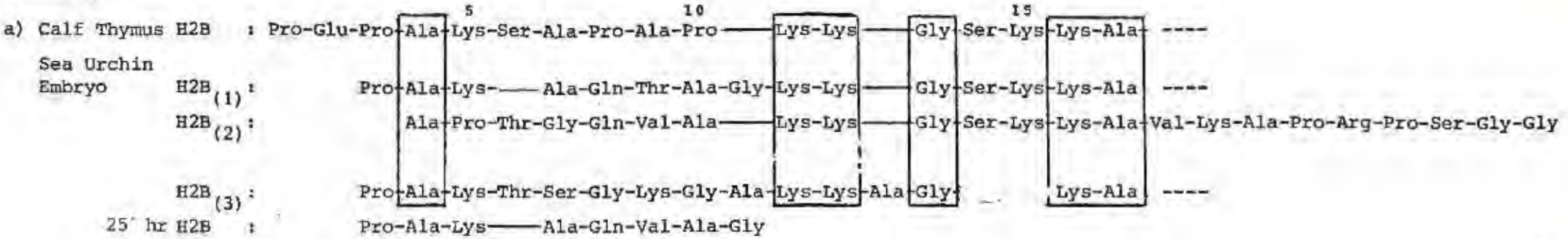


Figure a) Comparison of partial primary structures of Sea Urchin embryo H2B variants 1-3 to corresponding regions in histone H2B from Calf Thymus (Iwai et al. 1972) and 25 hr. Sea Urchin Embryo (Brandt and von Holt, 1978). The sequences have been aligned for maximum homology.
Numbering refers to alignment position and not to sequence positions.
Deletions in the alignment (——) regions not sequenced ---- homologous positions have been boxed.
A minor sequence in histone $H2B_{(2)}$ has been bracketed.

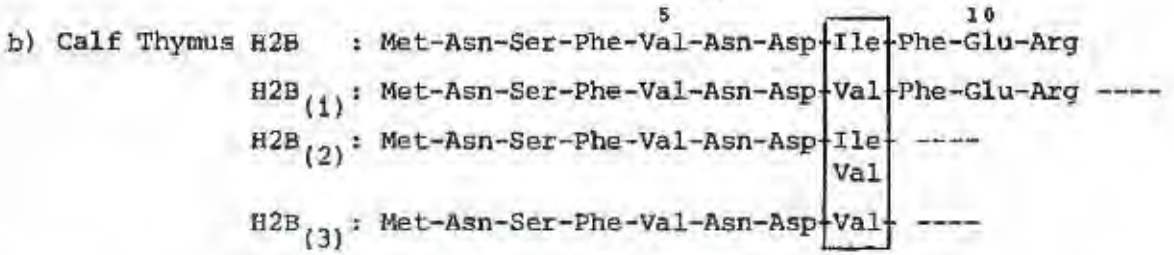


Figure b) Comparison of central region of H2B variants 1-3 from Sea Urchin to that of Calf Thymus H2B.
Numbering refers to position in CNBr fragment.
Heterogenous position has been framed.

$H2B_{(3)}$ is the most different of the H2B histones. It is still contaminated with $H2B_{(2)}$, marked as minor sequence in Fig. 36F pp. 110. Fig. 28b pp. 69 compares the sequence of the central H2B regions showing the sequence after the 2nd methionine. It appears that the C-terminal regions of all the three variants are much more conserved when compared to their N-terminal domains. $H2B_{(1)}$ and $H2B_{(2)}$ have valine in position 7 to isoleucine, suggesting the presence of two polypeptide chains (Fig. 28b pp.69 and Fig. 36C,E,G pp. 107,109,111). This result confirms the partial sequence of late gastrula H2B reported by Brandt and von Holt (1978).

At least 8 different H2B variants have been identified for the Sea Urchin embryo (Brandt and von Holt, 1978). Strickland et al., 1977 a,b, and 1978 sequenced 3 H2B histones from sperm cells, and one histone H2B from a diploid gut cell (Brandt et al., 1979). Therefore there are at least 12 different H2B variants in Sea Urchin *Parechinus angulosus* which have been identified on the basis of complete and partial sequences. Each of the variants is coded for by a different gene. The results reported here add further evidence to the notion that histones are synthesized at different stages of development, (Cohen et al., 1975; Brandt and von Holt, 1978; von Holt et al., 1979). It would also account for the multiple reiteration of histone genes (Kedes and Birnstiel, 1971).

6.4. Sea Urchin Embryo Histone H3 Variants

Sea Urchin embryo H3 histone variants have been isolated on preparative 6 M urea, 6 mM Triton polyacrylamide slab gels (Fig. 29a pp. 72). Two main fractions can be distinguished. They are in themselves heterogeneous (Fig. 29a pp.72), the acetylation of lysine residues may be the cause of this. Acetylation results in loss of one positive charge per acetyl residue resulting in a corresponding loss in electrophoretic mobility (Marztaff and McCarty, 1970). The recovered fractions $H3_{(1)}$ and $H3_{(2)}$ have been analysed on urea gradient gels (Fig. 30b & c, pp. 73) 6 M urea, 6 mM Triton-PAGE (Fig. 29b pp. 72) and SDS-PAGE (Fig. 29c pp.72). The separation achieved is not very good as judged by the analytical Triton and SDS gels. The amino acid analyses (Table 4 pp.74) do not show any striking differences but are characteristic for histones H3 when compared to the composition of Sea Urchin embryo histone H3* isolated by gel filtration (Brandt et al., 1974). The partial N-terminal sequence of the embryo isohistones is given in Fig. 31a pp.75. The yields of Pth-amino acids per degradation cycle are given in Fig. 36 H-K pp. 112-115

The first seven residues in histone $H3_{(1)}$ show no other contaminants and are homologous to Calf Thymus histone H3 (de Lange et al., 1973). Histone $H3_{(2)}$ is contaminated with histone $H2B_{(1)}$ and another minor sequence has also been marked (Fig. 31a pp.75). Fig. 31b pp.75 shows the resulting peptides after CNBr cleavage. Both histones $H3_{(1)}$ and $H3_{(2)}$ have 2 methionines each. In the CNBr-1 peptide, both $H3_{(1)}$ and $H3_{(2)}$ have serine in position 7 (von Holt et al., 1979), compared to cysteine in Calf Thymus H3. In the CNBr-2 peptide, Calf Thymus has a lysine residue in position 3. Although the rest of the sequence can be clearly identified for both $H3_{(1)}$ and $H3_{(2)}$ (Fig. 31b pp.75), and lysine is generally very easily identified, no amino acid can be identified for position 3 in CNBr-2.

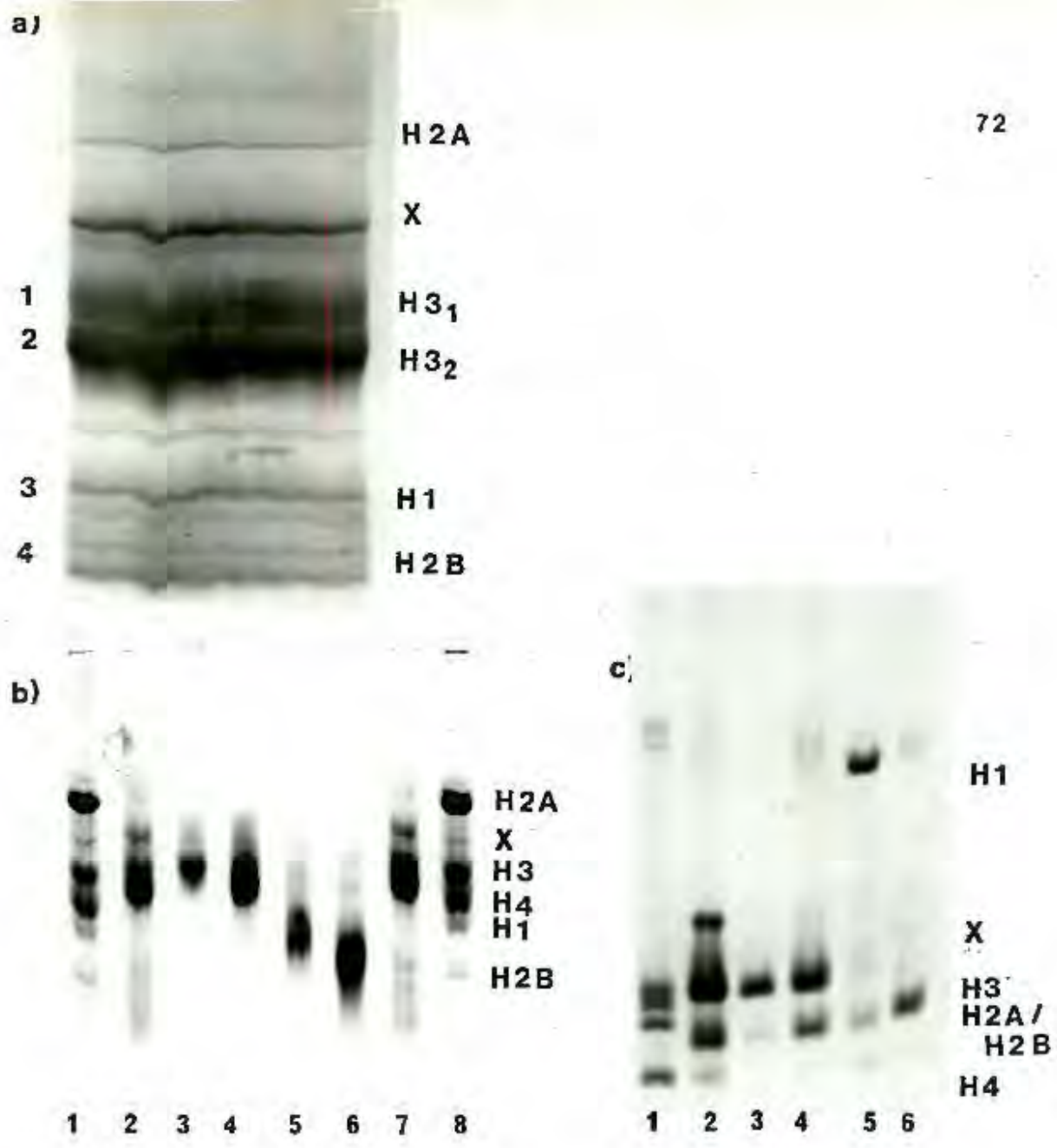


Figure **29** *Preparative Triton-PAGE of Sea Urchin embryo histones and identification of purified histone fractions by analytical PAGE.*

a) Separation of histone H3 isolated from preparative 8,7 M urea 6 mM Triton X-100 gels by preparative 6 M urea 6 mM Triton X-100-PAGE

b) Analytical 6 M urea 6 mM Triton X-100 PAGE of purified histone fractions,
lane 1 and 8: Sea Urchin embryo histone H2A enriched
" 2 " 7: Histone H3 as isolated from preparative 8,7 M urea 6 mM Triton gel (control)
" 3: Histone $H3^{(1)}$
" 4: Histone $H3^{(2)}$
" 5: Histone H1
" 6: Histone H2A

c) Analytical SDS-PAGE of purified histone fraction
lane 1 : Calf Thymus histone
" 2 : Histone H3 as isolated from preparative 8,7 M urea 6 mM
: Triton gel (control)
" 3 : Histone $H3^{(1)}$
" 4 : Histone $H3^{(2)}$
" 5 : Histone H1
" 6 : Histone H2B

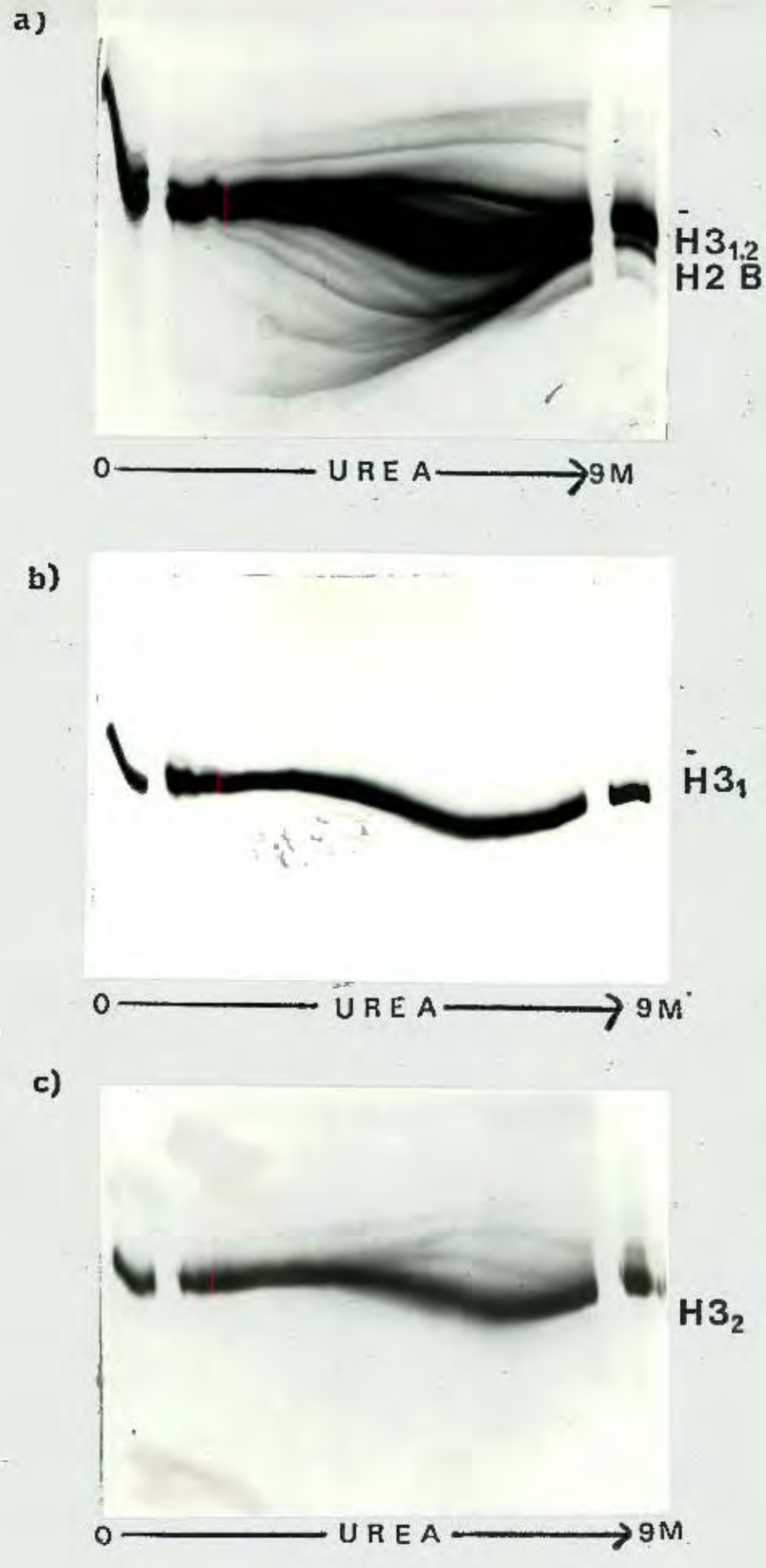


Figure **30** *Urea gradient Triton-PAGE of Sea Urchin embryo histone H3 variants*

0-9 M urea gradient 6 mM Triton X-100 PAGE

a) Sea Urchin embryo histone H3 (control)
b) " " " $H3_{(1)}$
c) " " " $H3_{(2)}$

TABLE 4 AMINO ACID COMPOSITION OF SEA URCHIN EMBRYO HISTONE H3 VARIANTS

Analyses are given in mole %
No corrections have been made for losses due to incomplete hydrolysis.

* Result of amino acid composition for Sea Urchin histone H3 purified by gel filtration, Brandt et al. 1974.

A.A.	$H3_{(1)}$ mole %	$H3_{(2)}$ mole %	H3* mole %
Asp	3,6	4,3	3,8
Thr	6,8	6,7	6,2
Ser	5,2	5,6	4,2
Glu	11,6	11,2	11,9
Pro	5,8	5,0	4,6
Gly	6,3	6,6	5,8
Ala	12,2	12,5	13,3
Val	5,7	5,6	4,7
Met	0,6	0,3	1,4
Ile	5,2	5,1	4,8
Leu	8,6	8,5	8,6
Tyr	2,1	2,5	2,2
Phe	2,3	2,6	3,1
Lys	10,3	10,7	10,2
His	1,7	1,3	1,5
Arg	11,7	11,3	13,0
$\frac{Lys+His+Arg}{Asp+Glu}$	1,55	1,5	1,5

```
                                                5                   10
Calf Thymus H3                  :   Ala-Arg-Thr-Lys-Gln-Thr-Ala-Arg-Lys-Ser-

Sea Urchin Embryo H3(1)         :   Ala-Arg-Thr-Lys-Gln-Thr-Ala-Arg-Lys-Ser-

Sea Urchin Embryo H3(2)         :   Ala-Arg-Thr-Lys-Glu-Thr-Ala-
                                        [Lys-   -Gly-Ala-Lys]

Contaminated H2B(1)             :   Pro-Ala-Lys-Ala-Gln-Thr-Ala-
```

Figure 31a: Comparison of Partial Amino Acid Sequences of Sea Urchin Embryo Histone H3 Variants to corresponding region on histone H3 from Calf Thymus (de Lange et al., 1973). A minor sequence in $H3_{(2)}$ besides the histone $H2B_{(1)}$ contaminant has been bracketed. Numbering refers to sequence position in the N-terminal region.

```
                                                    5                   10
Calf Thymus H3  CNBr 1              : Met-Ala-Leu-Gln-Glu-Ala-[Cys]-Glu-Ala-Tyr-

Sea Urchin Embryo H3(1) CNBr 1      : Met Ala-Leu-Gln-Glu-Ala-[Ser]-

Sea Urchin Embryo H3(2) CNBr 1      : Met-Ala-Leu-Gln-Glu-Ala-[Ser]-Glu-Ala-Tyr-
                                                    5
Calf Thymus H3 CNBr 2               : Met-Pro-Lys-Asp-Ile-Gln-Leu-Ala-

Sea Urchin Embryo H3(1) CNBr 2      : Met-Pro- X -Asp-Ile-Gln-Leu-Ala-

Sea Urchin Embryo H3(2) CNBr 2      : Met-Pro- X -Asp-Ile-Gln-Leu-Ala-
```

Figure 31b: Comparison of the two CNBr fragments in Sea Urchin Embryo Histone H3 Variants to corresponding CNBr peptides in Calf Thymus. Numbering refers to position in the CNBr fragment. Heterogeneous position has been framed.

The leucine residue in CNBr-1 has been identified on an isocratic run and the amount present does not account for the presence of leucine in both CNBr-1 and CNBr-2. The lysine residue may be modified or may have been substituted by some other more labile amino acid as the yield in both peptides is low.

In the preparative separation gels (Fig.29a pp.72), a band marked X with an electrophoretic mobility between H2A and H3 has been constantly observed. A protein with similar electrophoretic mobility was first noticed in Drosophila melanogaster by Alfageme et al., 1974, and has been designated D-2. Palmer et al., 1980, found that D-2 elutes together with H2A from Bio-Gel P-100 but can be distinguished electrophoretically from H2A and the other Drosophila core histones. The amino acid composition of D-2 resembles H2A as well as H2B (Palmer et al., 1980). The Sea Urchin histone observed here may be of the same identity as the Drosophila D-2 protein. This will be investigated at a later stage. The significance of the Drosophila D-2 protein is not known. Palmer et al., 1980, have reported that it is present in nuclei from embryos and adults, and appears not to be restricted to a narrowly defined developmental period.

6.5 Sea Urchin Embryo Histone H4 Variants

Histone H4 variants have been isolated on preparative 4,5 M urea 6 mM Triton polyacrylamide slab gels. The optimum concentration was chosen from 0-9 M urea gradient gel (Fig.13a pp.41). The starting material has been separated from a Johns H2A enriched fraction on Bio-Gel P-60 (Fig. 19 pp. 53). 120 mg histone H4 were applied to 12 preparative slabs, the four fractions marked in Fig. 32a pp. 78 have been excised and the corresponding histone fraction eluted. The recovery per fraction was:

F1 : 6,4 mg
F2 : 6,04 mg
F3 : 3,92 mg
F4 : 2,56 mg

The recovery is low. This is probably due to contamination of the starting material with other slower and faster mobility fractions (Fig. 32a pp. 78). In addition, histone H4 is the smallest of the histones (MW ∿ 11.000) and losses during dialysis in the final recovery step occurs . On analytical 4 M urea 6 mM Triton gels (Fig. 32d pp. 78) and analytical SDS gels (Fig. 32c pp. 78) F1 and F2 have been designated as histone $H4_{(1)}$ and $H4_{(2)}$ respectively. But it is obvious from the Triton gel (lane 2 and 3, Fig. 32d pp. 78) that the separation achieved for the two histones H4 is not very good. The two isohistones have only marginally separated in the preparative gels (Fig. 32a pp. 78). Though the separation achieved for histones $H2B_{(2)}$ and $H2B_{(3)}$ under similar conditions (Fig. 24b pp. 64) was not better, clear identification of distinctly different polypeptide chains had been possible in this case. If the amino acid composition of histones $H4_{(1)}$ and $H4_{(2)}$ is compared to H4 ex Bio-Gel P-60, in Table 5 pp. 79, methionine has apparently been destroyed during gel electrophoretic isolation procedure, but otherwise no clear differences can be detected. On SDS gel, Fig. 32c pp. 78 , the

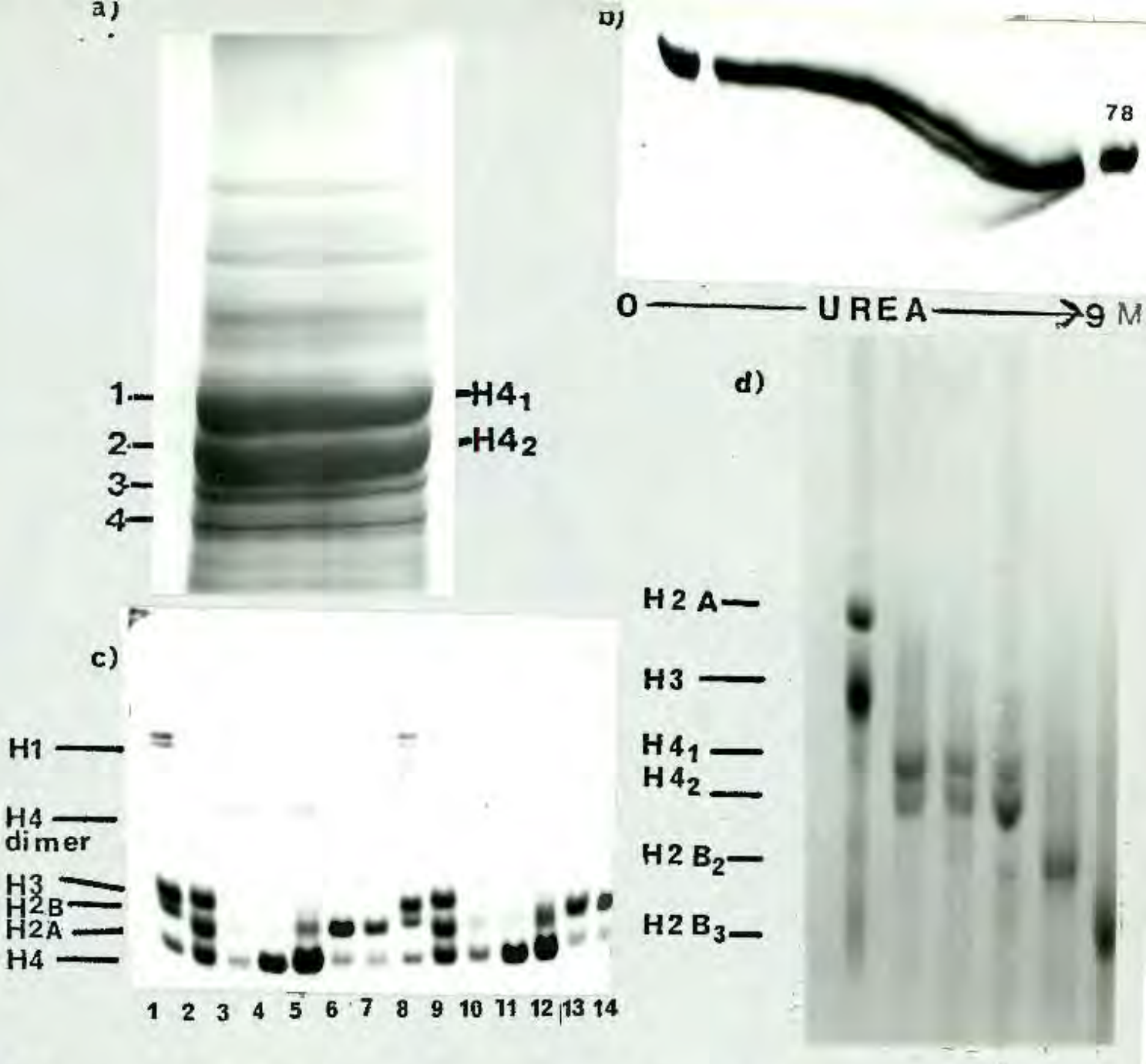


Fig. 32
Preparative Triton-PAGE of Sea Urchin embryo histone H4 and identification of purified histone fractions by analytical PAGE

a) Separation of Sea Urchin Embryo histone H4 isolated by gel filtration by preparative 4,5 M urea, 6 mM Triton PAGE.

b) 0-9 M urea gradient, 6 mM Triton PAGE of histone H4.

c) Analytical SDS PAGE of purified histone fractions
lane 1 and 8: Calf Thymus histone
" 2 and 9: Sea Urchin embryo H2A enriched histones
" 3: Histone H4 ex P-60
" 4: Histone $H4_{(1)}$
" 5: Histone $H4_{(2)}$
" 6: Histone $H2B_{(2)}$
" 7: Histone $H2B_{(3)}$
" 10 - 14: repeat of lane 3-7, except that samples have been reduced with mercaptoethanol.

d) Analytical 4,5 M urea, 6 mM Triton PAGE of purified histone fractions
lane 1: Sea Urchin embryo histones
" 2: Histone $H4_{(1)}$
" 3: Histone $H4_{(2)}$
lane 4: Histone H4(control)
" 5: Histone $H2B_{(2)}$
" 6: Histone $H2B_{(3)}$

TABLE 5 AMINO ACID COMPOSITION OF SEA URCHIN EMBRYO HISTONE H4 VARIANTS

The analyses are expressed in mole %; no corrections have been made for losses due to incomplete hydrolysis.

* results of amino acid composition for Sea Urchin histone H4 purified by extraction from preparative gel

** results of amino acid composition for Sea Urchin histone H4 purified by gel filtration.

A.A.	$H4_{(1)}$ mole %	$H4_{(2)}$ mole %	H4* mole %	H4** mole %
Asn	5,4	5,7	5,3	6,1
Thi	5,6	5,1	6,2	5,3
Ser	3,1	4,9	2,8	3,1
Glu	7,1	8,6	6,4	7,4
Pro	1,7	1,8	0,9	2,0
Gly	16,9	16,8	17,2	15,9
Ala	7,0	8,0	8,9	7,3
Val	7,8	7,6	6,5	7,8
Met	-	-	1,4	0,8
Ile	5,3	5,4	4,6	5,3
Leu	8,1	9,0	8,3	8,1
Phe	3,5	2,6	3,6	3,1
Tyr	2,2	1,3	2,0	1,6
Lys	9,8	9,7	10,3	10,1
His	2,6	2,2	2,7	2,4
Arg	13,5	12,1	12,9	12,1
Lys/Arg	0,7	0,8	0,8	0,9
$\frac{Lys+Arg+His}{Glu+Asp}$	2,0	1,7	2,45	1,8

histone H4 ex P-60, lane 3, and histone $H4_{(2)}$, lane 5, both show an H4 dimer, whereas this is not observed for $H4_{(1)}$. This may indicate the presence of a cysteine in $H4_{(2)}$. The dimer is reduced on addition of mercaptoethanol (lane 10 and 12, Fig. 32c pp. 78) with comcomitant increase in monomer H4. Carboxymethylation with iodoacetate was carried out to confirm existence of cysteine. The reaction product, S-carboxymethylcysteine is stable under hydrolysis conditions, and can be quantitated by amino acid analysis (Means and Feeney, 1971). Both isohistone fractions contain carboxymethylcysteine. However, this result may be due to cross-contamination mentioned earlier.

In Sea Urchin sperm histone H4, a substitution of threonine at position 73 by cysteine has been reported previously (Strickland et al., 1974; Wouters-Tyrou et al., 1976). It may be that the two isohistones from the Sea Urchin embryo differ from each other only in this position. If partial oxidation of a single methionine residue during the isolation procedures is the cause for the electrophoretic difference of the two fractions, then one could expect that a similar electrophoretic difference would be noticible in Calf Thymus histone H4, (Fig. 10 pp.36). The latter should split at a critical urea concentration into two fractions (i.e. the partially oxidized and the intact methionine containing fraction). However this is not the case. It is planned to investigate the presence of both cysteine and threonine at position 73, and a mixture of H4 will be carboxymethylated and then subjected to N-bromosuccinimide (NBS) cleavage (Brandt and von Holt, 1972). Sperm histone H4 has 4 tyrosine residues, one of them located at position 72, and therefore sequencing of resulting peptides from NBS cleavage should give an indication of the presence of either cysteine and/or threonine (at position 73).

CHAPTER 7

Conclusions

With the recognition that histones play more than a simple structural role in chromatin, it was desirable to investigate the histone complement of a wide variety of organisms on the evolutionary scale. PAGE lends itself to such an investigation, as one can examine changes in mobility and quantity of specific histone fractions. Panyim and Chalkley (1969) developed the acid-urea polyacrylamide gel system which distinguishes the five major histone groups in Calf Thymus. This system has since been adopted in many laboratories and has been used as a criteria to judge "homogeneity" of purified histone fractions as well as a reference for assigning histones to their respective classes according to their characteristic electrophoretic mobility. On the basis of their electrophoretic studies, Panyim and Chalkley (1971) maintained that histones H3 and H4 have electrophoretic mobilities, independent of their origin and histone H2A and H2B show only small changes in mobility. Support for their findings for the existence of histone variants of the H2A and H2B type by these criteria, comes from a number of electrophoretic studies by Bentinen and Comb (1971); Hill et al., 1971; Ruiz Carillo and Pallau (1973); Taub and Boeckman (1978) and Ozahi (1979).

There are, however, certain reservations and the only unequivocal proof for homogeneity of a certain histone fraction is its amino acid sequence, as it has been found that histones with identical electrophoretic mobility may nevertheless have different amino acid sequences. Triton X-100 PAGE has improved the detection of histone variants. Whereas the acid-urea system distinguishes between size and charge ratio, Triton X-

100 PAGE recognizes a difference according to size, charge and helical content, (Zweidler, 1972 ; Cohen et al., 1975; Hoffman and Trauben, 1978 ; Treigyte and Gineitis, 1979). The presence of Triton has made it possible to resolve isohistones, resulting from the substitution of a single neutral amino acid. No difference was detected between Calf Thymus and Chicken Erythrocyte histone H3 on acid-urea gels, sequence studies however, revealed a cysteine → serine substitution on histone H3 (Brandt et al., 1979). This difference can now be picked up with Triton-PAGE (Fig. 11, pp. 36). In the Triton acid-urea gel system, two factors contribute to the extent of Triton binding, which result in improved resolution of histone variants. One is the effect of urea on the helical region which leads to reduced Triton binding (Fig. 13a pp. 41), and the other is the increase in CMC of Triton with increasing urea concentration (Fig. 12 pp. 38). It is important to establish a Triton concentration which is high enough to maintain a critical CMC at all urea concentrations, such that the observed difference in mobility is only due to the effect of urea on the helical regions and not due to the limiting micellar Triton concentration. The urea gradient Triton gel, Fig. 13a pp. 41 , clearly demonstrates the improved resolution of histone variants, but it also shows pitfalls which can arise. A good example is the behaviour of Sea Urchin histone H2B and H4. At 0-5 M urea $H2B_{(2)}$ and $H2B_{(3)}$ have a faster electrophoretic mobility compared to histone H4, whereas histone $H2B_{(1)}$ moves between histone H3 and H4, at 7-9 M urea histones H2B now move slower than histone H4, this is also the order in which Calf Thymus and Chicken Erythrocyte histones H2B and H4 move, (Fig. 11 pp. 36). For the histones of these two organisms, no cross over is observed at any urea concentration contrary to the Sea Urchin histones (see above). Although the separation of histone variants from each other in Triton-PAGE is very good, there is the danger of wrong identification of a

histone due to an artefactual modification, e.g. oxidation of methionine (Fig. 10 pp. 34) as the result of the isolation procedure.

The preparative Triton gel system adopted is limited to certain dimensions of the slab for reasons mentioned in Chapter 2 . This restricts the amount of histone mixture to be separated. This limitation requires an additional purification step after the Johns selective extraction on Bio-Gel P-60, prior to the separation on the preparative gel. This is necessary as otherwise, two consecutive gel fractionations at different urea concentrations would become necessary to isolate variants. It is important to weigh the degree of homogeneity necessary for the particular purpose against the optimum yield. For sequence analysis, a certain level of contamination can be tolerated, as sequence analysis itself is not sensitive enough to pick up less than 5% impurities.

For most histones, the exclusion chromatography purification on P-60 is advantageous, particularly in combination with the analytical Triton-PAGE of the chromatographic fractions. The identification of the composition of chromatographic peaks becomes much more sensitive and allows a more precise pooling of fractions, Fig. 19 pp. 53.

This preparative use of Triton-PAGE has particularly facilitated the isolation of histone H2A and H2B variants. The purity of histone H2A variants can be improved upon by prior Bio-Gel P-60 purification, as this markedly reduces the contamination level due to adsorption of histones to acrylamide (Fig. 16 pp.47). Histone H2A is particularly affected by this source of contamination as it is the slowest moving histone. The isola-

tion of histone H2B variants poses no problem as long as histone H4 is not present, as it travels between $H2B_{(1)}$ and $H2B_{(2)}$. Sea Urchin isohistones H3 and H4, highly conserved, as judged from the urea gradient gel, are not easily separated at any particular urea concentration. The composition of the starting material for histone H3 variant separation is not as critical as the urea concentration, chosen for its isolation; most other histone groups are more than a bandwidth away. However, isolation of H3 variants itself is difficult, (Fig. 29 pp. 72). Histone H4 is easily obtained without contaminants from a Bio-Gel P-60 fraction, but variant separation is as difficult (Fig. 32 pp. 78), as in the case of histone H3.

The preparative PAGE methodology developed for Sea Urchin embryo histones allows unequivocal identification of protein fractions with different electrophoretic mobilities. It is anticipated that the detailed analysis of the biological significance of isohistones, particularly their contribution to the chromatin architecture, will be aided by these procedures.

CHAPTER 8

MATERIALS AND METHODS

8.1 Isolation of Histones by Preparative Polyacrylamide Gel Electrophoresis (PAGE)

8.1.1 Preparative PAGE

8.1.1.1 Stock Solutions

A : 60% acrylamide (w/v)

0,4% N,N' methylene bisacrylamide (w/v) in H_2O

B : 43,2% glacial acetic acid (v/v) in H_2O

4% Tetramethylethylenediamine (TEMED) (v/v) in H_2O

C : Urea, always make up fresh, variable molarity according to the different histones to be separated.

D : 10% Triton X-100 (w/v) in H_2O

E : 0,1% Riboflavin (w/v) in H_2O

F : Sample Application Buffer

8 M urea in 50 mM trihydroxymethylaminomethane (TRIS) adjusted to pH 8,5 with dilute HCl

1% mercaptoethanol (v/v)

0,001% pyronin Y (w/v) (as a tracking dye)

(made up fresh each time or store frozen)

G : Tray buffer

0,9 M acetic acid in H_2O

H : Staining Solutions

0,1% Amido Black (w/v)

7% acetic acid (v/v) in H_2O

and

0,25% Coomassie Brilliant Blue (w/v)

50% methanol (v/v)

10% acetic acid (v/v) in H_2O

I : Destaining Solutions

7% acetic acid (v/v) in H_2O

and

25% ethanol (v/v)

10% acetic acid (v/v) in H_2O

J : 70% formic acid (v/v) in H_2O

K : Dowex 1 x 8, mesh 50-100

50 g Dowex in a 250 ml conical flask, is washed twice with distilled H_2O and twice for 10 minutes with 500 ml 1 M NaOH. This is followed by a wash with distilled H_2O until the pH is neutral. The Dowex is then equilibrated with 40% formic acid, and packed into a column (1 x 20 cm), and washed with a further 2 column volumes of 40% formic acid.

8.1.1.2 Apparatus

The apparatus and its assembly are depicted in Fig.33 pp.88. The same equipment is used for both analytical and preparative gel electrophoresis. The glass plates (0,5 x 12 x 25 cm) and teflon spacers forming the gel shell are held in place with fold back clips. The spacers (especially their joining corners) are sealed from the outside with molten agar. Glass plates are used as they possess a greater affinity for the gel, as do apolar materials, such as Perspex (Maurer, 1974). Adhesion of gel matrix to supporting walls is important, as we are dealing with a larger gel mass in preparative gels,and inadequate adhesion will lead to creeping currents with resulting curved bands. The power supply used is a Shandon Model Power Supply, with a variable voltage(0-400V)and current(0-80 mA)output.

8.1.1.3 Gel Preparation

Acetic acid urea gels were made according to Panyim and Chalkley (1969), with the modification that Triton X-100 (Zweidler, 1972) was added. Gels were composed of 15% acrylamide 2/v, 0,1% N,N' methylene bisacrylamide w/v, 5% acetic acid v/v, 0,5% TEMED v/v and 0,375% Triton X-100 w/v. This gel mixture is also suitable for electrophoresis in the absence of Triton X-100. The urea concentration was varied to optimize the separation of the different histones. For a gel shell of the dimensions 0,17 x 10 x 20 cm, 40 ml of gel solution was prepared. Thiodiglycol was added to final concentration of 0,1% to prevent oxidation of methionine during the run (Zweidler, 1978). To initiate the polymerization of the gel riboflavin (5 µl/ml gel) was needed. The ready gel monomer mixture is poured gently into the glass shell under subdued light, a teflon comb is carefully inserted, trying to avoid bubbles at the interface. Normal light conditions in the laboratory are adequate to initiate the riboflavin mediated photoactivated polymerization in about 15 minutes.

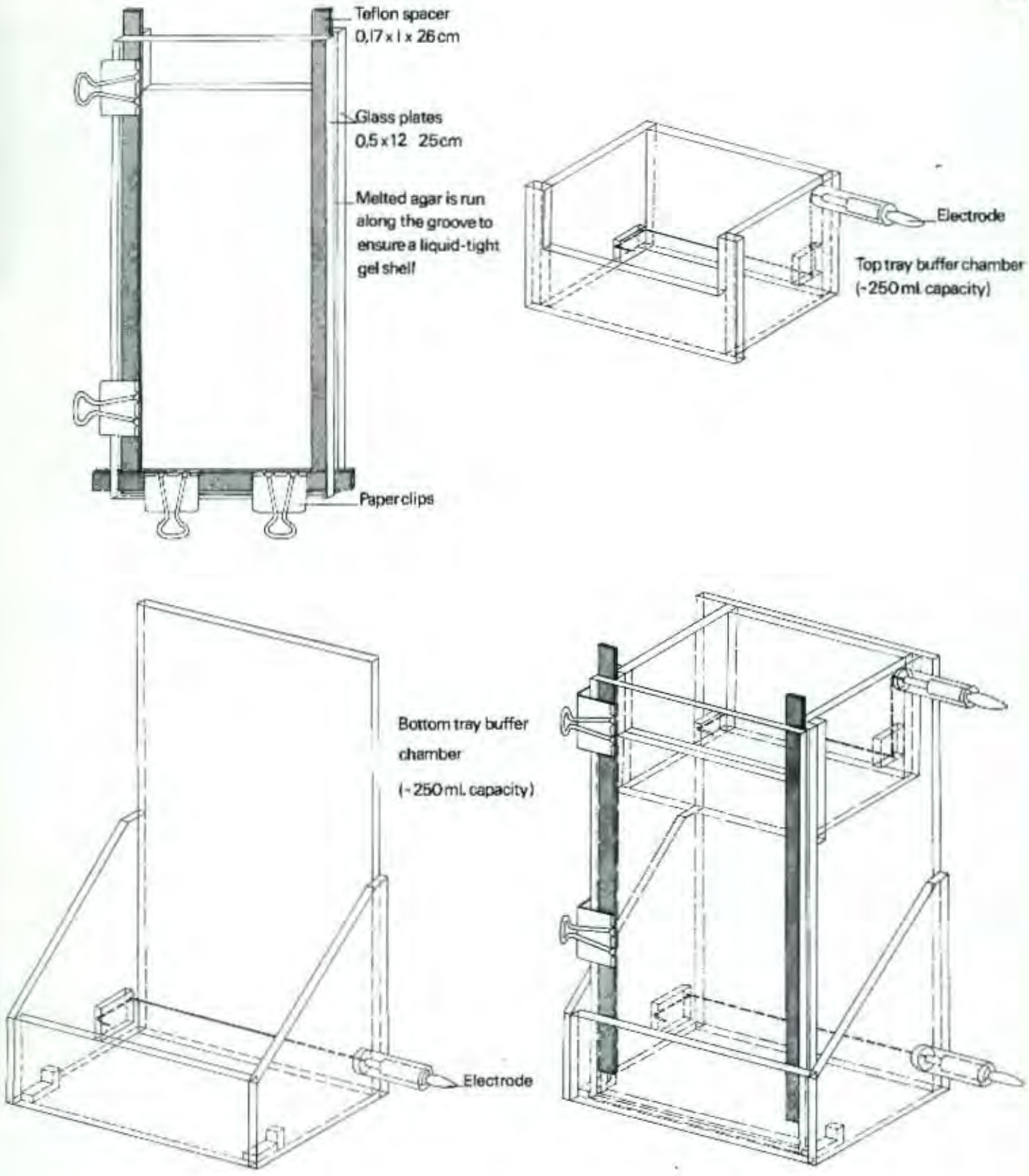


Figure 33 Analytical and Preparative Slab Gel Electrophoresis Apparatus

If delays are observed, the cast gel may also be placed on a sunny window sill (or a light box on rainy days). After polymerization is complete, (about ½ hour), the comb is carefully removed and the sample slot is rinsed out with tray buffer in a hypodermic syringe to remove unset gel and back diffusing urea. Setting with riboflavin makes pre-electrophoresis unnecessary, which is an advantage, as the sample tends to swell during pre-electrophoresis, and urea will diffuse out of the gel. Thus electrophoresis should start straight after the comb has been removed.

8.1.1.4 Sample Preparation

The amount of sample to be applied is in the region of 5-10 mg per slab, depending on the separation, and the number of histones present in the sample. The histones are dissolved for 0,5 h in 8 M urea, 1% mercaptoethanol, 50 mM TRIS-HCl, pH 8,5. Just prior to the application, the pH is dropped to 2 with dilute HCl. The samples are applied with a Hamilton syringe after the assembly of the apparatus. As the sample is in a solution of high density, it is underlayered under the upper buffer.

8.1.1.5 Electrophoresis

The buffer chambers and the glass shell are assembled as shown in Fig.33 pp. 88. The tray buffer is 0,9 M acetic acid. Preparative electrophoresis is carried out overnight for 16-17 hours at 10 mA constant current. The low current applied minimises heating and the separated bands move as straight lines.

8.1.1.6 Staining and De-staining

After electrophoresis the gel, removed from its glass shell via floating, is stained with Amido Black for 5 minutes and destained in 7% acetic acid. The acid is changed regularly, until the bands are clearly visible some hours later. During this time, urea and Triton diffuse out, which facilitates the subsequent recovery of the separated fractions.

8.1.1.7 Recovery of Histones from Gels

After partial destaining, the gel is placed on a light box. The Amido Black stained bands are excised with a sharp knife and placed in a conical flask. In a typical experiment, 4 preparative Triton X-100 polyacrylamide slab gels are run overnight and the corresponding gels (approximately 10 ml of gel per protein fraction) are combined and homogenised with an Ultra Turax in 70% (v/v) formic acid. The homogenate is finally made up to 50 ml. The gel slurry is shaken overnight at +4°C and filtered through Whatman No.1 filter paper. The filtrate which contained the Amido Black-histone mixture, is poured onto a column (1 x 20 cm) of Dowex 1 x 8 mesh, 50-100, previously equilibrated with 40% formic acid. The Amido lack is retained by the resin and the protein elutes with the formic acid (Wada and Snell 1972). The eluate is first dialysed against distilled H_2O and the freeze dried. The freeze dried sample is then dissolved in 2-3 ml 0.1N H_2SO_4 and precipitated with 6 volumes acetone. The precipitate is collected by centrifugation and washed twice with acetone. It is then redissolved and freeze dried again to result in a white fluffy power of histone sulfate.

8.2 Analytical Polyacrylamide Gel Electrophoresis of Sea Urchin Embryo Histones

8.2.1 0-24 Triton X-100 Gradient Polyacrylamide Slab Gel Electrophoresis Apparatus

Gel gradient maker, a linear Triton X-100 concentration gradient is formed in a two chamber gradient maker. The two chambers are separated by a stop cock, to allow separate filling of each chamber, The connection between the chambers should be as short as possible to avoid air locks.

Magnetic stirrers

Pumps

Connecting tubing

Procedure

The apparatus and the set up is depicted in Fig. 34 pp. . The stock solution listed under 8.1.1.1 are appropriately diluted and a linear 0,24 mM Triton X-100 gradient gel is poured from the following working solutions:

I) 15% (w/v) acrylamide, 0,1% (w/v) N,N' methylene bisacrylamide, 5,4% (v/v) acetic acid, 9 M urea, 0,5% (v/v) TEMED, 0,1% thiodiglycol, 10% (v/v) glycerol.

II) same as under I, but the solution also contained 24 mM Triton X-100 and the 10% glycerol was omitted. 8 ml of each of the polymerization mixture are poured into the two chamber gradient forms (stop cock closed), 10 µl riboflavin (1 mg/ml) is added to each side.

The stirrer is activated and simultaneously the stop cock is opened and the pump activated. The polymerization mixture is pumped into the slab gel shell at 1 ml/min. via connecting tubing, from the gradient chamber

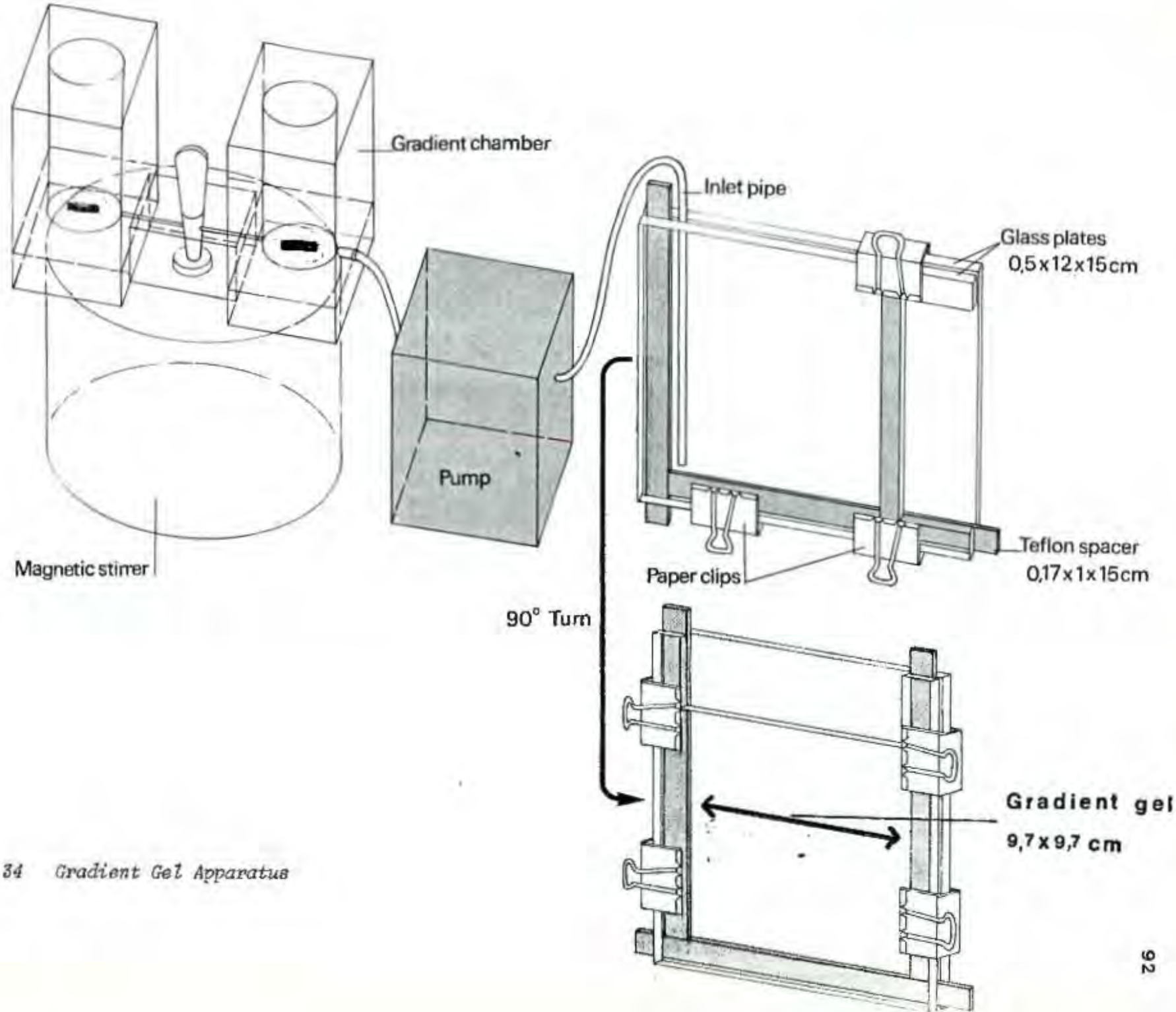


Figure 34 Gradient Gel Apparatus

with the low Triton concentration. The inlet tubing is continuously raised in the slab shell as the level of the gel rises. The illumination level is subdued to avoid polymerization while pouring. The pump delivery speed should not be faster than 1 ml/min in order to avoid mixing of the layers. The polymerization time is about 15 minutes. Solution I is also used for the gel and poured on top of the gradient to form the sample slots. The sample is dissolved for ½ hour in 8 M urea, 1% mercaptoethanol in 50 mM TRIS-HCl, pH 8,5. Just before application, the pH is lowered to 2 with dilute HCl. 100 µl of sample solution (2 mg/ml) is applied to the centre well and 10 µl to the smaller side wells as pilot samples. Electrophoresis is carried out at 16 mA constant current until Pyronin Y has run off completely (about 3½ hours). 0,9 M acetic acid is used as tray buffer. The slabs are stained for a minimum of 3 hours in Coomassie Brilliant Blue and destained until the background is clear.

8.2.2 0-9 M Urea Gradient Polyacrylamide Slab Gel Electrophoresis

The stock solutions are the same as listed under 8.1.1.1. The apparatus and procedure for pouring the gel have been described in 8.2.1. A linear 0-9 M urea gradient slab gel is poured from the following solutions:

I) 15% (w/v) acrylamide, 0,1% (w/v) N,N' methylene-bisacrylamide, 6 mM Triton X-100, 5,4% (v/v) acetic acid, 0,5% (v/v) TEMED and 0,1% (v/v) thiodiglycol.

II) same as under I, except that the solution also contains 9 M urea. The glycerol may be omitted as the gradient is poured by pumping from the high urea concentration. The urea is dense enough for producing a reproducible gradient.

Otherwise the pouring, the setting, the sample application and the running of the gel are the same as in the Triton gradient gel (8.1.2).

8.2.3 Analytical Triton-Acetic Acid-Urea Polyacrylamide Gel Electrophoresis

The stock solutions, the apparatus and the gel composition are the same as for the preparative gel electrophoresis (8.1.1.2). The gel monomer solution is poured, but now a multislot comb is inserted. The samples are dissolved at 2 mg/ml as described before (8.1.1.4) and 5-10 µl are applied depending on the homogeneity of the sample. The tray buffer is 0,9 M acetic acid and electrophoresis is at 10 mA constant current overnight (16-17 hours). The gels can also be run at 20 mA constant current for 8-9 hours, but must then be cooled. A fan placed in front of the gel has been found to be adequate. The analytical gel is stained in Coomassie Brilliant Blue to increase the sensitivity, and destained until the background is clear. Fig. 35 pp.95 shows a comparison between staining with Amido Black and Coomassie Brilliant Blue. The optimum concentration of a total histone mixture for Coomassie stained gels is between 2-5 µg, whereas for Amido Black, 10 µg are needed with a sample well diameter of 0,3 cm.

8.2.4 Analytical SDS Polyacrylamide Gel Electrophoresis

Stock solutions:

a) Running gel

A : 30% acrylamide (w/v)

0,8% N,N' methylene bisacrylamide (w/v) in H_2O

a) Amido Black b) Coomassie

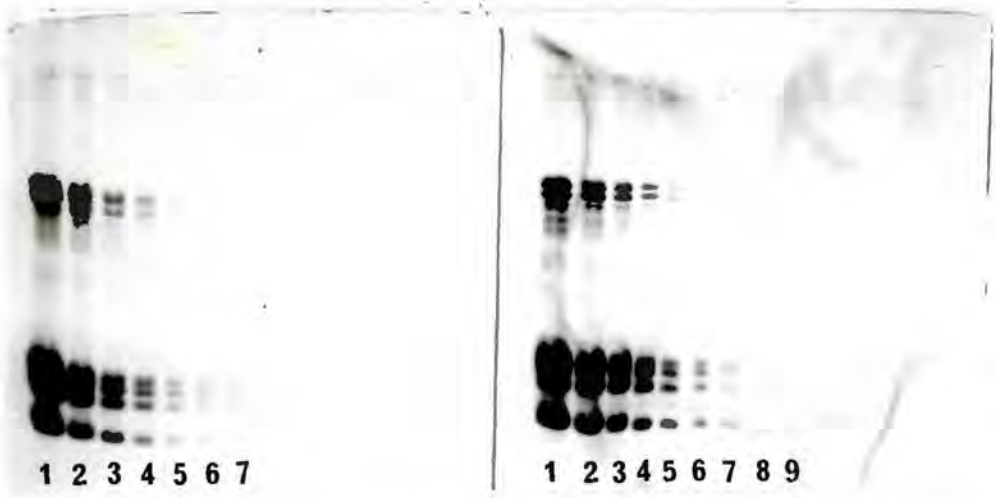


Figure **35** *Staining sensitivity of Amido Black and Coomassie Brilliant Blue*

SDS-PAGE of total Calf Thymus (C.T.) histones;

1	40 µl	C.T. histones dissolved at 1 mg/ml
2	20 "	
3	10 "	
4	5 "	
5	2 "	
6	1 "	
7	10 "	C.T. histones dissolved at 0,1 mg/ml
8	5 "	
9	2 "	

B : 1,125 M TRIS-HCl pH 8,8
0,3% Sodium Dodecylsulfate (SDS) (w/v)

C : 10% ammonium persulfate in H_2O

D : TEMED

b) Stacking gel

A : 30% acrylamide (w/v)
0,8% N,N' methylene bisacrylamide (w/v) in H_2O

B : 0,375 M TRIS HCl pH 8,8
0,3% Sodium Dodecylsulfate (SDS) (w/v)

C : 10% ammonium persulfate (w/v) in H_2O
(make up fresh, or keep frozen)

D : TEMED

Sample Application Buffer

0,0625 M TRIS HCl pH 6,8

2% Sodium Dodecylsulfate (SDS) (w/v)

10% glycerol (v/v)

0,001% bromophenol blue (w/v)

Tray Buffer

0,25 M TRIS-HCl pH 8,3

0,192 M glycine

0,1% Sodium Dodecylsulfate (SDS)

Apparatus

The same as that used for the Triton slab gel (8.2.3)

Gel Preparation

The separating gel is composed of 15% (w/v) acrylamide, 0,4% (w/v) N,N' methylene bisacrylamide, 0,375 M TRIS-HCl pH 8,8 and 0,1% (w/v) SDS. The polymerization is induced with 0,1% (w/v) ammonium persulfate and 0,6% (w/v) TEMED. The gel monomer solution is poured up to a level which leaves enough space for a 1,5 cm spacer gel. The gel is over-layered with water to ensure a straight interface. The setting time is about 15-20 minutes. The stacking gel is composed of 3% (w/v) acrylamide and 0,125 M TRIS-HCl pH 8,8. A multiwell comb is inserted into the stacking gel leaving about 1,5 cm of stacking region before the separating gel. The sample is dissolved at 2 mg/ml in the application buffer 5% (v/v) mercaptoethanol is added to the sample which is then immersed into a boiling water bath and boiled for 2 minutes just before application. Electrophoresis is carried out at 90 volts (constant) for 16-17 hours, or at 200 volts (constant) for 8-9 hours, until the bromophenol blue has run off. The gels are stained in Coomassie Brilliant Blue and destained until the background is clear.

8.3 Amino Acid Analysis

Between 0,2-0,5 mg histones were used for amino acid hydrolysis. The sample was dissolved in 200 µg 2 x distilled HCl (GN) containing 0,25% phenol. The glass tubes were sealed under nitrogen. Hydrolysis was carried out at +110°C for 24 hours. The hydrolysate was analysed with a Beckman amino acid analyser by Miss M. Chauhan from this department.

No corrections were made for incomplete hydrolysis and destruction of residues during hydrolysis.

8.4 Amino Acid Sequencing

Sequence analysis was carried out by Dr. Brandt from this department, using automated sequential Edman degradation (Edman, P. and Begg, G., 1967), on a Beckman sequencer. Analysis of the Pth-amino acids was by high liquid pressure chromatography, HPLC, using a Hewlett Packard Model 1080 A liquid chromatograph. The separation of Pth-amino acids was on a Merck LiChrosorb RP-18 column, 4,6 x 250 mm with a particle size of 10 µm using a 5-48% methanol gradient (Strickland et al., 1978). The Pth-Lys, Pth-Phe and Pth-Ile were not resolved under these conditions and an isocratic run using 50% methanol was necessary to separate them, (Strickland et al,m 1980). The residues were quantitated for each step and are presented using a computer drawing routine (Brandt et al., 1980). Blocked histones were first subjected to Cyanogen bromide cleavage (CNBr) (Gross, E., 1967) with 70% formic acid, either inside the cup (Brandt et al., 1979) or outside the cup (Brandt et al., 1974) depending on how much sample was available for sequencing. A special peptide program was used to analyse the sequence of the CNBr - derived peptides.

8.5 Photography

The film used is Kodak Technical Pan (speed ASA 100).
Gels are placed on a light box The loaded camera, with a 50 mm macro lens is mounted onto a copy stand with a red filter attached to the lens. Exposures are generally in the order or 1/30 sec. at 16. The developer is Kodak HC 110, and is diluted 1 to 9 with deionized water. At 20°C, developing time is 8 minutes. The stop bath consista of 2,5% (v/v) acetic acid (30 seconds), and the film is fixed with M and B Amfix, diluted with 1 to 3 parts deionized water (2 minutes). The film is washed for 15 minutes and then dried.

The printing is done of Ilford photographic paper (Ilospeed, 3.1 M). This is a soft paper and has been chosen to bring out even the faintest bands of interest as well as contaminating bands. Often, however, this is done at the cost of increasing the general background when contrast is not very high or gels have not been fully destained.

8.6 Fractionation of Sea Urchin Histones

8.6.1 Preparation of Sea Urchin Embryo Histone Fractions

8.6.1.1 Solutions

-Ca^{2+}, Mg^{2+} - free sea water

NaCl 28 g

KCl 0,8 g

$NaHCO_3$ 0,2 g

0,01% EDTA

Make up to 1 litre with H_2O and adjust pH to 8,2 with 1 M NaOH.

-0,5 M Sucrose, 10 mM TRIS-HCl pH 7,5

171,15 g sucrose

1,21 g TRIS

Make up to 1 litre with H_2O and adjust pH to 7,5 with HCl

-0,02 M NaCl, 0,01 M $MgCl_2$, 1% Triton, 0,02 M TRIS-HCl pH 7,5

1,16 g NaCl

2,03 g $MgCl_2$

2,42 g TRIS

100 ml 10% Triton X-100

Make up to 1 litre with H_2O and adjust to pH 7,5 with HCl

Saline Sodium Citrate (SSC)

0,15 M NaCl	8,77 g
0,015 M tro sodium citrate	4,41 g
thiodiglycol TDG	1 ml

Make up to 1 litre with H_2O

10% Perchloric Acid (PCA) (w/v)

143 g of 70% PCA

Make up to 1 litre with H_2O

1,25 M HCl

142,5 g of 32% HCl

Make up to 1 litre with H_2O

0,25 M HCl in Ethanol

1 part 1,25 M HCl : 4 parts absolute ethanol

0,25 M HCl

1 part 1,25 M HCl : 4 parts distilled H_2O

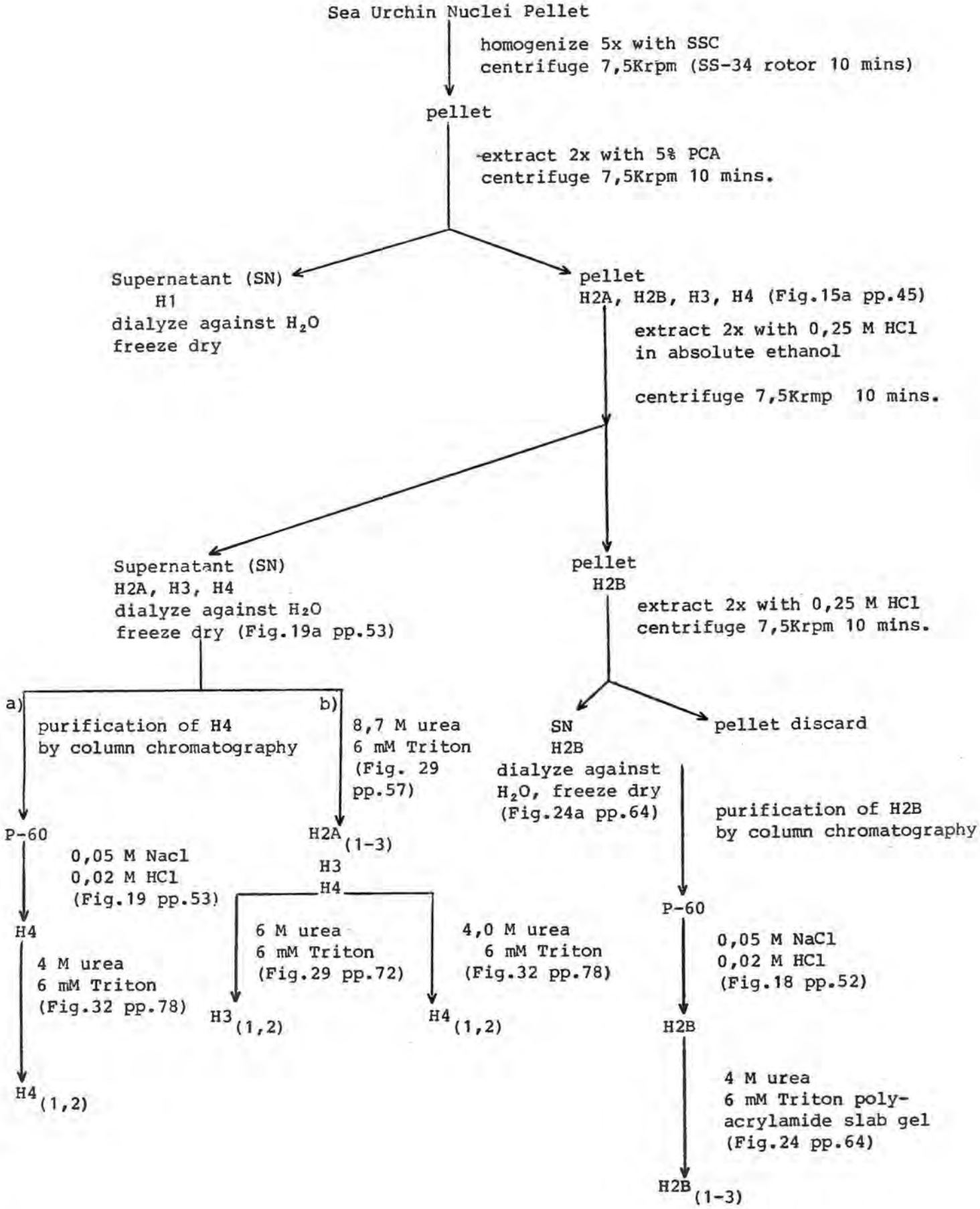
8.6.1.2 Isolation of Sea Urchin Nuclei

Sea Urchin nuclei were prepared according to Keichline and Wasserman (1977). The eggs were collected from female sea urchin by injection of 0,5 M KCl and washed twice with Millipore filtered fresh sea water. 10 ml of the egg suspension was centrifuged in a calibrated centrifuge tube to determine the packed cell volume. 4 ml of packed eggs were transferred to 100 ml of sea water, 10 mg penicillin and 5 mg streptomycin per 100 ml. For fertilization, 100 µg of fresh sperm was added for 100 ml of egg suspension. The growing embryos were kept in suspension by gentle stirring and aeration. Success of fertilization was monitored by the appearance of the fertilization membrane with a phase contrast microscope. The embryos were grown for 24 hours at 22°C, at which time they had reached the late gastrula stage. The embryos were settled by the addition of 3 ml of 10% sodium dodecylsulfate (SDS) per 10ℓ of sea water. They were washed five times with Ca^{2+}, Mg^{2+} - free sea water containing 0,1% EDTA and centrifuged for 10 minutes. The suspension was then homogenized with about 10 strokes in a Dounce homogenizer in 0,5 M sucrose, 10 mM Tris pH 7,5, at a concentration of 1:20 relative to packed cell volume to result in a complete deintegration of the embryo into cells. The cells were in turn lysed by adding an equal volume of 20 mM NaCl, 10 mM $MgCl_2$ in 1% Triton X-100. The lysis of the

cells was monitored with a phase contrast microscope. The nuclei were pelleted at 2000 rpm for 10 minutes. At this stage the nuclei can be stored in liquid nitrogen.

8.6.1.3 *Extraction of Histones from Nuclei*

Sea Urchin Histones were isolated as subfractions using Johns' selective extraction procedure (Johns, E.W., 1964). The following flow diagram indicates the procedure and shows the resulting starting fractions and the conditions for further purification of the histone variants on preparative Triton polyacrylamide gel electrophoresis.



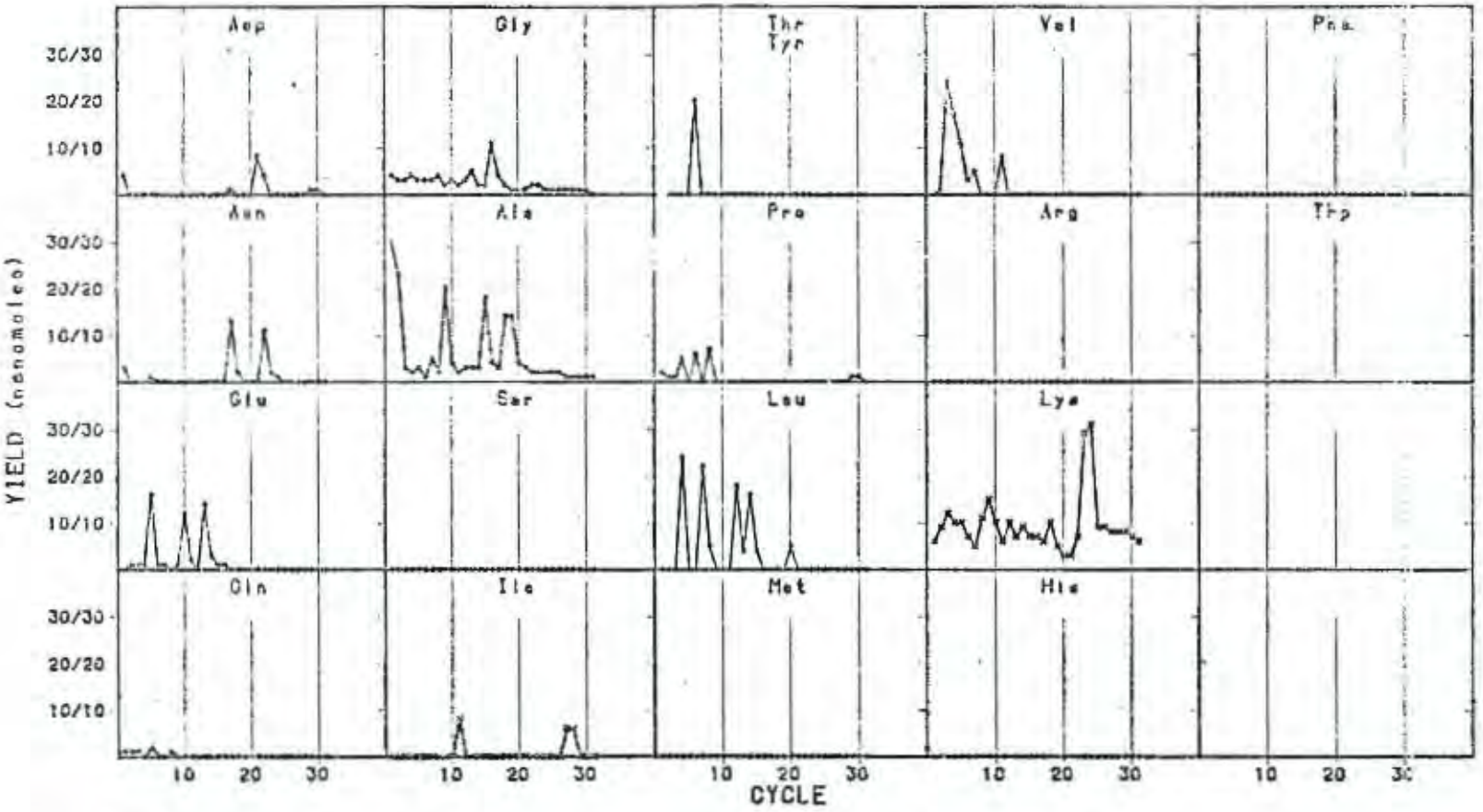
APPENDIX

Figure 36 *Recovery of phenylthiohydantoins (PTH) amino acids per Sequencing step.*

Analysis of PTH-amino acids by high pressure liquid chromatography (HPLC) (●——●) yield, changes in yield are indicated by a break in the connecting line. The separation of PTH-amino acids is achieved on a Merck Li-Chrosorb RP-18 column using a 5-48% methanol gradient (Strickland et al., 1980). The PTH-Lysine and PTH-isoleucine are not resolved under those conditions, they are however indentified in an isocratic run at 50% methanol (X——X).

A) Sea Urchin Embryo Histone H2A3 CNBr2 Peptide

Ala-Ala-Val-Leu-Glu-Tyr-Leu-Thr-Ala-Glu-Ile-Leu-Glu-Leu-Ala-Gly-Asn-Ala-Ala-Arg-
Asp-Asn-Lys-Lys-Thr-Arg-Ile-Ile-Pro-Arg-His-

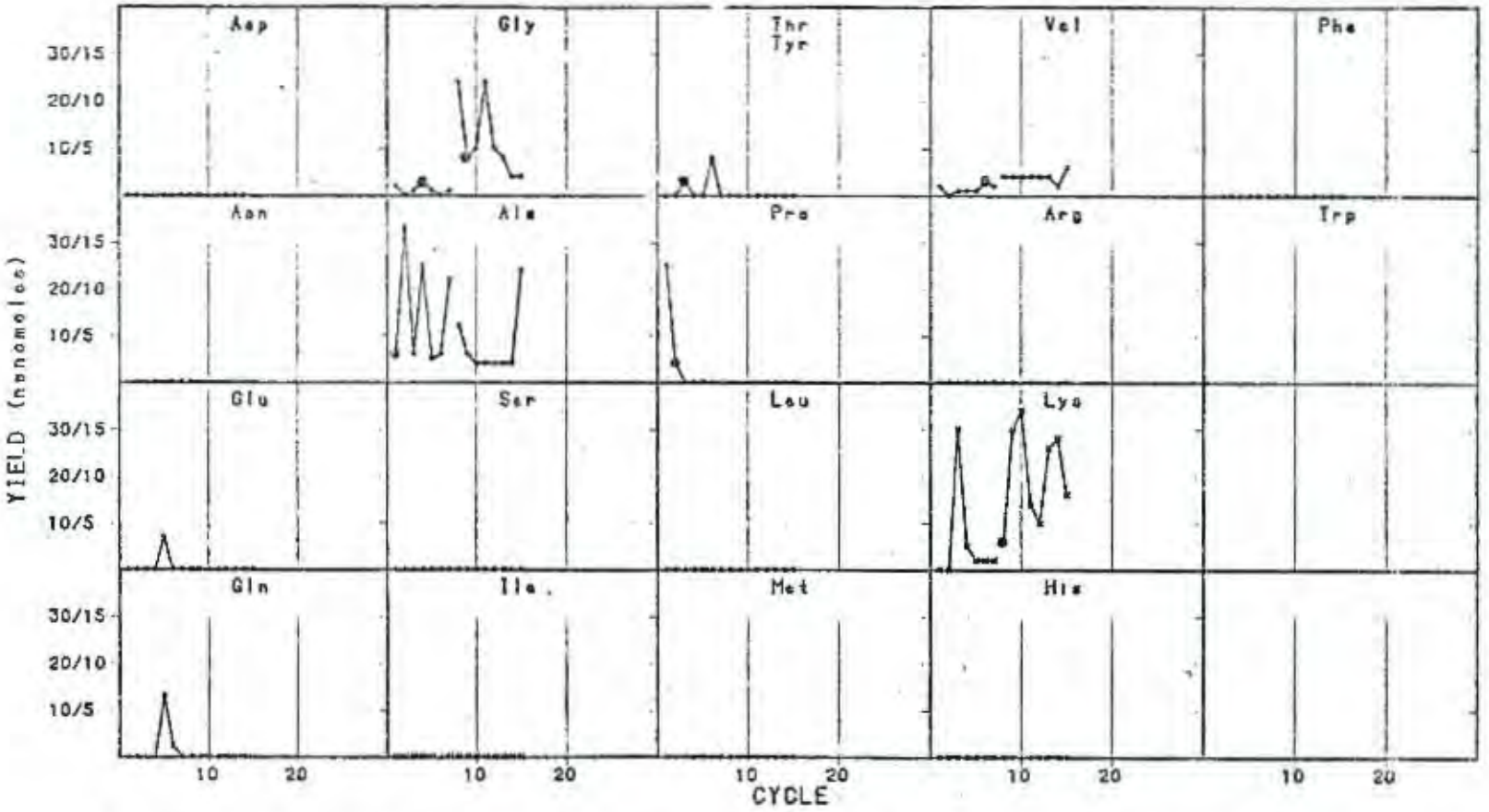


Yields of PTH-amino acids recovered by degrading Sea Urchin Embryo $H2A_{(3)}$ histone variant after CNBr cleavage using the peptide program.

B) Sea Urchin Embryo Histone $H2B_1$ N-Terminal Sequence

Pro-Ala-Lys-Ala-Gln-Thr-Ala-Gly-Lys-Lys-Gly-Ser-Lys-Lys-Ala
(5, 10, 15)

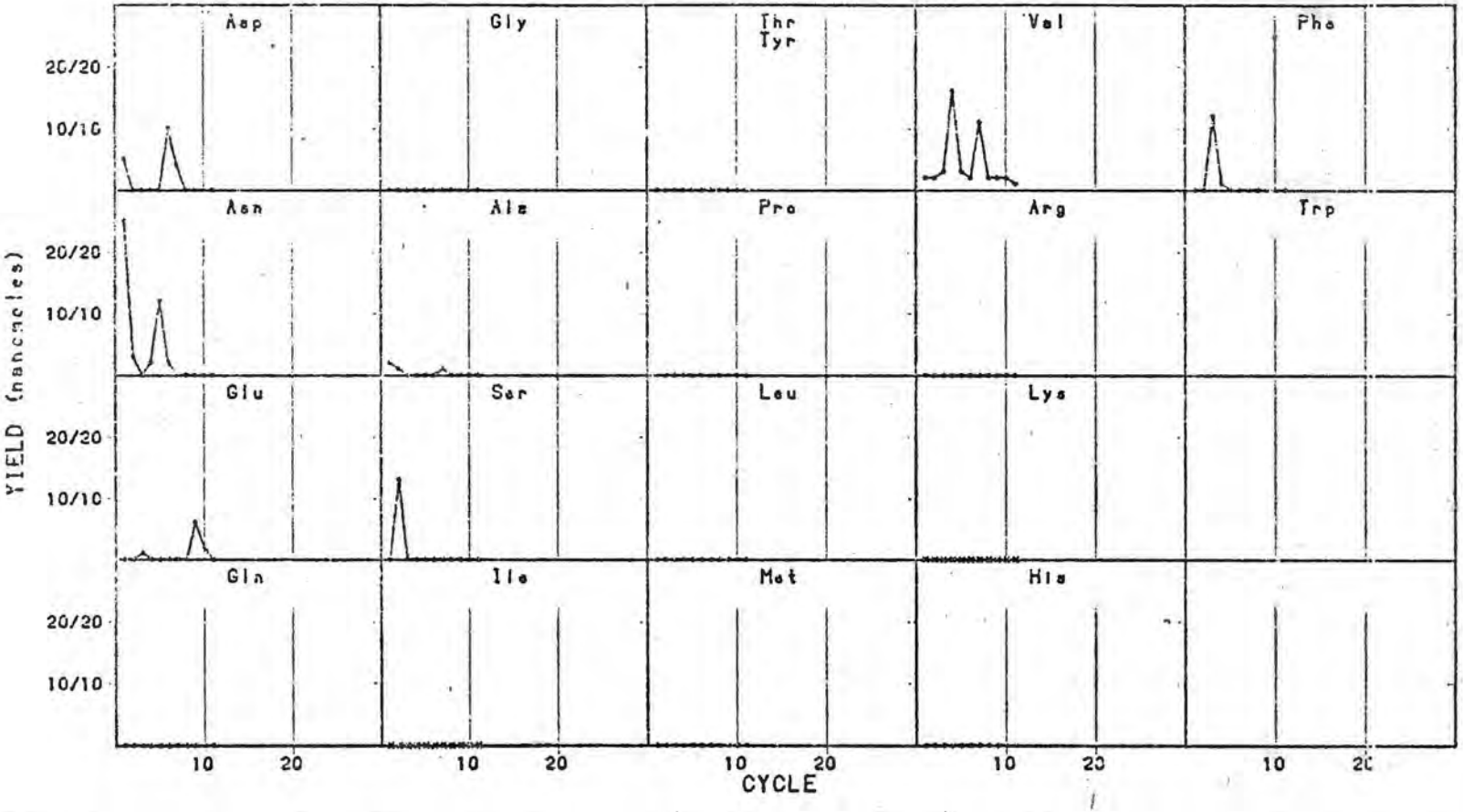
Ala-Pro-Thr-Gly- (5) -Val- -Lys-Gly



Yields of PTH-amino acids recovered by degrading Sea Urchin Embryo $H2B_{(1)}$ histone variant. The heavy dots (O) represent the minor contaminating sequence of Sea Urchin $H2B_{(2)}$ histone variant

C) Sea Urchin Embryo Histone $H2B_1$ CNBr2 Peptide.

-Asn-Ser-Phe-Val-Asn-Asp-Val-Phe-Glu-Arg-
5 10



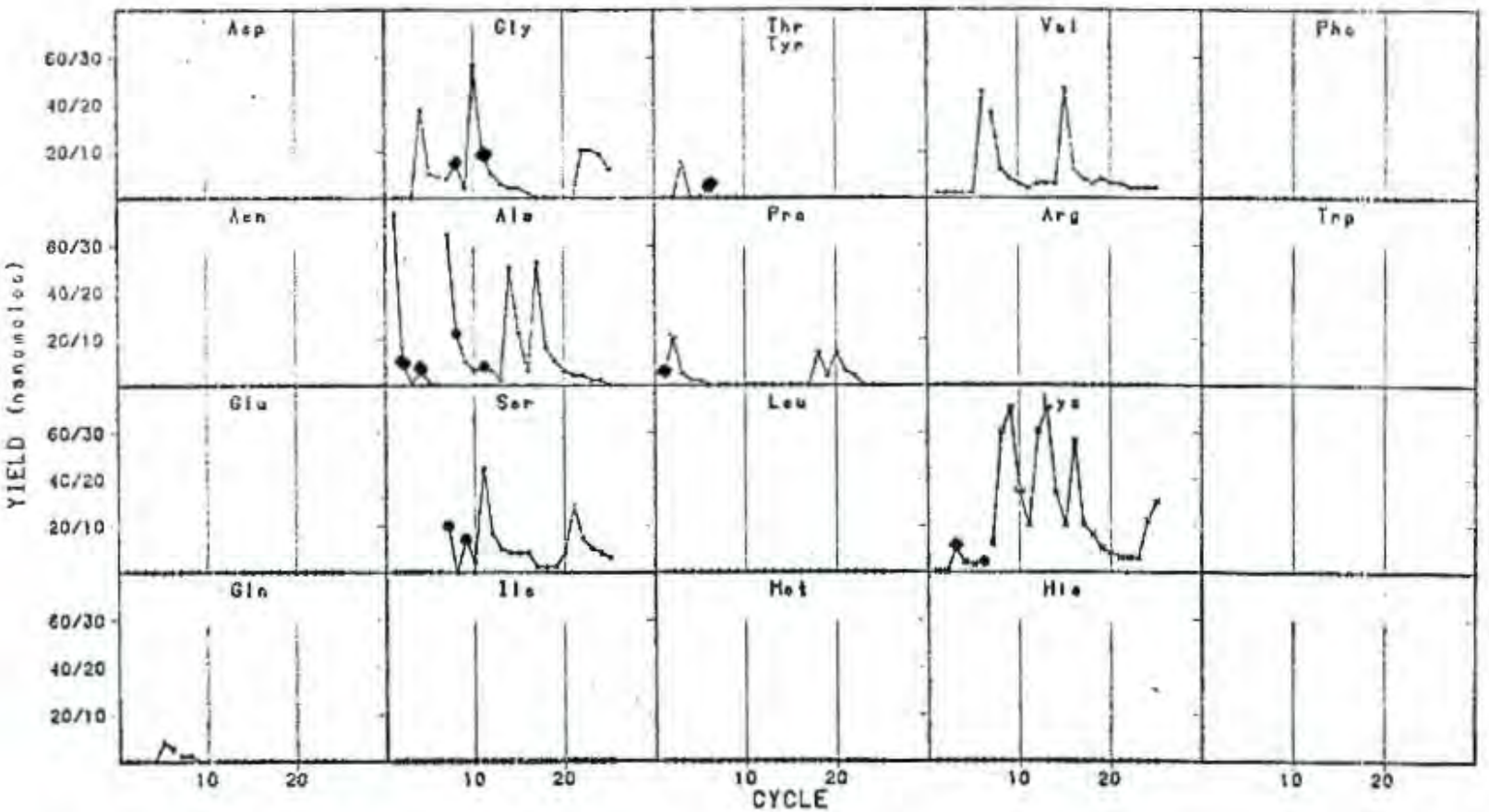
Yields of PTH-amino acids recovered by degrading Sea Urchin Embryo $H2B_{(1)}$ histone variant after CNBr cleavage using the peptide program.

D) Sea Urchin Embryo Histone $H2B_2$ N-Terminal Sequence

Ala-Pro-Thr-Gly-Gln-Val-Ala-Lys-Lys-Gly-Ser-Lys-Lys-Ala-Val-Lys-Ala-Pro-Arg-Pro

Pro-Ala-Lys-Ala-Thr- -Gly- -Lys-Gly-

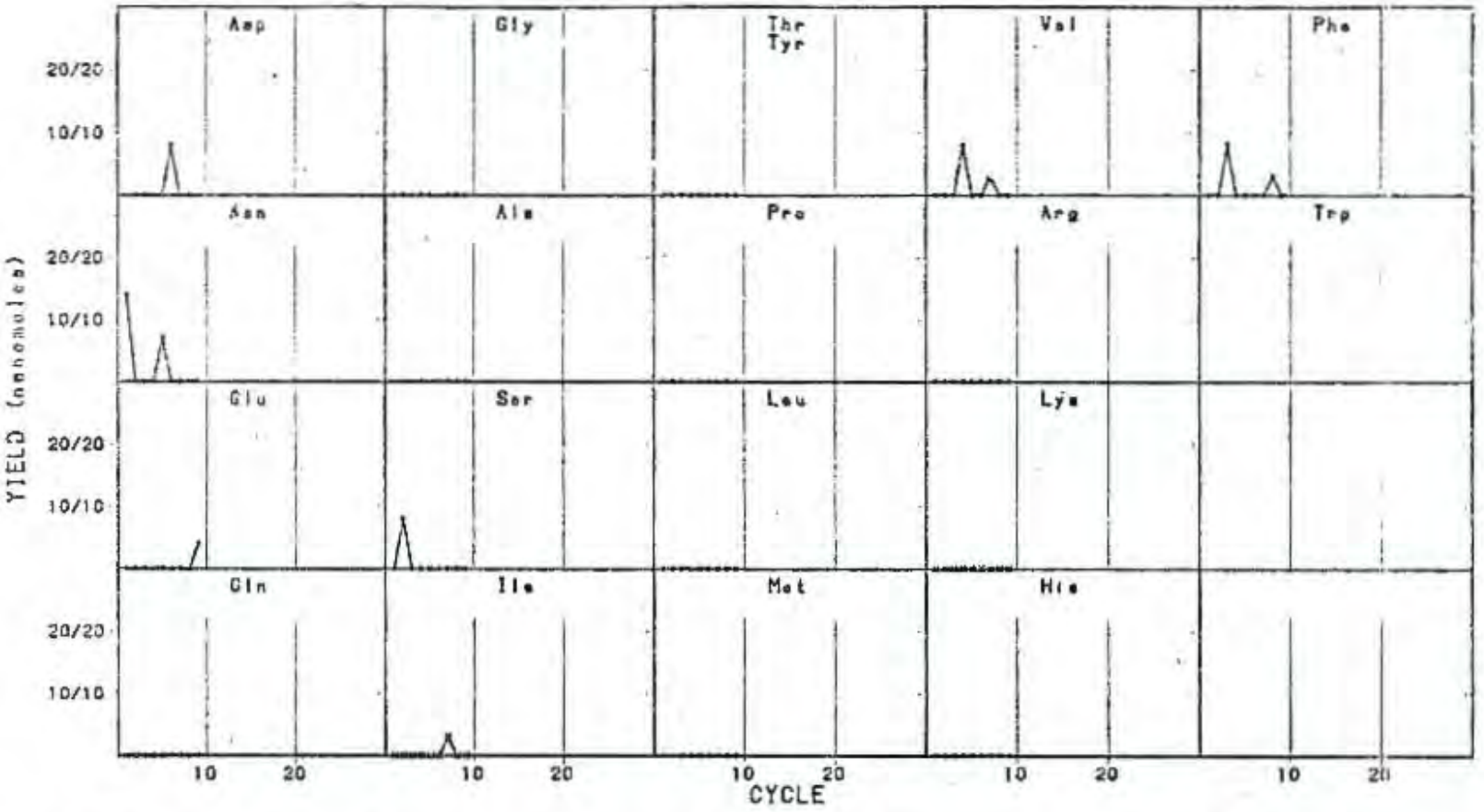
Lys-Ser-Ala-Ser- -Ala-



Yields of PTH-amino acids recovered by degrading Sea Urchin embryo $H2B_{(2)}$ histone variant. The heavy dots (●) represent contamination with a minor unknown component, the squares (◆) represent contamination with $H2B_{(3)}$ histone variant.

E) Sea Urchin Embryo Histone $H2B_2$ CNBr2 Peptide.

-Asn-Ser-Phe-Val-Asn-Asp-Ile-Phe
5

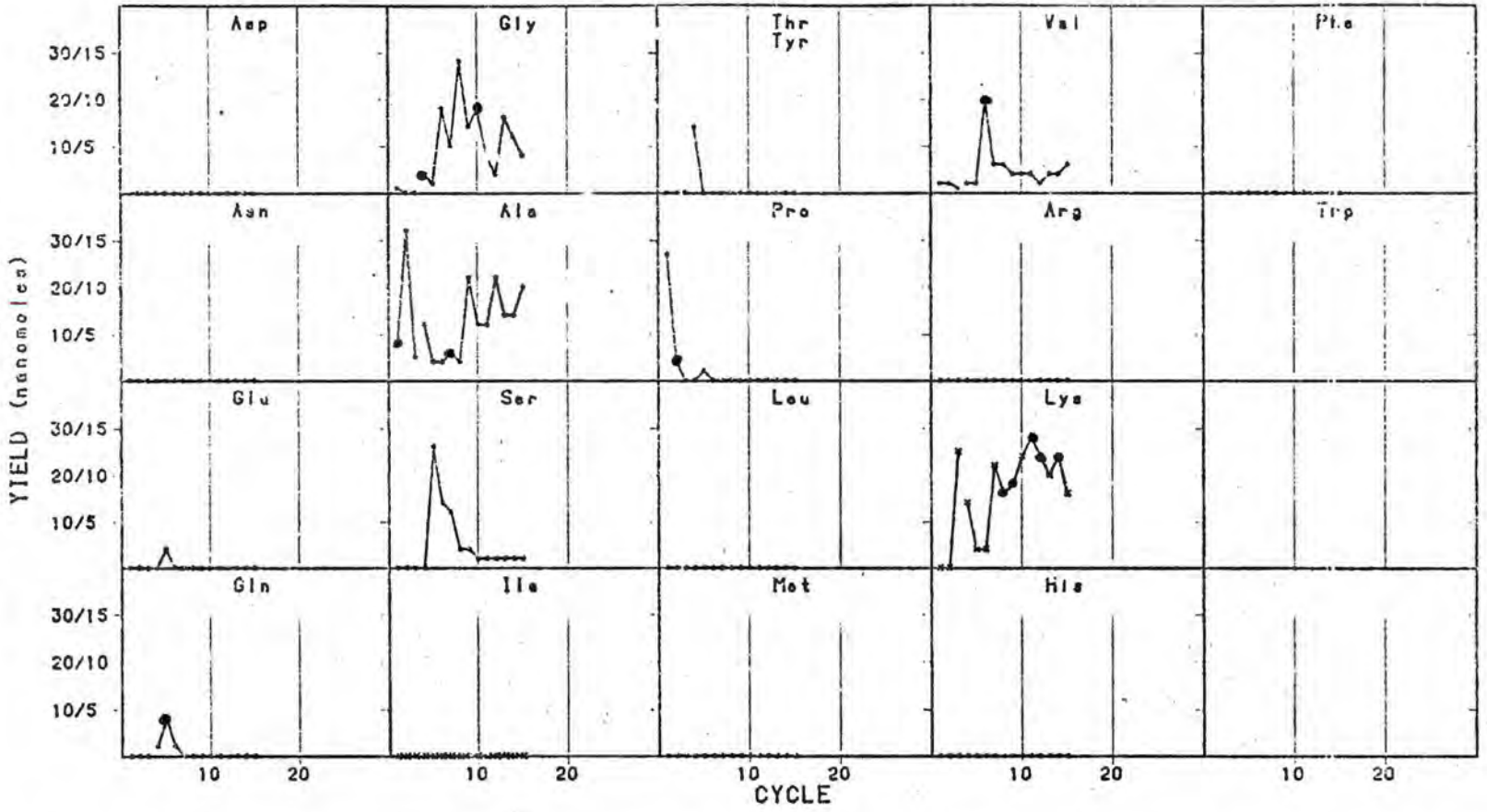


Yields of PTH-amino acids recovered by degrading Sea Urchin embryo $H2B_{(2)}$ histone variant after CNBr cleavage using the peptide program.

F) Sea Urchin Embryo Histone $H2B_3$ N-Terminal Sequence

Pro-Ala-Lys-Thr-Ser-Gly-Lys-Gly-Ala-Lys-Lys-Ala-Gly-Lys-Ala

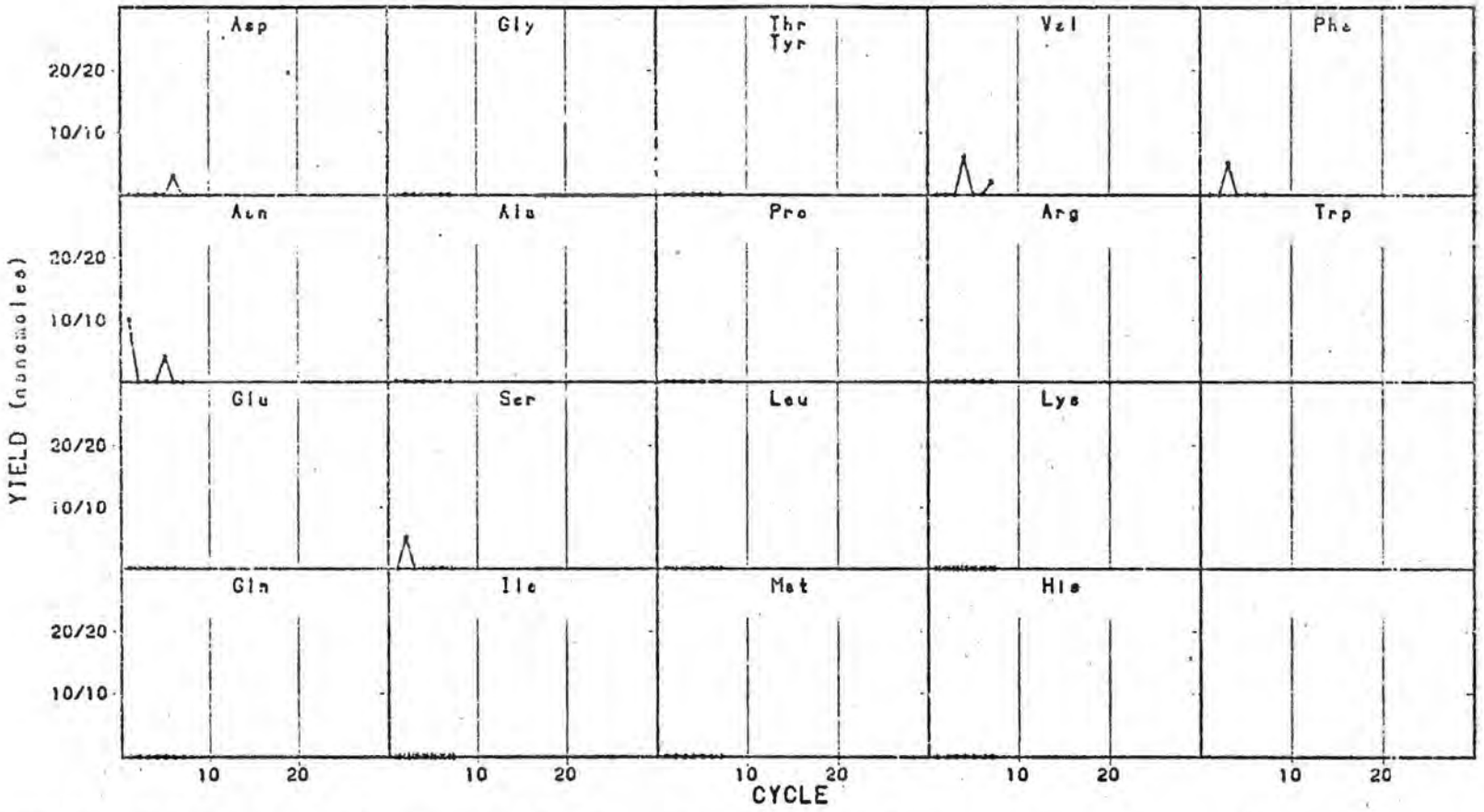
Ala-Pro- -Gly-Gln-Val-Ala-Lys-Lys-Gly- -Lys-Lys-



Yields of PTH-amino acids recovered by degrading Sea Urchin embryo $H2B_{(3)}$ histone variant. The heavy dots (●) represent contamination with $H2B_{(2)}$ histone variant.

G) Sea Urchin Embryo Histone H2B3 CNBr2 Peptide.

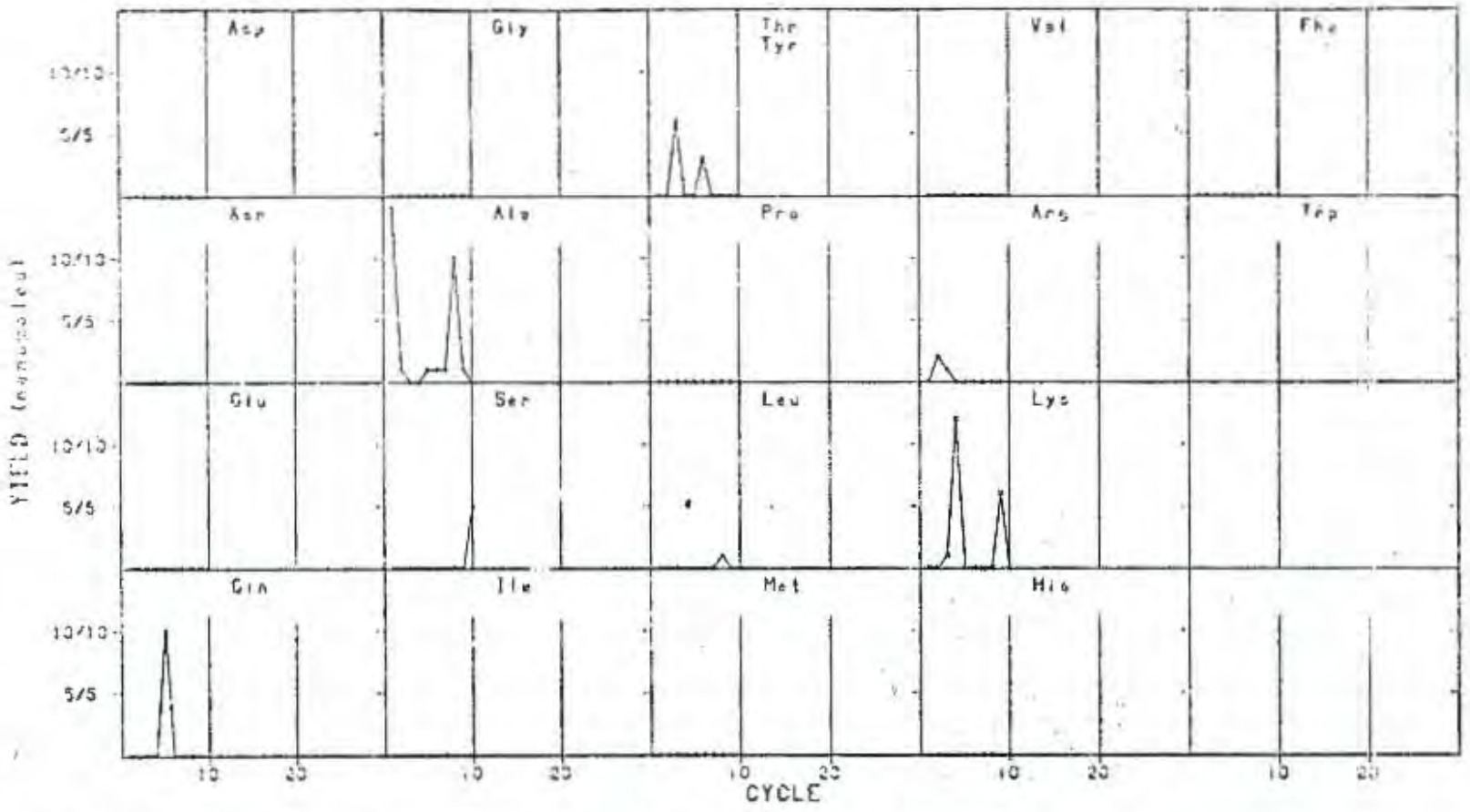
-Asn-Ser-Phe-Val-Asn-Asp-Val
5



Yields of PTH-amino acids recovered by degrading Sea Urchin embryo $H2B_{(3)}$ histone variant after CNBr cleavage, using the peptide program.

H) Sea Urchin Embryo Histone $H3_1$ N-Terminal Sequence

Ala-Arg-Thr-Lys-Gln-Thr-Ala-Arg-Lys-Ser
(5, 10)

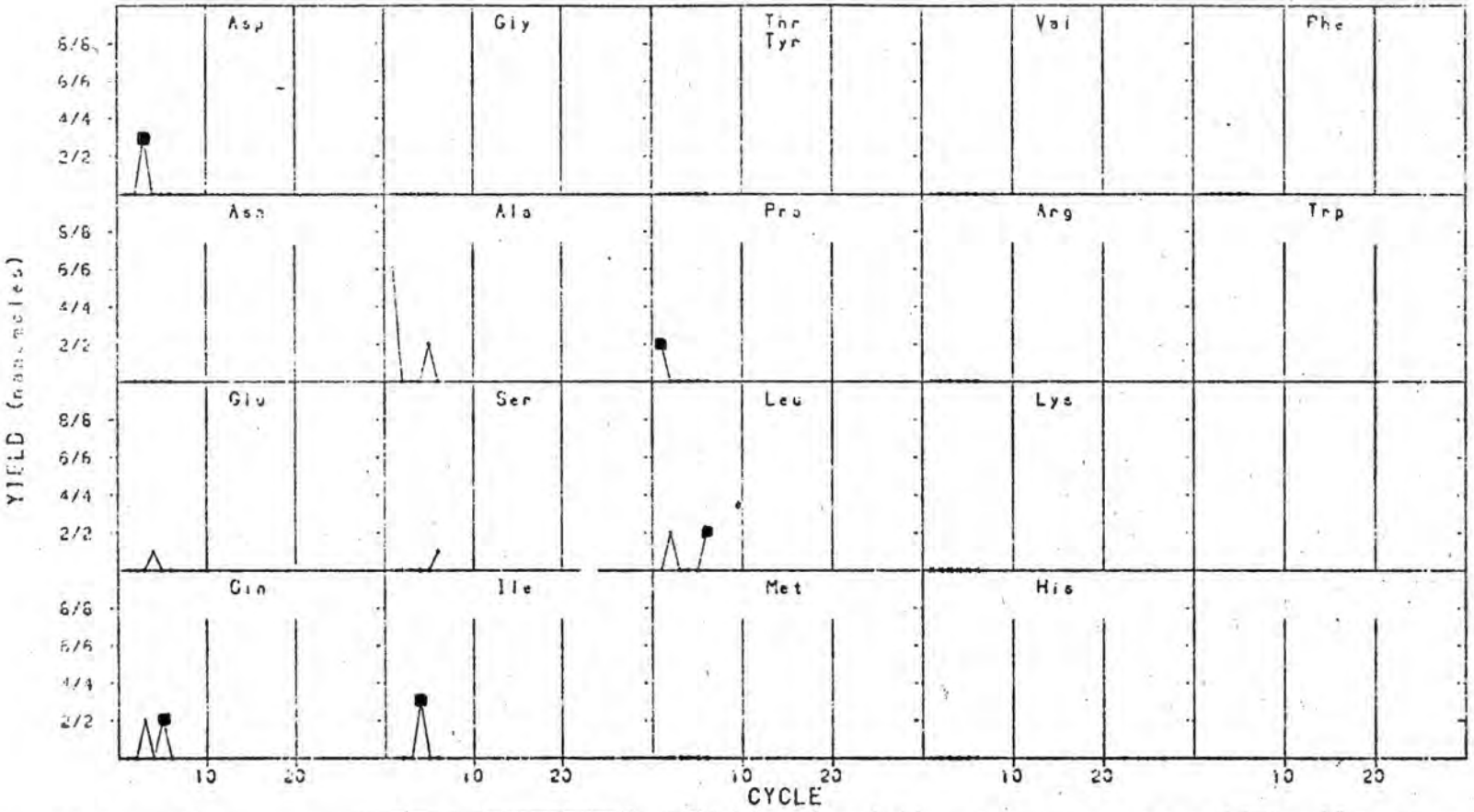


Yields of PTH-amino acids recovered by degrading Sea Urchin embryo $H3_{(1)}$ histone variant.

I) Sea Urchin Embryo Histone $H3_1$ CNBr Peptides

1) Ala-Leu-Gln-Glu-Ala-Ser

2) Pro- **X**-Asp-Ile-Gln-Leu



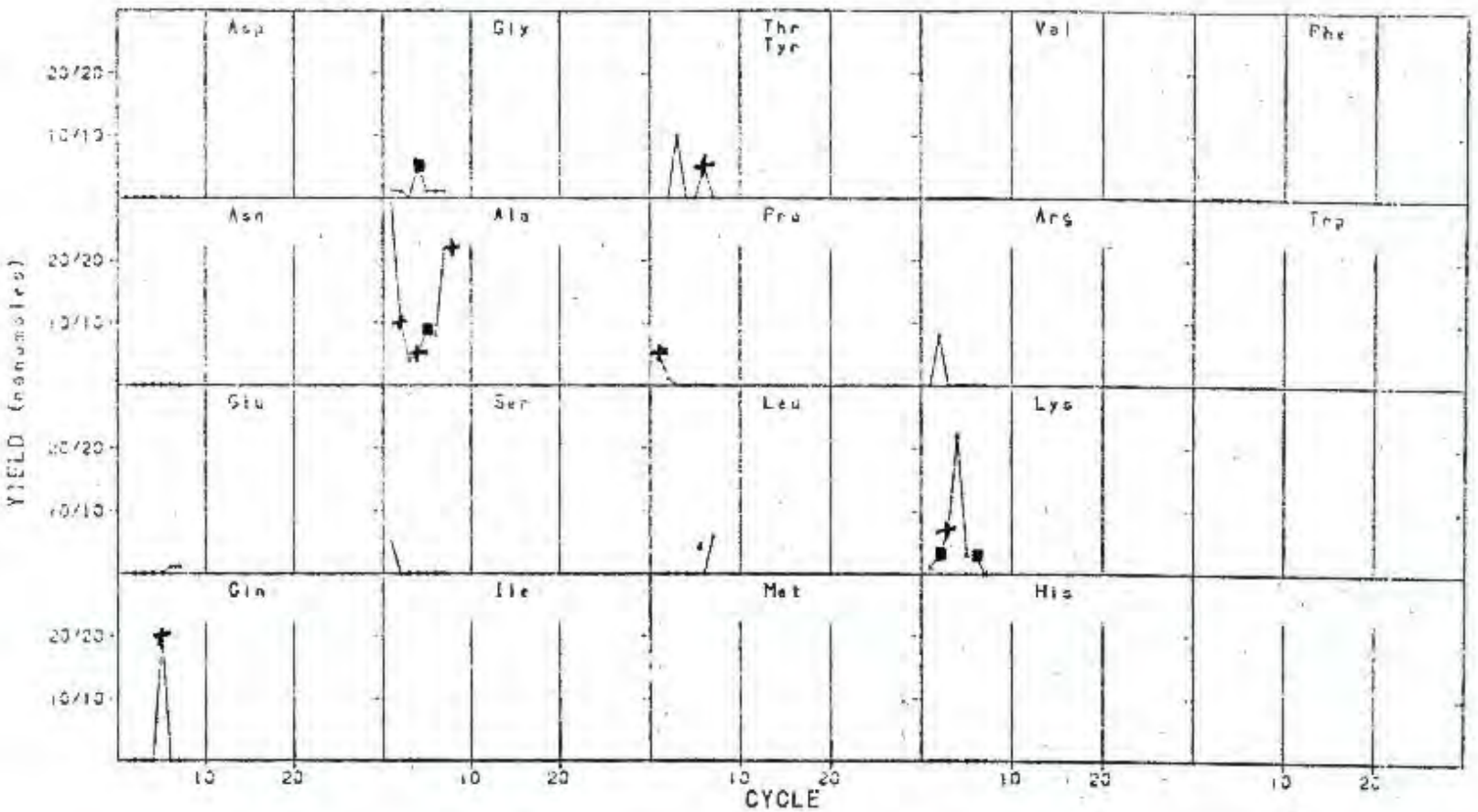
Yields of PTH-amino acids recovered by degrading Sea Urchin embryo $H3_{(1)}$ histone variant after CNBr cleavage.

●——● CNBr 1 peptide

■——■ CNBr 2 peptide

J) Sea Urchin Embryo Histone $H3_2$ N-Terminal Sequence

Ala-Arg-Thr-Lys-Gln-Thr-Ala-
5
-Lys- -Gly-Ala-Lys-
Pro-Ala-Lys-Ala-Gln-Thr-Ala-

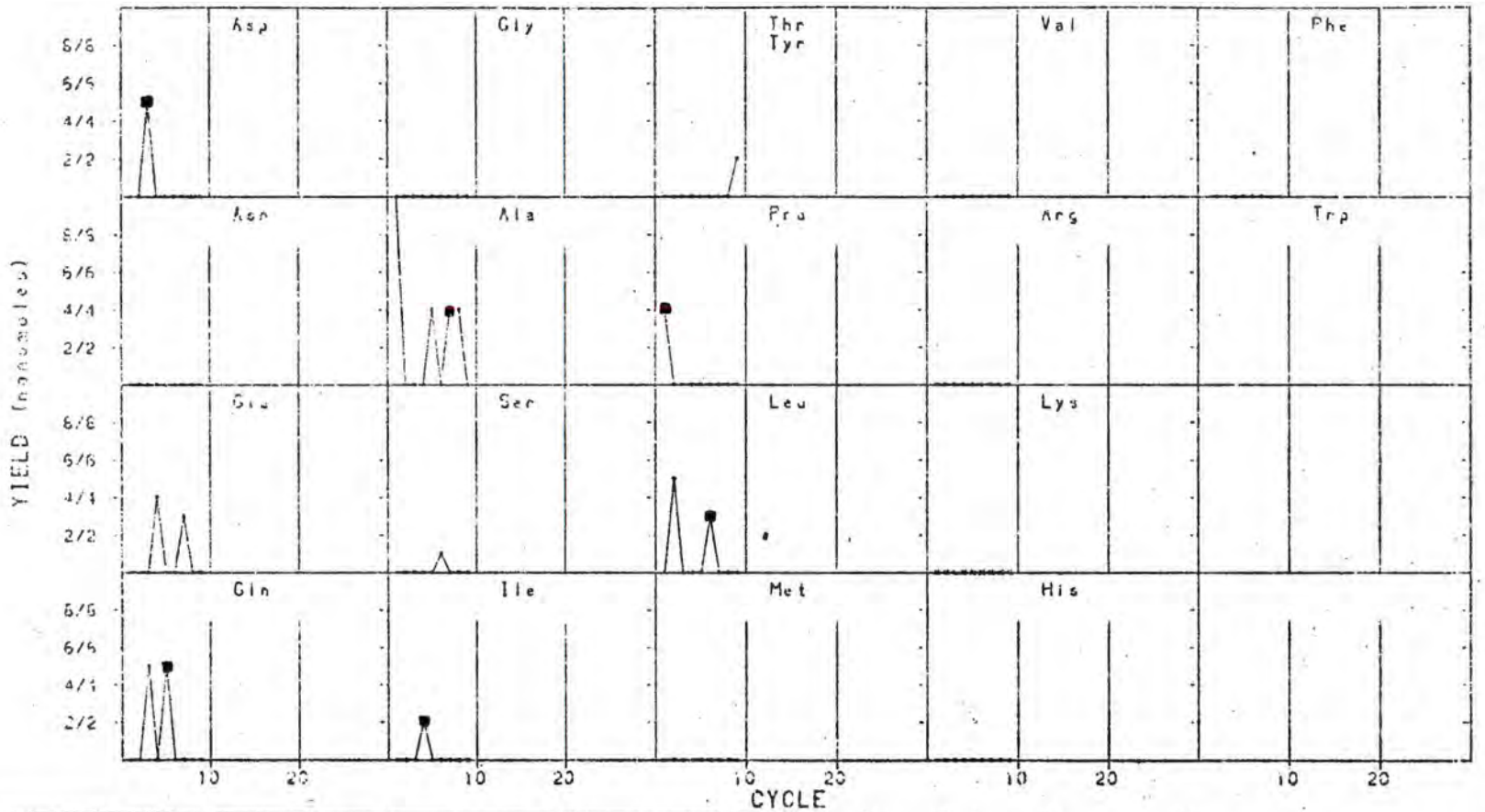


Yields of PTH-amino acids recovered by degrading Sea Urchin embryo $H3_{(2)}$ histone variant. The square (■) represents comtamination with a minor unknown component. The plus (+) represents contamination with $H2B_{(1)}$ histone variant.

K) Sea Urchin Embryo Histone $H3_2$ CNBr Peptides

1) Ala-Leu-Gln-Glu-Ala-Ser-Glu-Ala-Tyr

2) Pro-X-Asp-Ile-Gln-Leu-Ala



Yields of PTH-amino acids recovered by degrading Sea Urchin embryo $H3_{(2)}$ histone variant after CNBr cleavage.

●——● CNBr 1 peptide

■——■ CNBr 2 peptide

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