THE HISTONE H1 OF THE SEA URCHIN EMBRYO, PARTIAL STRUCTURES, ENZYMATIC MODIFICATIONS AND DEVELOPMENTAL PROGRAMME.

by

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Submitted in fulfilment of the requirements
for the degree of
Masters of Science
in the
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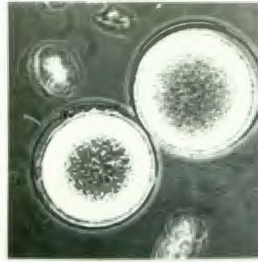
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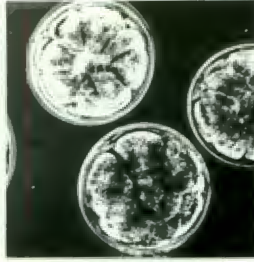
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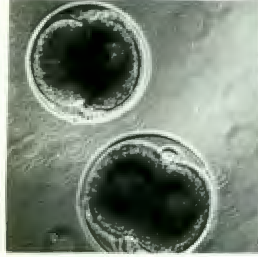
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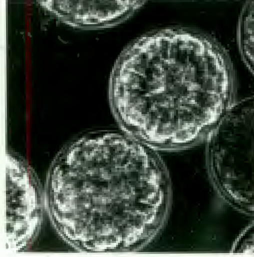
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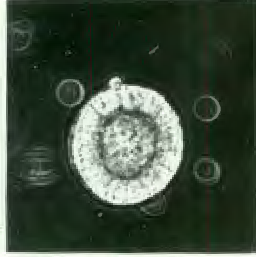
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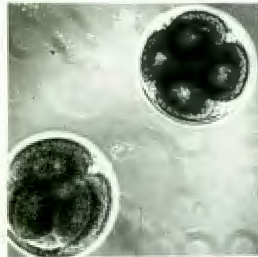
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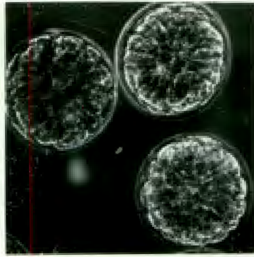
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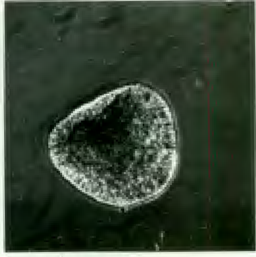
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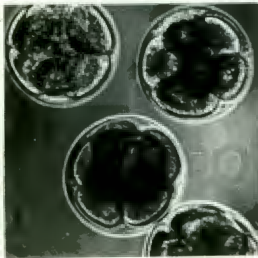
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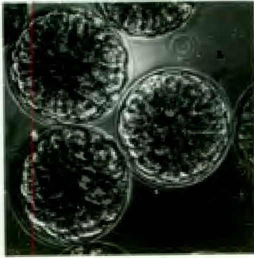
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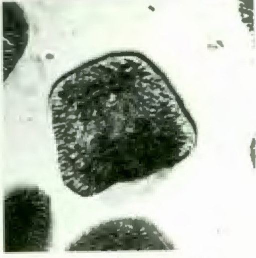
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Sea Urchin development in synchronized culture (*Parechinus angulosus*).

Phase contrast at 104 times microscope magnification.

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Certificate of Supervisors

In terms of paragraph five of "General regulations for the degree of M.Sc." we, as supervisors of the candidate, P.C. de Groot, certify that we approve of the incorporation in this thesis of material that has already been published or submitted for publication.

Professor C. von Holt

Head of the Deaprtment of Biochemistry

Associate Professor W.N. Strickland

Associate Professor of Genetics

SUMMARY

Developmental biology owes a tremendous debt to sea urchins. These animals have proved to be experimental jewels, and since they are distributed abundantly along the Peninsula coastline, they are readily at hand.

Sea urchin embryos have been shown to be extremely well suited for analysis of developmental processes at the ultrastructural, biochemical and molecular levels.

This project is a study of the very lysine-rich histone fraction or H1-histone fraction of Parechinus angulosus embryo.

The first part deals with the characterization and separation of the H1-variants present in the late gastrula embryo.

The second part describes the determination of the partial primary structure of the three chomatographically separated H1-fractions. The amino acid composition and sequences of the H1-histone variants are compared to those of H1-histones from other sea urchin embryo species and from various other sources.

The third part is a study of the histone variant synthesis program during the early development.

[^{3}H] Lysine incorporation into newly synthesized histones was utilized to examine the histone synthetic program. This part also describes the examination of histone acetylation and phosphorylation occurring during the ninth cell cycle of development.

Examination of the modification of the different histone variants and modifications of the histone variants at the different cell stages are discussed.

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PART 1

1.1 INTRODUCTION

The class of basic eukaryotic chromosomal proteins, known as the very lysine-rich or H1-histones, though closely related, is quite distinct from other histones on several counts.

H1 is clearly an accessory protein in the sense that it is on the outside of the nucleosome and is not essential for the coiling of the DNA around the core particle. Each nucleosome consists essentially of nine histone molecules, two each of H2A, H2B, H3 and H4 and one H1 and approximately 190 base pairs of DNA (Thoma et al., 1979; Smerdon and Lieberman, 1981). Kornberg and Klug (1981) proposed that H1 is bound to the DNA at the sites where the DNA enters and leaves the nucleosome. The entrance and exit of the DNA are close together and on the same side of the nucleosome. Kornberg and Klug (1981) proposed that with increasing ionic strength the inter nucleosomal H1 regions form a helical polymer, giving rise to the geometrical form of the solenoid with 5 or 6 nucleosomes per turn (Kornberg and Klug, 1981). Therefore, it is the aggregation of H1 which promotes, and may even control, the formation of the solenoid. The H1 molecules are situated in close proximity on the inside of the solenoid of the nucleosome and touch each other (Thoma et al., 1979) forming a type of poly-H1.

Removal of H1 from chromatin followed by nuclease digestion results in a decrease in the size of DNA fragments isolated, from approximately 169 to 145 base pairs (Spadafora et al., 1979) indicating that H1 binding to linker DNA lends a partial protection to the linker DNA.

The histones which form the core of the nucleosome are relatively conservative, particularly H3 and H4. However the lysine rich histones show a far greater variability (von Holt et al., 1979; Hohmann 1980; Seyedin and Kistler, 1979, 1980) which is a potential mechanism for a coarse gene regulation by variation of the structure of chromatin.

1.2 The Role of H1

Several functions have been ascribed to the H1 histone fraction. Two lines of evidence have indicated that H1 is involved in the condensation of chromosomes. Firstly, at physiological ionic strength, H1 caused a contraction of the chromatin and, secondly, during the cell cycle H1 phosphorylation proceeded chromatin condensation (Bradbury et al., 1973a; Bradbury, et al., 1973b). The phosphorylation of H1 proteins in synchronized cell cycles of the slime mould Physarum polycephalum was found to correlate with the initiation of mitotic chromosome condensation (Bradbury et al., 1973; Bradbury et al., 1974). The phosphorylation of the H1 fraction was also associated with new DNA synthesis (S-phase), as demonstrated in the H1 of normal and tumour cells (Balhorn et al., 1972a).

Some H1 variants, namely avian erythrocyte H5 (Johns and Diggle, 1969), sperm specific H1 (Paoletti and Huang, 1969) and H1° (Panyim and Chalkley, 1969) were found in non-dividing tissues. The chromatin in these tissues is in a highly condensed state and is mitotically and transcriptionally inactive.

It has been suggested that the variability of linker DNA length is determined by the structure of the proteins which interact with the internucleosomal DNA. H1 is one of such proteins since it is bound to at least a part of the linker DNA (Shaw et al., 1976; Varshavsky et al., 1976; Noll and Kornberg, 1977). Zalenskaya et al. (1981) have proposed that the structure of H1 determines the length of linker DNA.

1.3 Structure of H1

In early sequence studies Bustin and Cole (1970) recognized three distinct regions of the H1 molecule, based on amino acid composition. Firstly a region of about 40 residues starting from the N-terminus which was highly variable in sequence between subfractions. The next 80 residues were highly conserved and included nearly all of the

hydrophobic residues in the molecule. The remainder (about 95 residues) was highly basic, and contained repetitive sequences, rich in lysine, alanine and proline.

In solution under physiolgical conditions the H1 histone molecule indeed consisted of three separate structural domains (Bradbury et al., 1975a; Bradbury et al., 1979b; Chapman et al., 1976; Hartman et al., 1977). These were the random coil N-terminal "nose" of the molecule that extended at least as far as residue 34; a globular "head" involving the next approximately 80 residues; and a random coil "tail" of the remainder of the molecule. It was demonstrated that the N-terminal portion of the molecule was not required for the formation of the H1 globular structure (Hartman et al., 1977).

The conformational picture obtained from studies done by Aviles et al., (1978) on histone H5 was found to be similar to histone H1 in that both contained three domains. The important difference between H1 and H5 was that the N-terminal domain of H5 was shorter by about 12 residues and had a much lower net positive charge (Aviles et al., 1978). Later Puigdomenech et al., (1980) emphasised that this three domain structure of H1 molecules existed in chromatin and was not merely a free solution artefact. The central portion of the H1 molecule was shown to contain all the secondary and tertiary structure of intact H1 and moreover to retain this structure after removal of the N-terminal and C-terminal regions.

1.4 Variability of H1

Histone H1 is known to be the most variable in primary structure among the histones. It is noteworthy that even in single tissues there are several variants of this protein and that the relative proportions of the variants vary from tissue to tissue. (Bustin and Cole, 1968b, 1969; Kinkade, 1969; Hohmann, 1980).

1.4.1 The Variability of H1 in Fully Differentiated Somatic Tissue

The H1 class of histones contains several molecular species, even within a single organism. This became apparent when the H1 class was chromatographed on columns of Amberlite IRC-50 (Kinkade, 1969; Bustin and Cole, 1969) or subjected to polyacrylamide gel electrophoresis (Panyim et al., 1971). Kinkade and Cole (1966) did a structural study on the four lysine-rich histone variants found in calf thymus. They found that the H1 variants were closely related in primary structures and molecular weight. They concluded that these variants do have specific functions to fulfill in the chromatin. Kinkade (1969) extended his study of the lysine-rich histone variability. He demonstrated species-specific differences and differences in the distribution of the lysine-rich histones of different tissues of the same species. His results also showed qualitative differences in the complement of the H1 histones from the same tissues of different species (e.g the spleen tissue of calf, cat, rat and chicken), and quantitative differences between the H1-fractions of different tissues of the same species.

Bustin and Cole (1968a) demonstrated similar species- and tissue-specific variation of the H1-histone in mammary gland tissue.

Further evidence for species specificity came from the fact that H1-variants from one species did not crossreact immunologically with those of others in the micro-complement fixation test (Bustin and Stollar, 1973; Sluyser and Bustin, 1974).

Sodium dodecyl sulfate (SDS) electrophoresis applied to vertebrate histones revealed that the lysine-rich histone fraction exhibited molecular weight heterogeneity within a given tissue and between the respective tissues of the various animals studied (Panyim and Chalkley, 1971).

The number of H1 histone variants in different animals and tissues varies between 2-5 types per tissue (Bustin and Cole, 1968b; Kinkade, 1969; Sluyser and Hermes, 1973). Chicken erythrocytes contain six H1-fractions and GR-mouse mammary tumours contain at least eight chromatographically separable H1 histone (Sluyser, 1977). The exception is sea urchin sperm which contains one H1 histone (Zalenskaya and Zalensky, 1980).

Hohmann et al., (1971) found that the lysine-rich histones from normal mouse tissues matched those from neoplastic mouse tissues. Quantitative differences were found to be present among the H1-variants of the liver, thymus and spleen of the mouse tissues but similar H1-patterns existed between the respective normal and neoplastic tissues.

Hohmann (1980) presented studies where he utilized a combination of chromatographic and electrophoretic techniques to demonstrate that the H1-histones of 25 tissue culture cell types expressed the same level of H1 histone complexity as seen in the tissues. The relative quantity of the H1 histone subtypes has been shown to change during the development of animals (Newrock et al., 1977) and in the hormone dependent differentiation of specific cell types in tissues of animals (Hohmann and Cole, 1971; Hohmann et al., 1972; Seyedin and Kistler, 1979).

To demonstrate that the chromatographic heterogeneity of the histones H1 was not due to varying degrees of phosphorylation or to the formation of RNA-histone complexes, Evans et al. (1970) showed that the chromatographic resolution of lysine-rich histones was unaffected by phosphatase and ribonuclease treatment. Hohmann (1980) confirmed that neither the species- nor cell-specific properties of the H1 histones could be abolished by alkaline phosphatase treatment.

These chromatographic (Kinkade and Cole, 1968 a & b; Kinkade, 1969) and electrophoretic (Panyim et al., 1971; Rall and Cole, 1971) studies demonstrate that the H1 histones are a group of closely related proteins with different primary structures and with species- and cell-

specific characteristics. Structural studies (Kinkade and Cole, 1966; Rall and Cole, 1971; MacLeod et al., 1977) and immunological evidence (Bustin and Stollar, 1973; Sluyser and Bustin, 1974) suggest that individual H1 variants show complex inter- and intraspecies relatedness.

Although the ratio of DNA to total H1 has been found to be relatively constant, H1 variants were found to be present in various amounts among tissues (Hohmann et al., 1971).

The observed changes in ratios of H1 histones to each other in different tissues, may reflect changes in the structure of large areas of the cellular genome related to phenotype-specific gene expression (Hohmann et al., 1971).

Varshavsky et al. (1976) studied the distribution of histone H1 on the chromatin subunits. They found that a heterogeneous population of nucleosomes was obtained from a micrococcal nuclease digest of chromatin: two discrete structural types of nucleosomes could be indentified by electrophoresis and they differed by both the length of their DNA and H1 content. Two other reports showed that nucleosomes could be distinguished by the H1 subfraction distribution (Bakayev et al., 1977; Albright et al., 1979).

Recent experiments suggest that different H1 subfractions may protect chromatin from nuclease digestion to different degrees (Gorka et al., 1979).

In 1969 Panyim and Chalkley reported the occurrence of a histone in ox liver which they named H1°. This H1° histone bears a similarity to H1 in amino acid composition. This H1 protein is interesting since its presence seems to correlate with the absence of DNA synthesis (Panyim and Chalkley, 1969; Marsh and Fitzgerald, 1973). In a study of H1° Smith and Johns (1980) reported the fractionation of this histone into

three components. The different forms of H1° were separable on acid-urea gels and ran as a single band, just ahead of the H1 band, in SDS-gel electrophoresis.

The amino acid composition of the H1° proteins (from calf or ox) were very similar to each other. The tryptic peptide maps of the H1° variants also demonstrated a close resemblance. These results (Smith and Johns, 1980) showed that H1° was readily distinguishable from H1. They suggested that if H1° is to be considered as an H1 subfraction, it should be regarded as different from other H1 subfractions, and therefore also functionally different. Pehrson and Cole (1980) showed that the H1° content of growth-inhibited cultured cells accumulated substantially along with the arrest of cell division. The comparisons were made between the H1° content of rapidly growing Hela cells and cells whose growth had been inhibited by high density. These results suggest that the H1° present in cells is correlated to functional changes in individual cells.

An H1-like histone , called H5, is present only in nucleated erythrocytes and reticulocytes from birds, fish, amphibians and reptiles (Yaguchi et al., 1977). Tsai et al., (1975) reported the presence of H5 in turtle nucleated erythrocytes which he could not detect in other turtle nuclei. He noted that the amino acid composition of the H5-histone corresponded well to that of bovine H1°. The H5 histone fraction was found to show species variability. This species specificity among the H5 histones was shown by a comparison made of the N-terminal amino acid sequences of H5 isolated from chicken, quail, duck, goose and pigeon (Seligy et al., 1971). This comparison showed considerable sequence variation. Yaguchi et al., (1979) found that the H5 species variability was limited mostly to the N-terminal domains, whereas variability between H1 and H5 protein was within both N- and C-terminal domains. Despite this, there seemed to be a conservation of the central domain between H1 and H5 histones (Yaguchi et al., 1979). Comparative work done on the primary structure and conformation (Crane-Robinson et al., 1976; Aviles et al., 1978) of H1 and H5 histones clearly demonstrated that H1 and H5 are

homologous proteins (Greenaway et al., 1971) and that the genes for H1 and H5 histones have evolved from a common ancestral gene (Yaguchi et al., 1977).

Smith, Walker and Johns (1980) reported homologies between the amino acid sequences of one of the bovine H1° subfractions and the avian erythrocyte H5 histone. The sequence homologies existed in the N-terminal parts of the globular regions of the molecule. Though this homology was apparent from sequence data (Smith et al., 1980) the total amino acid analysis of the H1° subfractions (Panyim and Chalkley, 1969; Smith and Johns, 1980) did not correspond well with the H5-like histones, for it lacked the high arginine content usually found in H5 histones from avian erythrocytes (Johns and Diggle, 1969). However, the H5 histone from non-avian sources, such as fish, contained less arginine (Miki and Neelin, 1977).

Evidence for another lysine rich species specific histone has been found in rat tissues (Sluyser and Hermes, 1973). This rat specific H1 histone could not be detected in other species and was present in normal and tumour rat tissues.

Kharchenko et al. (1980) compared the electrophoretic fractionation of histones of cells with a completely inactive genome (crayfish-, newt-, and frog spermatozoa, avian erythrocytes, rabbit and cow neutrophils) and homologous somatic cells with a template-active genome. The most common characteristic feature in cells with an inactivated genome was the presence of several more subfractions of histone H1. The authors proposed that this may be due to the need for total condensation of the chromosomal fibrils in the repressed cells.

Borun et al., (1977), using mRNA from HeLa Cells, demonstrated that the subtypes of all histones resolved on Triton-acid-urea-gels could be synthesized in cell-free systems. The separation of H1 into multiple species of Triton-acid-urea gel systems was not due to post-translational modification, since the same translational system made different histone subtypes in response to different mRNA preparations.

Important though these findings are, their significance will only become apparent once the function of these variants is understood.

1.4.2 The Variability of H1-histones in Embryo Tissues

Actively differentiating embryonic tissue represents a developmental step between gametic and somatic tissues. It is important to know whether or not the embryonic nucleus contains unique types of histones.

1.4.2.1 Histone H1 of Sea Urchin Embryos

Vorobyev et al. (1969) reported dramatic changes in histone patterns and synthesis in developing embryos of the sea urchin Strongylocentrotus droebachiensis. The lysine-rich histones were found to increase significantly in the gastrula stage.

Earlier studies of histone synthesis during embryogenesis did not show any increase in transcriptional activity until the late blastula stage (Hnilica and Johnson, 1970; Arceci et al., 1970). These largely negative results were due to the less sensitive procedures unable to detect transcriptional activity and the absence of powerful separation techniques allowing the electrophoretic fractionation of histone variants. These earlier studies nevertheless indicated changes in the composition of the histones at different stages (Thaler et al., 1970; Benttinen and Comb, 1971) and a differential rate of synthesis (Fambrough et al., 1969; Vorobyev et al., 1969; Orengo and Hnilica, 1970; Thaler et al., 1970; Crane and Villee, 1971).

In later studies of the sea urchin embryo, the number of H1 histones has been shown to increase from one component at the morula stage to three components at the gastrula stage (Hill et al., 1971; Cohen et al., 1973; Seale and Aronson, 1973; Brandt et al., 1979). The appearance of new and electrophoretically distinct H1 components in sea urchin gastrula embryos, coincided with the cessation of synthesis of the initial H1 component (Ruderman and Gross, 1974; Poccia and Hinegardner, 1974). Although the morula type of H1 (H1m or H1)

ceased to be synthesized at the blastula stage it was shown to be present in the chromatin long afterwards, even through metamorphosis (Poccia and Hinegardner, 1974). The changes in H1 patterns were shown not to be the result of post-translational modifications nor degradation (Panyim et al., 1968), but due to differences in primary structure (Ruderman, Baglioni and Gross, 1974). The switch in synthesis of the H1 variants during early embryogenesis took place at the late blastula/early gastrula stage. The change in histone H1 synthesis has been observed in various sea urchin species, Lytechinus pictus (Arceci et al., 1976; Poccia and Hinegardner, 1975); Arbacia punctulata (Ruderman and Gross, 1974); Strongylocentrotus purpuratus (Seale and Aronson, 1973); Arbacia lixula (Ruiz-Carrillo and Palau, 1973); Parechinus angulosus (Brandt et al., 1979) - with the timing of the switch characteristic for each species.

The early claims concerning the absence of newly synthesized histones in early cleavage nuclei and pre-blastula nuclei have been questioned by Seale and Aronson (1973). They observed a full set of histones in nuclei of 16-cell and later stage embryos but found only the slightly lysine-rich histones in the 4- and 8-cell embryo nuclei.

Ruderman and Gross (1974) showed that newly synthesized histones were in fact present in the chromatin as early as the first cell division, and that chromatin of pre-blastula stage sea urchin embryos contains a typical complement of histones. In their investigation of histones during early embryogenesis, Newrock et al. (1977) showed that there were three histone-like proteins that were synthesized and incorporated into chromatin even earlier than the α-subtypes. One of these resembled H1 in behaviour and these proteins were referred to as cleavage stage proteins. The cleavage stage (CS) proteins are the same as those reported earlier by Cohen et al. (1975) and Seale and Aronson (1973). The radio-active CS subtypes made during the first round of replication were still detectable at the prism stage 40 hours later (Newrock et al., 1977).

The determined properties of the lysine-rich H1 histones of sea urchin embryos were comparable to those of mammals. They were selectively extracted with 5% perchloric acid, had a high molecular weight relative to that of other histones, relatively slow migration in sodium dodecyl sulfate (SDS) gels and significant heterogeneity within the class (Seale and Aronson, 1973; Ruderman and Gross, 1974; Brandt et al., 1979).

Orengo and Hnilica (1970) found that the amino acid composition of the 5% perchloric acid extracted proteins from Strongylocentrotus purpuratus closely resembled the H1 fraction derived from calf thymus nuclei. The H1 histones of sea urchin embryos were remarkably different from that of sperm. Ruiz-Carrillo and Palau (1973) found that the embryo H1 fraction had a higher electrophoretic mobility and a lower content of arginine and proline.

Brandt et al., (1979) also demonstrated the divergence in amino acid composition between sperm and embryo H1-fractions, with the embryo H1-variants containing more lysine and glutamic acid residues and lower arginine and proline content than the sperm H1 fraction. Based on a comparison of the first five post methionine amino acid sequences of embryonic H1 to the sequences following the four methionine residues in sperm histone H1, it was obvious that the embryonic histones had amino acid sequences distinctly different from those present in the sperm H1 (Brandt et al., 1979). Hnilica and Johnson (1970) found no qualitative differences in the electrophoretic patterns displayed in the embryos of three sea urchin species - Strongylocentrotus purpuratus, Sphaerechinus granulosus, Paracentrotus lividus.

The electrophoretic pattern of histones revealed species specific differences among the very lysine-rich embryo histones of different sea urchin species (Ruderman and Gross, 1974). The switch-over of H1 variants occurred earlier in Arbacia punctulata embryo than in Lytechinus pictus embryos.

The late and early histone genes of Strongylocentrotus purpuratus were found to be considerably more diverged from one another than the early histone gene classes of two distantly related sea urchins, Strongylocentrotus purpuratus and Lytechinus pictus (Kunkel and Weinberg, 1978).

Arceci et al., (1976) found that maternal mRNA contained templates for the synthesis of H1m, but appeared to lack those for H1g, since the cell-free system used, yielded only H1m when challenged with sea urchin egg RNA. There was no evidence of mRNA for H1g present in the egg, which indicated that H1g was probably a very early developmental protein controlled at the level of transcription in the embryo.

Triton-acid-urea electrophoresis of histones translated "in vitro" showed that there was a shift in the H1 complement made on mRNA preparations from different developmental stages (Weinberg et al., 1977). The mRNA's from mesenchyme blastula and gastrula did not direct the synthesis of H1 α, but made the H1β,γ. This observation of different mRNA's coding for different H1 variants, which could be resolved on Triton X-100 urea gels, demonstrated that post-translational modifications were an unlikely explanation for the observed H1 variation on gels, but that the variants differed in primary structure.

1.4.2.2 Histone in the Embryonic Tissues of Mammalian, Amphibian, Insect and Plant Species

Apart from histone studies done on sea urchin embryo species, there have been several reports on the embryonic histones from mammalian, amphibian, insect and plant species.

For amphibian embryos, there are contradictory reports, one showing profound changes in histone composition depending on stages and regions of the embryos (Asao, 1969; 1970) and the other reporting constancy of histone composition with or without exception of the H1 fraction (Destrée et al., 1973; Imoh and Kawakami, 1973).

Histones were found to be absent during the early development of newt embryos and the amounts extracted from early developmental stages (blastula and gastrula) were shown to be very small (Asao, 1969). Histone appearance started at blastula and later stages. The H1 histone fraction was found to appear at the time of morphogenic movement and continuously increased in amounts throughout the subsequent developmental stages (Imoh, 1977). A change in H1 histone mobility was also observed on Triton gels (Imoh, 1977). In a re-examination of the histone changes during development of newt embryos, Imoh (1978) confirmed his previous observation that the content of H1 histone increased during development, but did not confirm his previous results of displacement of blastula H1 by other H1 variants in later stages.

Adams and Woodland (1977) found that the oocytes of Xenopus laevis synthesized histones more slowly than the unfertilized eggs and embryos. Histone synthesis increased in the egg at the time of maturation of the oocyte. When the embryonic development had reached the late blastula stage, histone synthesis was found to be about three times that of the egg. Adamson and Woodland (1974) did not detect the presence of newly synthesized H1 before gastrulation in Xenopus nuclei but this finding was not substantiated by Byrd and Kalinsky (1973). The electrophoretic analysis study done by Risley et al. (1980) on the lysine-rich H1 histone form embryos and various adult tissues of Xenopus laevis, revealed a previous unrecognized degree of developmental and cell specific variation in these histones. A minimum of five H1 histones (H1A; H1B.1, H1B.2; H1C; H1D) were identified by different mobilities on acetic-acid-urea gels. SDS gel electrophoresis showed them to have varying molecular weights. These H1 variants were present in varying amounts in the different tissues and H1C and H1D were found to be present only in adult tissue. The H1 pattern from sperm stem cells was found to resemble that of embryos rather than adult tissue, since it was deficient in H1C and H1D and contained greater amounts of H1A relative to H1B.

The blowfly, _Calliphora_, had five histone fractions that were comparable to the histones in _Drosophila._ The changes in the basic proteins of chromatin during the larval development of the blowfly, _Calliphora,_ showed no qualitative changes in the histones during the larval development. A quantitative increase in basic proteins H1 and H3 was demonstrated from the feeding stage to the wandering stage in the tissues examined (Sin, 1973).

The H1 fraction from _Drosophila melanogaster_ embryos was electrophoretically distinct from corresponding mammalian and echinoderm histones and substantially different from calf thymus H1 in amino acid composition and molecular weight (Alfageme et al., 1974).

A significant increase of the specific arginine-rich histone (H5) was observed in chick erythropoiesis, during maturation (Moss et al., 1973). The study of nuclear histones during early embryogenesis of chick revealed no qualitative and only small quantitative differences which could not be attributed to a particular embryonic stage of development (Kischer, 1966).

Enea et al., (1978) re-examined changes that occurred during maturation of primitive and definitive embryonic red blood cell lines of the duck. Their results indicated that immature red blood cells contained little if any of the tissue specific histone H5 and that H5 accumulated in the nucleus during maturation of the cell. Their results suggested that histone H5 was absent at early times of development.

Turning to the very lysine-rich histones in plants, Fambrough et al. (1966) found striking similarities between pea bud and calf thymus histones. Fambrough et al. (1968) found differences in the quantities of the lysine-rich histones in different pea organs and maturing pea seedlings. Developing pea seedlings contained three electrophoretic H1 variants, the concentration of which changed on maturation of the seedlings.
These studies suggested that the synthesis of H1 histone variants was programmed during embryogenesis.

1.4.3 The Variability of H1 in Sperm Cells

Not only during embryonic development are histone variants observed but sperm specific histones are synthesized in the adult sea urchin (Easton and Chalkley, 1972).

Spermatozoa of animals exhibit remarkable variation in the chemical composition of the basic nuclear proteins (for review see Bloch, 1969). They range from protamines in fish spermatozoa (Marushige et al., 1969; 1971) to keratin- like proteins in mammals (Coelingh et al., 1969, 1972). In sperm of sea urchin there are five electrophoretically distinct histone proteins (Paoletti and Huang, 1969; Ozaki, 1971; Easton and Chlakley, 1972). In addition histones, "intermediate" in size and composition between protamines and histones, are found in the sperm of amphibians, some echinoderms, molluscs and fish (Subirana et al., 1973; Kasinsky et al., 1978). There are acrosomal basic proteins in the sperm of the crustaceans (Vaughn and Thomson, 1970, 1971, 1972; Langreth, 1969) and "typical somatic" histones in the sperm cells of the Rana type frog (Bloch, 1962; Alder and Gorovsky, 1975). Insect spermatozoa contain the normal complement of somatic histones in addition to a unique basic protein, which is not a protamine (Kaye and McMaster-Kaye, 1966; Tessier and Pallotta, 1973, 1976). In plants, highly basic, small proteins have been described (Reynolds and Wolf, 1978).

Bloch (1969) discussed a number of reasons for the separate nature of the sperm histones:

1. Sperm histones permit condensation and consequent streamlining of the sperm head.

2. Sperm histones inhibit template activity of the chromatin.

3. Sperm histones have a protective role such as withstanding a hostile environment.

4. The sperm histone may "erase" the developmental history of the cell thereby allowing the sperm cell to be highly differentiated and specialized.

5. The nature of the sperm basic protein may reflect requirements of early embryonic development.

6. The variation in basic protein of sperm might be an indication of their non-functional role. Their role may be a primitive one and their structural requirements relatively unexacting.

In sea urchin species, the lysine-rich fraction of sperm chromatin was found to be quite different to the embryonal lysine-rich H1 histone (Paoletti and Huang, 1969; Johns et al., 1973; Brandt et al., 1979). The sperm chromatin H1 histone fraction was found to be high in arginine content, but contained less lysine, twice the amount of serine and half as much glutamic acid when compared to the lysine-rich fraction in embryos (Johnson et al., 1973; Brandt et al., 1979). The complete primary structure of sea urchin sperm H1-histone from P. angulosus has been determined. This clearly showed the arginine-rich character of this sperm specific H1-variant (Strickland et al., 1980). The amino acid composition of the basic protein found in the sperm of the sea urchin Arbacia punctulata was very similar to somatic type histone (calf thymus), differing only in its higher arginine content (Poaletti et al., 1969).

In sea urchin sperm a consistently true lysine-rich histone is absent. Instead, it is replaced by a protein which has some properties of the slightly lysine-rich histones and other properties of the very lysine-rich histones. Paoletti et al. (1969) found the template activity of sea urchin sperm chromatin very low and proposed that this might be the result of the presence of these sperm H1 histones. Apart from the somatic-like histones present in sea urchin sperm, there have

been reports on the occurrence of "intermediate" basic proteins in the spermatozoa of echinoderms (Subirana and Palau, 1968; Palau et al., 1969).

These proteins are probably identical to a group of proteins called the Ø1 proteins, and have been described to occur in the sperm cells of the echinoderm *Arbacia lixula* (Palau et al., 1969) and *Holothuria tubulosa* (sea cucumber). All these basic proteins can be considered homologous to histone H1 since they resemble calf thymus histone H1 in amino acid composition (Palau et al., 1969). Furthermore the Ø1 protein from sea urchin *Arbacia lixula* has an almost identical amino acid composition to that of H1 from sperm cells of *Parechinus angulosus* (Strickland et al., 1980). This group of proteins has an arginine-, lysine- and alanine-rich composition. The conformation studies of both *Arbacia lixula* and *Holothuria tubulosa* histone Ø1 suggested homologies between their secondary and tertiary structure and that of calf thymus. The conformation of *Mytilus edulis* basic spermine (which has been mistakenly classified as Ø1) appeared to resemble a protamine more than a histone (Puigdoménech et al., 1975). From the primary structure of the sea urchin H1 (Strickland et al., 1980) one would expect a three domain structure which has been confirmed by tryptic digestion of H1 resulting in a preferential digestion of the 35 N-terminal residues and all the C-terminal half of the molecule beyond residue 120 (Hartman et al., 1977). Later studies also showed that the Ø1 histone from sea urchin *Arbacia lixula* sperm had a three domain structure similar to that of calf thymus H1 and chicken erythrocyte H5 (Puigdoménech et al., 1980).

Sea urchin sperm cells and nucleated erythrocyte cells are highly differentiated cells with unique properties, though they bear some common characteristics: mitotic activity is suspended, the chromatin is highly condensed and each contains a new class of basic histone which has a characteristically high arginine content. Puigdoménech et al. (1976) showed that the highly contracted state of sperm chromatin was directly related to the increased arginine content of the Ø1 (i.e. H1) histone as demonstrated by nuclear-magnetic-resonance studies. They

suggested that the sperm-specific arginine-rich histones were responsible for chromatin contraction and protection in the spermatozoa.

In sperm cells from species of the Phylum Mollusca (Mytilus and Chiton) there is a mixture of somatic-like histones and proteins intermediate in size and composition between protamines and histones. The sperm cells of other molluscs (Ostrea, Spisula and Patella) also contain proteins intermediate in composition between protamines and histones, but their molecular weight appears to be larger than histones. In the octopus Eledone, a mixture of proteins containing cystine was found, with some components rich in arginine (Subirana et al., 1973). The H1-protein from the sperm of the sea urchin Echinolampas crassa was found to contain a cysteine (W.N. Strickland - personal communication).

Colom and Subirana (1979) characterised the mixture of histones and protamines present in spermatozoa of molluscs in detail and concluded the following: in all species, one or more arginine-rich (protamine-like) components were present. The molecular weight of these arginine-rich components was found to be variable and some species contained several protamine-like components which appeared to be multiples of a basic unit.

In the mature spermatozoa of some species there was also a lysine-rich protein of low molecular weight in addition to the somatic histones were found to be present in the mature spermatozoa of many species. The protamine- like basic proteins from molluscs are microheterogeneous, constituted by a mixture of proteins with closely related composition as in fish (Bretzel, 1973) and histone H1 (Kinkade and Cole, 1966).

The mature sperm of the sand crab Emerita analoga was found not to contain nuclear basic proteins (histones or protamines) (Vaughn, 1968; Vaughn et al., 1969). These sperm, however contain abundant acrosomal basic proteins. General absence of histones and protamines in mature

crustacean sperm nuclei has also been reported for various species of the crab genus Cancer (Langreth, 1969), and for the crab Libinia emarginata (Vaughn and Hinsch, 1971).

A marked diversity of sperm histone types exists among species of the class Amphibia (Alder and Gorovsky, 1975; Bols et al., 1976). The principal testis specific histones from Anura are varied, as shown by an electrophoretic study, ranging from somatic-like histones in Rana to "intermediate" basic proteins in Xenopus to a number of bands migrating on the protamine region of the gel coming from sperm of Bufo (Kasinsky et al., 1978). In the Anurans, Xenopus and Scaphiopus, testis specific histone patterns were found to be different even among several congeneric species (Kasinsky et al., 1978).

The electrophoretic pattern of the testis-specific proteins of the newt Notophthalmus viridescens showed three components migrating more rapidly than somatic histones (Bols et al., 1976).

Species from Squamata (snakes and lizards) showed a relative conservation of testis specific basic protein electrophoretic patterns among the species. Two principal protamine-like proteins were found to represent sperm-specific proteins in Squamata (Kasinsky et al., 1978).

Tessier and Pallotta (1973) found that cricket (Acheta domestica) spermatozoa contained the normal complement of somatic histones in addition to a unique basic protein. Amino acid composition of the sperm specific protein from the house cricket spermatozoa revealed that it contained relatively high amounts of arginine, low amounts of lysine plus a high content of serine, glutamic acid and glycine (Pallotta and Tessier, 1976). This protein showed unique features which distinguished it from testis specific histones present in sea urchins, trout and mussels (Pallotta and Tessier, 1976).

Histone-protamine transition during spermatogenesis occurs in many fish species and the amino acid compostition of protamines remains remarkably constant for the same species, regardless of geographical location or age (for review, see Hnilica, 1967). Protamines appear in the nucleus relatively late during spermatogenesis.

Protamine has been found to be synthesized in the cytoplasm of the spermatids and to be transported rapidly into the nucleus where it eventually replaces the histones completely (Ingles et al., 1966; Ling et al., 1969; Ling and Dixon, 1970).

Analysis by ion exchange chromatography, gel exclusion chromatography and electrophoresis showed conclusively that histones in the nucleoprotamine portions of spermatid chromatin were highly enriched in H1-histone, suggesting that this histone was the last histone to be replaced during the biological replacement (Marushige and Dixon, 1971).

A single-banded electrophoretic pattern of a protamine-like protein has been observed in the sperm of various mammals, e.g. human, rabbit, guinea pig, bull, mouse and rat (Coelingh et al., 1969; Bellvé et al., 1975; Kumaroo et al., 1975; Goldberg et al., 1977).

Sperm proteins of several eutherian mammals resemble protamines in being rich in arginine residues, but unlike protamines, are relatively rich in cysteine residues (Coelingh et al., 1969, 1972; Kistler et al., 1973, 1976).

Seyedin and Kistler (1980) have recently determined that rat testis chromatin contains an unusual H1 variant (H1t). The existence of this variant had been overlooked for some time because H1t was not extracted in significant amounts by 5% (w/v) perchloric acid. H1t and H1a (prominent rat sperm H1-histone) were found to co-migrate in acetic-acid-urea gels, but resolved well in the presence of SDS (Seyedin and Kistler, 1980).

In their report Seyedin and Kistler (1980) showed that H1t, unlike H1a, was not detectable in somatic organs or in the very immature testis. The amino acid composition of H1t was found to be markedly different from other mammalian H1 forms but it had certain similarities to the H1 variant found in sea urchin spermatozoa (Strickland et al., 1980).

The nuclei from bull spermatozoa were found to contain only a single type of basic protein (Coelingh et al., 1969). This protein has a molecular weight of about 6 100 and was rich in arginine (24 residues) and half cystine (6 residues). The sperm histones from four other mammals, namely boar, ram, stallion and man have been isolated and partially characterized. They varied in amino acid composition and carboxy-terminal residue, but all were rich in arginine and half-cystine.

Protamines in humans werre more heterogeneous than in other mammals. There are three main fractions HP1, HP2, HP3 and two minor fractions, HPS1 and HPS2 (Puwaravutipanich and Panyim, 1975). Human protamines were found to be high in arginine content, but unlike other mammals, were also rich in glutamic acid and histidine (Pongswasdi and Svasti, 1976).

Despite the collection of information available on histone replacement in animal spermiogenesis, there is little known concerning the basic proteins of plant pollen cells. Brandt and von Holt (1975) demonstrated that cycads retained somatic histones in pollen nuclei. Reynolds and Wolf (1978) made a study of the motile sperm of a lower plant, the liverwort Marchantia polymorpha. This study provided an electrophoretic and amino acid analysis of the basic complement from the sperm of liverwort. Their study demonstrated that during spermatogenesis, several highly basic, small proteins displaced somatic histones. The plant sperm proteins were found to be comparable in basicity to the protamines of mammalian sperm.

These findings reporting histone replacements in plants and animals over a very widely separated evolutionary range, indicates that there are selection pressures for the replacement of somatic histones in sperm cells, and that the change does indeed have biological significance.

Reynolds and Wolf (1978) suggested that the important selection pressures met by sperm in animals and plants are for reduction in the volume and weight of the sperm nucleus and that histone replacement by protamines represents the most efficient pattern developed in nature for this reduction.

1.5 Changing Patterns During Development

Early development in the sea urchin embryo is characterized by exponential cell division resulting in a complex series of metabolic activations.

The growing sea urchin embryo assembles new protein rapidly, so that early in gastrulation the over-all pattern of protein is distinctly different from the early cleavage stages (for review, see G. Giudice, 1973).

The development from a fertilized egg proceeds by the orderly activation of a genetic program which selects from identical genomes specific genes to be transcribed, that in turn will determine the specialized properties of different cell types. Evidence accumulated during recent years suggests that histone synthesis and modification during embryogenesis might be involved in regulating the translation of genes where the products of the genes are closely related in their function in different cells.

DNA synthesis is one of the most important processes occurring during development. The DNA was found to increase dramatically during early development of the sea urchin embryo (for review, see Giudice, 1973). DNA synthesis was found to start immediately after fertilization as demonstrated by thymidine incorporation (Fansler and Loeb, 1969) and [2-^{14}C] cytidine incorporation (Hinegardner et al., 1964). Each sea urchin embryo must synthesize a considerable amount of DNA and histone in the short period of development from a single cell to an 800 cell gastrula. Even though there is a marked increase in the DNA content per embryo (Giudice, 1973), early observations have demonstrated that the DNA content of the single nucleus does not vary during development (McMaster, 1955; Whiteley and Baltzer, 1958).

Diversity in chromatin structure is generated as development proceeds and may be related to the functional diversification of chromatin leading to cell differentiation. Histones as major determinants of chromatin structure, are likely to be involved in some way in this diversification of chromatin structure.

As previously noted (Cohen et al., 1975; Newrock et al., 1977), the existence of multiple subtypes of H2A and H2B, synthesized at different developmental stages, would indicate there is variation among nucleosomes, increasing as development proceeds from fertilization to gastrulation, that might lead to diversification of chromatin structure and function during embryonic development. It is striking that the onset of synthesis of multiple late subtypes occurs quite rapidly just before and during the mesenchyme blastula stage, a period of cell migration and differentiation. This has led to the suggestion that the generation of multiple cell lineages might involve specific subtype replacement within the genome with different patterns being laid down in different cells (Newrock et al., 1977).

The histone gene system is characterized by a high degree of developmental regulation. In sea urchins the histone genes are repeated several hundred to a thousand times (Weinberg et al., 1972; Grunstein et al., 1974; Grunstein and Schedl, 1976) and are arranged in

a unit which contains the genes for each of the five histones interspersed with non-coding spacer sequences (Weinberg et al., 1975; Kedes et al., 1975; Cohn et al., 1976; Holmes et al., 1977).

It is clear from the studies done on the sea urchin embryo that during cleavage and blastula stages, only the α- forms of H2A, H2B and H1 are synthesized. During the mesenchyme blastula stage the synthesis of these subtypes ceases and a new set of H2A, H2B and H1 histones is made. The multiplicity of histone proteins demands that there be sequence heterogeneity of the multiple histone gene copies and the type of gene which is active is switched at some point prior to the mesenchyme blastula (Weinberg et al., 1977; Hieter et al., 1979).

Sea urchin sperm nuclei display the longest chromatin repeat length yet determined, while chromatin for developing sea urchin embryos has smaller repeats (Spadafora et al., 1976; Keichline and Wassarman, 1977; Keichline and Wasserman, 1979). These differences reflect variations in spacer DNA length, since both sperm and embryonic chromatins have the normal amount of DNA in the core nucleosome (Spadafora et al., 1976; Keichline and Wasserman, 1977; Keichline and Wasserman, 1979).

Savic et al. (1981) found that in contrast to the long repeat distance in sperm, chromatin loaded with cleavage stage histones has a much smaller repeat. Later stages containing predominantly α - histones, displayed an intermediate spacing. They found that the decrease of the repeat length of cleavage stages began at about the time of DNA synthesis.

Keichline and Wasserman (1979) presented data showing that in S. purpuratus, the repeat length did not change significantly between 32-cell embryos and pluteus larvae. Arceci and Gross (1980), however, reported a progressive increase in spacing from blastula to larvae in L. pictus.

Savic et al. (1981) found in both these sea urchin species that three general classes of repeat lengths could be correlated with three general classes of histone variants: (i) sperm-specific histones and long spacers; (ii) cleavage stage histones and short spacers; and (iii) α- and possibly β, γ and δ histones and intermediate spacers.

A number of authors have described a correlation between alterations in nucleosome spacing and changes in the lysine-rich histone composition of the chromatin (Noll, 1976; Morris, 1976; Weintraub, 1978; Schlegel et al, 1980).

Little RNA synthesis occurs in developing sea urchins before the 16-cell stage (Wilt, 1970). It was found that maternal histone mRNA, transcribed during oogenesis and stored in the unfertilized egg, was utilized for translation during early development (Gross, 1967; Skoultchi and Gross, 1973; Gross et al., 1973; Lifton and Kedes, 1976; Galau et al., 1976). This maternal mRNA encoded the major classes of histones synthesized during the cleavage period and it formed a large part of the mRNA present in the cytoplasm (Kedes and Gross, 1969; Hogan and Gross, 1971, 1972).

However, increasing amounts of RNA synthesis occurred after the fourth cleavage and by the time the morula stage was reached, 9-12 S RNA was the predominant pulse labelled species (Farquhar and McCarthy, 1973). Humphreys (1971) showed that by 90 minutes after fertilization, about 15% of the mRNA contained in the embryo polysomes was newly transcribed. The remainder was maternal at that stage, but at later stages, the mass contribution of maternal mRNA appeared to be much smaller. It was also shown that the bulk of the message in the polysomes of mesenchyme blastulae and plutei was newly transcribed (Brandhorst and Humphreys, 1971, 1972; Galau et al., 1977).

During development of sea urchin embryos from blastula to pluteus, striking changes in RNA transciption have been described. (Glisin et al., 1966; Whiteley et al., 1966). During the period covering the blastula and mesenchyme blastula stages, there was a virtually total

switch of histone mRNA that was associated with polysomes from those encoding α-subtypes to those encoding β, σ and δ subtypes (Seale and Aronson, 1973; Ruderman and Gross, 1974; Cohen et al., 1975; Newrock et al., 1977; Newrock et al., 1978).

The switch did not appear to be the result of initiation of translation of functionally competent mRNAs already present in the embryo, since mRNAs coding for the late subtypes were not detected prior to the blastula stage, and the timing of their appearance in the embryo was found to parallel that of their presence in the polysomes (Newrock et al., 1978). Moreover, histone gene transcripts synthesized in the early blastula have been found to differ in nucleotide sequence from those synthesized in the late mesenchyme blastula (Kunkel and Weinberg, 1978). Recently, Hieter et al. (1979) demonstrated that the accumulation of the late histone mRNA in the cell began well before the mesenchyme blastula stage. These data suggest that the changing patterns of transcription could have resulted from the histones performing a regulatory function. The sea urchin embryonic development is accompanied by the sequential incorporation of histone variants into the chromatin.

The sea urchin embryo demonstrates striking developmental changes in synthesis among the various forms of three histone classes; H1 (Seale and Aronson, 1973; Ruderman and Gross, 1974), H2A and H2B (Cohen et al., 1975; Newrock et al., 1977). The sea urchin embryo chromatin contains CS histones up to the 16-cell stage (Cohen et al., 1975; Newrock et al., 1977) while during the morula and blastula stages of development, synthesis of α-variants become predominant and these are followed, still later, by synthesis of β, γ and σ variants (Cohen et al., 1975; Newrock et al, 1977).

All the subtypes made early are retained in the chromatin long after their synthesis ceases (Newrock et al., 1977). The retention of early subtypes demonstrates that the high affinity of histones for the genome

and any special functional properties that a subtype may have will continue to influence the cells, and indeed the region of the genome, that inherits that subtype later in development (Newrock et al., 1977).

The observed histone changes represent only the most prominent features of chromatin remodeling and the possibility that other proteins are involved is not excluded.

Seale and Aronson (1973) found that the chromatin associated non-histone proteins of the sea urchin embryo are heterogeneous, and undergo qualitative as well as quantitative changes throughout early development. The rate of synthesis of these proteins was found to be fairly constant to the pluteus stage and in contrast to histone synthesis, did not parallel changes in the rate of synthesis of DNA.

The sum of the rates of synthesis of total chromatin proteins reaches a maximum in mid-cleavage and accounts for as much as 50% of the total protein synthesis in the embryo at that time (Seale and Aronson, 1973).

The changes in sea urchin chromatin proteins as a function of embryonic development show a significant increase in the relative proportion of non-histone to histone protein during development from blastula to pluteus (Hill et al., 1971).

1.6 Histone Modifications During Development

1.6.1 The Sea Urchin Cell Cycle

During the cleavage stage of development of *Arbacia punctulata*, the cell cycle (28 to 32 minutes at 23°C) was characterized by almost continuous DNA synthesis (S), followed by mitosis (M). Gap phases (G_1 and G_2), characteristic of growing cells, were brief or found to be missing entirely (Hinegardner at al., 1974). Synthesis of histone and DNA was found not to coincide during the early stages of embryogenesis

(Seale and Aronson, 1973; Ruderman and Gross, 1974; Woodland, 1980), but the bulk histone synthesis was restricted to the S-phase of the cell cycle (Oliver et al., 1974; Jackson et al., 1976).

In the literature differential histone modifications have been correlated with cell cycle phases. There is evidence that gene activation, DNA replication and mitotic condensation of chromatin all require phosphorylation of histones, in particular histone H1.

Synchronized cultured mammalian cells have been found to contain the highest level of phosphate in histones H1 and H3 during mitosis (Lake and Saltzman, 1972; Lake, 1973; Gurley et al., 1975; Hohmann et al., 1975; 1976). Super-phosphorylation of H1 and phosphorylation of H3 have been specifically correlated with condensed chromosome structure in mammalian cells (Gurley et al., 1978). The phosphorylation of H1 during mitosis was found to be distinctly different from the H1 phosphorylation observed during interphase (Lake, 1973; Hohmann et al., 1975, 1976; Ajiro et al., 1975; Gurley et al., 1978).

In Physarum the H1 phosphate content was found to rise during G_2, reaching a maximum just before mitosis and dropping as the cell went through the cell cycle towards metaphase (Bradbury et al., 1973, 1974; Inglis et al., 1976). These results led to the hypothesis that H1 phosphorylation activity might be the mitotic trigger (Bradbury et al., 1973, 1974a, 1974b; Hardie et al., 1976; Inglis et al., 1976).

Histone deacetylation was found to be strictly correlated with the condensation of chromosomes at metaphase, while the interphase population showed the known phenomenen of hyperacetylation (D'Anna et al., 1977; Simpson, 1978; Bode et al., 1980; Gõmez-Liba and Bode, 1981). The acetylation of the core histones was found to lead to an unravelling of the nucleosome (Bode et al., 1980) and to a general decondensation of chromatin (Simpson, 1978).

Methylation of histones was found to start immediately after histone biosynthesis. It proceeded more slowly than histone synthesis and the rates of methylation among different histones were found to differ (Thomas et al., 1975). Most reports dealing with histone methylation in synchronized or partly synchronized cell systems, placed this modification at late S or at G_2 phase (Tidwell et al., 1968; Shepard et al., 1971; Lee and Paik, 1972; Borun et al., 1972). Histone methylation was functionally connected with the final arrangement of newly replicated histones in the nucleoprotein complex rather than with gene activation of the cell (Thomas et al., 1972, 1975).

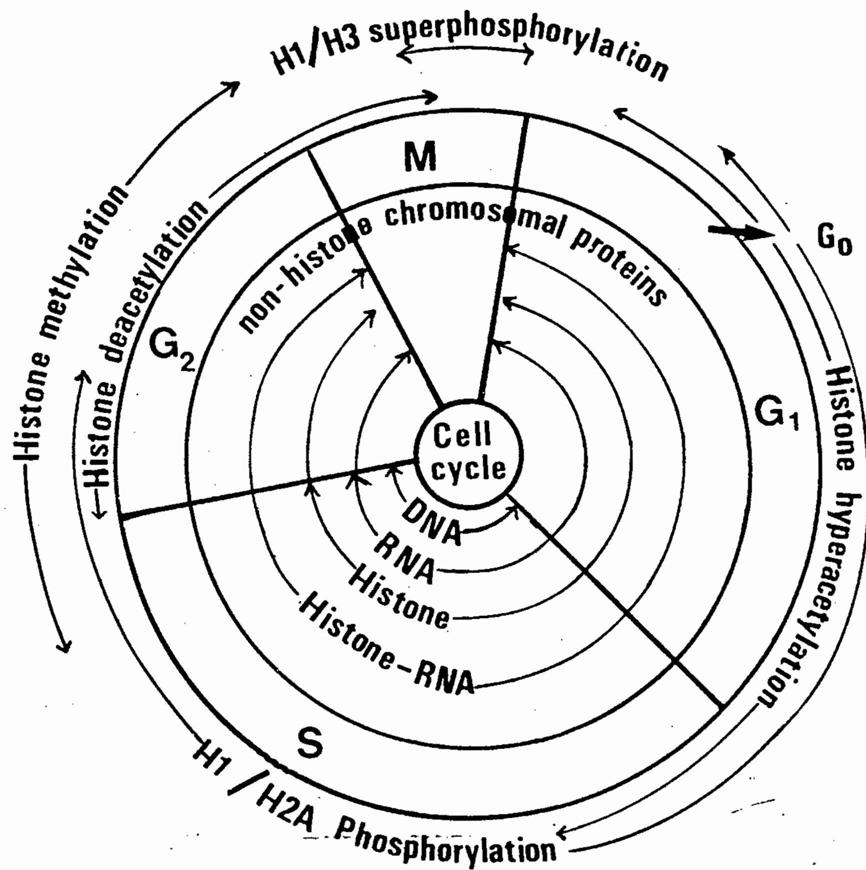


Figure 1.1 Diagramatic representation of changes occurring during the eukaryotic cell cycle.

G_1, G_0, S, G_2 and M are the 5 phases into which the cell cycle of normal eukaryotic cells is conventionally divided (Hinegardner et al., 1974; Rudermann and Gross, 1974; Thomas et al., 1972, 1975 D'Anna et al., 1977; Pardee et al., 1978; Gurley et al., 1978; Bode et al., 1980).

1.6.2 Histone Phosphorylation

Interest in histones has grown since the discoveries that these proteins can be enzymatically modified by phosphorylation (Ord and Stocken, 1966) and acetylation (Gershey et al., 1968; De Lange et al., 1968).

Changes in the structural organization of chromatin are necessary for the progression of the cell cycle. These changes are thought to be regulated mainly by the modifications of chromosomal proteins by reactions such as phosphorylation (Lake et al., 1972; Bradbury et al., 1973; 1974b; Inglis et al., 1976; Gurley et al., 1974, 1978), acetylation (Sung and Dixon, 1970; Sealy and Chalkley, 1978), methylation (Lee et al., 1973; Thomas et al., 1975) and poly (ADP-ribosyl)ation (Ueda et al., 1975; Tanuma et al., 1977).

There is a lack of definite data as to the kinds of histones modified, and the levels, times and sites of modification. Also the influence of modification on chromatin structure and function is not well defined.

Newrock et al. (1977) described H1 as a clear example of a histone that displayed different subtypes which have different roles in the dynamic as well as the stable aspects of chromatin conformation. Evidence that H1 phosphorylation was correlated with gene activation, DNA replication and mitotic condensation of chromatin (Langen, 1969; Lake et al., 1972; Marks et al, 1973; Bradbury et al., 1974; Hohmann et al., 1976) probably indicates that phosphorylation of H1 occurred at different intra-molecular sites (Newrock et al., 1977).

There is evidence that H1 phosphorylation in mammalian cells is a complex of several phenomena that are intimately related to modulations of chromosome structure (Dolby et al., 1979), and reflect processes relating to at least three kinds of chromosomal functions: gene

transcription (Langen and Hohmann, 1975); DNA replication (Balhorn et al., 1972b; Marks et al., 1973; Gurley et al., 1975), and mitosis (Lake et al., 1972; Bradbury et al., 1973; 1974; Gurley et al., 1975).

In mammalian cells, H1 subtypes differ from one another in the number of phosphorylation sites (Langen et al., 1971; Ajiro et al., 1976), providing a possible means for regional control of these chromatin functions. There are at least two levels where phosphorylation may occur in the living cell: the immediate post-translational phosphorylation of histones in the cytoplasm and the phosphorylation of histones already assembled into the nucleosomes. The former process may be a prerequisite for the proper assembly of histones into chromatin. Louie et al., (1973) speculated that since most of these modifications occur in cells actively synthesizing DNA, and hence histones, these modifications may be involved in one of the following: (a) the attachment of newly synthezised histones to DNA; (b) changes in chromosomal coiling; (c) changes in chromosomal activity during the cell cycle or differentiation; or (d) maintenance of the correct conformational relationship of histones to DNA.

All histones are phosphorylated, but lysine-rich histones were found to be the most active substrates for cyclic AMP-dependent protein kinase (Johnson et al., 1974; Bradbury, 1979). Phosphorylation of H1 occurring at serine and threonine residues (Bradbury, 1979) was found to be the most important modification observed in H1. The major phosphorylation sites in all the assayed histones were generally located within predicted β-turns (Small et al., 1977; MacLeod et al., 1977) and in the basic region of the proteins, which is involved in electrostatic interactions with the phosphate groups of the DNA.

It is well known that H1 phosphorylation is cell-cycle dependent. Early studies concerning the phosphorylation of H1 histones of Chinese hamster cell line, CHO, revealed a sequence of phosphorylation events which began in late G_1, continued at an increased rate through S phase, and accumulated in a superphosphorylation event corresponding with chromosome condensation (Gurley et al., 1973a, 1974a, 1974b, 1979).

Other reports showed distinct interphase and mitotic phosphorylation of H1 histones in several other mammalain cell lines (Balhorn et al., 1971, 1972, 1973; Lake, 1973). The inhibition of DNA synthesis was shown to result in significant inhibition of phosphorylation of lysine-rich histones, with little effect on the phosphorylation of other histones and non-histone proteins (Lea et al., 1973).

Hohmann et al., (1976) demonstrated that cell-cycle dependent H1 phosphorylation in synchronized Chinese hamster cells, CHO, occurred in different, distinct regions of the H1 molecule as well as on distinct amino acids during different stages of the mitotic cycle. Hohmann et al. (1975) demonstrated that the serine and threonine amino acids in the H1 histone in CHO cells became phosphorylated in a cell-cycle dependent fashion.

The H1 phosphate content of mammalian cells was observed to be much greater in metaphase cells than in G_2 cells (Gurley et al., 1975; Hohmann et al., 1976). However, observations of H1 phosphorylation in synchronized nuclei of the slime mould Physarum polycephalum differed from those made in mammalian cells. The H1 phosphate content was observed to rise during G_2, reaching a peak at the beginning of early prophase (Bradbury et al, 1973,, Bradbury et al., 1974a, 1974b). As a result of these observations, it was proposed that H1-phosphorylating activity might be the mitotic trigger causing chromosome condensation (Bradbury et al., 1973a, 1974a, 1974b).

In proliferating mammalian cells the phosphorylation of H1 was divided into two classes: type $H1_I$ phosphorylation was found predominantly in interphase cells, and type $H1_M$ phosphorylation was found predominantly in mitotic cells (Gurley et al., 1978). $H1_I$ phosphorylation involved only serine residues and occurred in both the COOH-terminal and NH-terminal N-bromo-succinimide fragments of H1 (Hohmann et al., 1975, 1976).

Gurley et al. (1978) correlated these two different H1 phosphorylations and the phosphorylation of H3 with the chromatin structural changes which occurred during mitosis in mammalian cells. They examined the process of mitosis by electron microscope and concluded that entry into mitosis occurred in two stages; (1) the gathering of chromatin into aggregates during preprophase, followed by (2) the condensation of these aggregates into chromosome structures during prophase. Exit from mitosis followed the reverse process, (1) chromosomes disorganized into dense chromatin clumps during telophase, followed by (2) dispersion of these aggregates into early G_1. Correlation of these structural changes with histone phosphorylation revealed that $H1_I$ phosphorylation (1-3 phosphates per molecule) existed in interphase and during the chromatin aggregation stages of mitosis (preprophase and telophase). During the second stage of mitosis (prophase, metaphase and anaphase), when chromosome structures were fully condensed, most of the histone H1 existed as superphosphorylated molecules ($H1_M$) containing 3-6 phosphates, and all histones H3 molecules were phosphorylated. Exit of cells from anaphase correlated closely with dephosphorylation of H3 and of $H1_M$ (0-3 phosphates) (Gurley et al., 1978)

Gurley et al. (1978) proposed that H1 and H3 may impose a restriction on chromatin structure which prevents chromosome condensation during interphase and that the $H1_M$ and H3 phosphorylations remove this restriction during mitosis.

The kinase-containing extract obtained by Langan from Ehrlich ascites cells was capable of advancing the time of mitosis in Physarum (Bradbury et al., 1974a; Inglis et al., 1976) thereby further correlating H1 histone kinase activity and the timing of mitosis (Bradbury et al., 1974b).

In their studies of cell cycle changes in Physarum polycephalum histone H1 phosphorylation, Fischer and Laemmli (1980) found that the accumulation of phosphate in H1 increased markedly before the onset of mitosis, but found no significant dephosphorylation of the histones

either during or shortly after mitosis, suggesting that non-specific postmitotic dephosphorylation was not a prerequisite for chromosome decondensation.

The interactions of H1 from plasmodia of Physarum with the DNA from chick erythrocyte nuclei was examined using turbidity measurements. The results showed that H1 from early prophase was more effective at causing turbidity with DNA than was H1 from S phase, which was consistent with the proposed role of H1 phosphorylation in chromosome condensation (Corbette et al., 1980).

Marushige and Marushige (1978) proposed that phosphorylation of the very lysine-rich histone in sperm of mammals might be involved in the proper condensation of spermatid chromatin during spermiogenesis since phosphorylation of sperm histone occurs on the chromatin during the packaging of DNA. They found a considerable variation of phosphorylation of sperm histones from species to species and suggested that this observed difference of sperm histone phosphorylation may be a reflection of the differences of structure of sperm histone in various mammals. Phosphorylation of trout protamine (Marushige et al., 1969; Louie and Dixon, 1973) and of rat sperm histone (Marushige and Marushige, 1975) during chromosomal changes in spermiogenesis have been reported.

Morris and Cohen (1979) made a study of the phosphorylation of the two H1 subfractions present in the liver of premetamorphic tadpoles (Rana catesbiana) and found that they differ in net incorporation of [^{32}P] phosphate in N- and C- terminal regions of the molecules.

Depending on the tissue and the phase of the cell cycle, phosphate incorporation was found to occur only in C- terminal (Hohmann et al., 1976), only in N-terminal (Lamy et al., 1977) or in both C-terminal and N-terminal regions of H1 histones (Lake, 1973; Hohmann et al., 1976). Considering the differences in growth states of the cells studied, as

well as possible species variations in turnover rates of individual phosphate groups and sequence location of phosphorylatable sites, the absence of a consistent pattern is not surprising.

In addition to species-specific variations in primary structures of H1 histones (Rall and Cole, 1971; Strickland et al., 1976; MacLeod et al., 1977), chromatographic (Kinkade, 1969), and electrophoretic (Panyim et al., 1971) methods have been used to show that these same H1 histones exhibit species-specific variation in their phosphopeptides (Hohmann, 1979). The studies presented by Hohmann (1979) demonstrated that the phosphopeptides derived from the H1 histones of four different mammalian species were about as divergent as tryptic peptides in general, i.e., about 20%.

Ajiro et al., (1981) found that the two H1 subtypes present in Hela S.3 cells differed in phosphorylation states throughout the cell cycle and within a single cell type. This was in accord with the hypothesis that the H1 subtypes are functionally distinct, namely, that the subtype-specific phosphorylations contribute to the control of chromatin organization. This chromatin organization is required for gene regulation, orderly chromosome replication, and orderly chromosome condensation at mitosis.

1.6.3 Histone Acetylation

Different extents of histone acetylation have been observed in various cell states and suggestive correlations have been made between histone acetylation and several metabolic reactions.

Acetylation is restricted to the four histones comprising the nucleosomal core particle (H2A, H2B, H3 and H4) and it has been shown convincingly that it serves to release structural constraints within the individual nucleosomes (Bode et al., 1980) and also for inter-nucleosomal contacts (Simpson, 1978).

This modification is dominant in the loose chromatin structures responsible for transcription and replication processes (Davie and Candido, 1978; Chahal et al., 1980; Perry and Chalkley, 1981).

Histones also became rapidly acetylated after synthesis (Jackson et al., 1975, 1976; Ruiz-Carrillo et al., 1975; Sheperd, 1973; Sealy and Chalkley, 1979). Jackson et al. (1975, 1976) proposed that extensive acetylation of histones immediately after synthesis may play a role in the deposition of specific histone fractions. They hypothesised that this acetylation might be characteristic of newly synthesized material and perhaps distinct from the modifications of pre-existing histones.

It has been difficult to define the level of initial acetylation with precision because of the rapid deacetylase reaction. The use of sodium butyrate to induce hypermodification of histones by inhibiting the histone deacetylas(s) (Sealy and Chalkley, 1978; Candido et al., 1978; Vivaldi et al., 1978) has provided a way of analysing the degree of acetylation more accurately over short intervals.

Sodium butyrate ultimately inhibits DNA synthesis and cell replication (apart from hyperacetylation) (Hagopain et al., 1977; Sealy and Chalkley, 1978). Butyrate treatment induces the dephosphorylation of CHO (Chinese hamster cells) histones (D'Anna et al., 1980). Butyrate also induces changes in several molecular species such as an increased production of non-histone protein, IP_{25} (Candido et al., 1978), increased quantities of histone H1 (Riggs et al., 1977) and induction or modulation of several other enzymes and proteins, some of which have been associated with cellular differentiation (Griffen et al., 1974; Rastl and Swetly, 1978; Rubenstein et al., 1979).

The highly acetylated chromatin from butyrate-treated cells has been found more susceptible to digestion with DNase I (Vivaldi et al., 1978; Sealy and Chalkley, 1978; Simpson, 1978) and there are changes in the accessibility of particular DNA sites within the nucleosome core particle to attack by DNase I (Simpson, 1978).

The extent of acetylation in response to butyrate has been found to vary, depending upon whether the histones were newly synthesized or not (Sealy and Chalkley, 1978). Histone acetylation reactions can be separated readily into those occurring on histones before or after they have been assembled into the nucleosomes (Ruiz-Carrillo et al., 1975), those on new or old nucleosomes, and those on three classes of nucleosomes which differ in regard to their susceptibility to acetylation (Cousens et al., 1979). It is possible that each of these types of histone acetylation has a separate function.

Various experimental approaches have been utilized in attempts to elucidate the role which acetylation has in the structure and function of chromatin.

One approach has been to correlate histone acetylation temporally or spatially with alterations in gene expression or cell specific events. Another experimental approach has been to acetylate chromatin chemically *in vitro* and to compare the structural and functional properties of the modified nucleoprotein with those of native chromatin.

Modifications of histones immediately following synthesis showed H4 to be rapidly and extensively acetylated and to some degree H3 (Jackson et al., 1975, 1976; Sealy and Chalkley, 1979). The histones returned to a steady-state level of acetylation during the ensuing 30 minutes, and the steady state level of parental and modified histone appeared (Jackson et al., 1976; Ruiz-Carrillo et al., 1975). With the use of sodium butyrate to inhibit deacetylation, Sealy and Chalkley (1979) showed that histone H4 first appeared in the nucleus in the deacetylated form and during the ensuing 135 minutes became slowly further modified. A significant amount of H2A, H2B and H3 appeared in the nucleus in the parental unmodified form (Sealy and Chalkley, 1979).

It is known that the parental forms of H4, H2A and H1 contain a NH - terminal acetate group (Hnilica, 1974; Jackson et al., 1975). Since this acetate group becomes rapidly associated with the molecule, and does not turn over, it provides a measure of the extent of incorporation of acetate onto newly synthesized histone.

The amino acid residues in histone H3 which are known to undergo phosphorylation in vivo, were located in the amino terminal region of the protein (Dixon et al., 1975); this was found to be the same region which became acetylated in vivo (Dixon et al., 1975). Whitlock et al. (1980) demonstrated with the use of sodium butyrate (deacetylase inhibitor) that an acetylated form(s) of histone H3 were particularly susceptible to phosphorylation in vitro. These results revealed a potential link between H3 acetylation and phosphorylation, modifications which are thought to have important effects on chromatin structure and function.

Studies of H4 acetate content as a function of the cell cycle in synchronously growing Physarum polycephalum revealed that (1) tetra-acetylated H4 correlated with transcription; (2) highly acetylated H4 (2 to 4 acetates per molecule) was inversely correlated with H1 phosphorylation and initiation of chromosome condensation in prophase (Chahal et al., 1980).

Other evidence of a more direct nature suggested that acetylation might be involved in the genetic activity in the cell.

The transcriptionally active macronucleus of Tetrahymena pyriformis has been shown to contain acetylated histones, whereas the repressed micronucleus did not (Gorovsky et al., 1973).

Studies on histone acetylation during the development of the sea urchin embryo Strongylocentratus purpuratus (Johnson et al., 1973), demonstrated that the pattern of histone acetylation changed only slightly during development. At all stages of early embryogenesis, the H1 histone was minimally acetylated in comparison to other

fractions. H3 and H4 were the most highly acetylated histones at the early cleavage stage (32-64 cell stage). The extent of acetylation of H2B-H2A histones was found to vary somewhat during development and was highest at the mesenchyme blastula and gastrula stages. H4 acetylation showed a slight drop at prism stage (Johnson et al., 1973).

Burdick and Taylor (1976) reported a 2.5 fold increase in the rate of acetylation of the slightly lysine-rich and arginine rich histones between the gastrula and blastula stages of the sea urchin embryo *Arbacia punctulata*. This was followed by a decrease in histone acetylation in the fully differentiated pluteus stage. Burdick and Taylor (1976) proposed that the increased histone acetylation may be at least one preparative factor for the activation of new genes at gastrulation.

Elevated activities of histone acetylation and deacetylation were reported only at the early-blastula stage of the sea urchin embryo (Horiuchi et al., 1980). Changes in histones fractions and acetylated fractions of histones were found between early-blastula and hatching-blastula stages (Horiuchi et al., 1980).

In contrast to sea urchin embryo histones, the sea urchin sperm histones were not acetylated *in vivo* (Easton and Chalkley, 1972). This is consistent with the absence of genetic activity in sperm chromatin. When sea urchin sperm chromatin was added to DNA-free calf thymus and sea urchin sperm histones *in vitro* (Wong, 1980), acetylation was inhibited.

Histone acetylation led to an increase in the rate of transcription of chromatin by E. *coli* DNA-dependent RNA polymerase (Marushige, 1976) and increased rate of digestion of chromatin by both DNase I and staphylococcal nuclease (Wallace et al., 1977).

Other studies showed that major alterations in histone-DNA interactions have occurred due to the modifications (Simpson, 1971; Wong and Marushige, 1976), and that the modifications *in vitro* are not confined to those histones or sites which are known to be modified *in vivo* (Simpson, 1971; Wong and Marushige, 1976).

The diffuse, extended regions of the chromatin, which are the predominant sites of chromosomal RNA-synthesis (Littau et al., 1964) show the highest levels of histone acetylation (Ruiz-Carrillo et al., 1975; Berlowits and Pallotta, 1972) and were digested more rapidly by DNase I than the transcriptionally inert, condensed areas of chromatin (Berlowits and Pallotta, 1972).

The suppression of transcription in maturing avian erythrocytes was found to be paralleled by a decreased acetylation of histones H3 and H4 (Ruiz-Carrillo et al., 1976). In the mature sperm of *Arbacia lixula*, where no RNA synthesis takes place, the histones appeared only in their nonacetylated forms (Wangh et al., 1972); after fertilization, the acetylated forms of H3 and H4 reappeared with the resumption of RNA synthesis at the blastula stage (Wangh et al., 1972).

Vivaldi et al. (1978) found that increased acetylation of HeLa histones labilized the associated DNA sequence to DNase I digestion and suggested that this might indicate that a significant structural change had occurred at the nucleosomal level. This structural change, which clearly influenced DNA accessibility to DNase I, may have permitted DNA interactions with other enzymes and regulatory proteins involved in transcription. This would account for the many spatial and temporal correlations between histone acetylation and RNA synthesis (Allfrey et al., 1977) and the observation that chemical acetylation of histones increased the transcriptional activity of cell free systems (Marushige, 1976).

Work done by Cousens et al. (1979) showed that a substantial sub-class of each histone remains totally unacetylated, even at the highest concentration of n-butyrate and that a readily acetylatedsub- class of histones responds to n-butyrate treatment much more rapidly and to a larger extent than the bulk of the histones.

The manner in which the acetylated histones accumulate suggests the existence of special nucleosome environments within the cell, which differ markedly in their accessibility to the histone acetylase enzyme(s) (Cousens et al., 1979).

Acetylation of histones is known to occur not only in dividing cells (Allfrey et al., 1964; Sanders et al., 1974; Jackson et al., 1975) but also in non-dividing cells containing template inactive nuclei (Sanders et al., 1973). The acetylation may thus represent a general cellular activity which would function differently in different cell types as well as at different phases of the cell cycle.

PART 2

2.1 ISOLATION AND CHARACTERSATION OF H1-HISTONES FROM SEA URCHIN EMBRYOS OF PARECHINUS ANGULOSUS

2.1.1 Preparation of the H1 Histones by Selective Extraction

Sea urchins were collected at various sites along the Cape Peninsula coast (5.1.1). The embryos were prepared and grown under laboratory conditions (5.1.3 and 5.1.4) in the presence of Penicillin G and Streptomycin sulfate.

Batches of eggs showing more than 95% fertilization were used for experiments.

The synchronized development of the embryos was monitored under a phase contrast microscope in order to determine whether development proceeded in a normal synchronized fashion.

Histones were prepared from purified nuclei of the sea urchin embryos (5.2.1). Embryos were washed (4 times) in calcium-magnesium-free sea water (CMFSW) containing 5 mM EDTA, whereupon the sea urchin embryo cells dissociated. Nuclei were obtained from the cells by the method of Keichline et al. (1979). Histones extracted from nuclear preparations were found to be cleaner preparations than the histone extracted from whole cells.

The selective extraction method of Johns (1964) was used to prepare H1-histones. Most of the H1 was removed with 5% (w/v) perchloric acid (5.2.2). Polyacrylamide gel electrophoresis of the perchloric acid extracted H1-fraction showed three H1-variants present in the gastrula stage embryo (Fig. 2.1).

After the removal of the H1-histones with 5% perchloric acid (w/v), the rest of the histones, H2A, H2B, H3 and H4 were extracted with 0.25 N HCl (5.2.2). An acid extract (0.25 N HCl) of the purified nuclei contained all the histones.

Figure 2.1 shows the electrophoretic pattern of Parechinus angulosus embryo whole histone extract compared to P. angulosus whole sperm histones and H1-histones from the embryo extracted with perchloric acid.

Typical yields after selective extraction of 100 ml of centrifuged eggs were: average of 27 mg of H1 histones from the perchloric acid extract and about 120 mg (H3, H4, H2A, H2B) in the 0.25 N HCl extract. The H1-fraction was 18-25% (w/w) of total histones.

2.1.2 Analytical Polyacrylamide Gel Electrophoresis of the Sea Urchin Embryo H1-histone Fraction

The H1-variants were run on various polyacrylamide gel systems in an attempt to determine the optimal separation of the variants and to establish the number of variants present.

The effect of different Triton X-100 and urea concentrations on the separation of the H1-variants was examined on slab gels. Transverse urea gradients from zero to 6 M urea were used and transverse Triton X-100 gradients ranging from zero to 20 mM (Figures 2.2 and 2.4, Section 5.3.3).

The combined effect of Triton and urea on the separation of the H1-variants was examined by preparing a urea gradient gel with 6 mM Triton present in the gel and a Triton gradient gel with 2.5 M urea present in the gel (Figures 2.3 and 2.5).

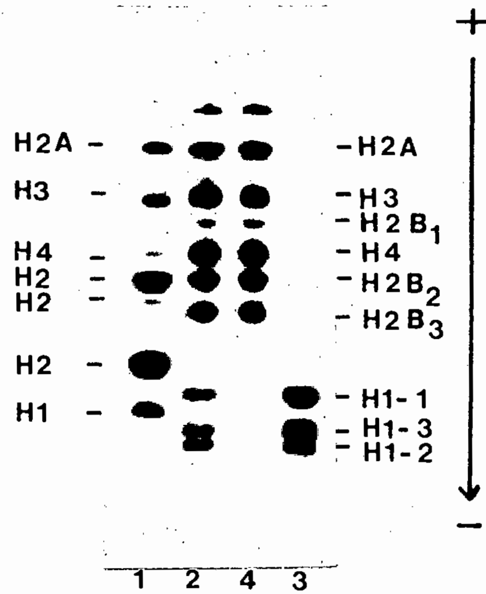


Figure 2.1 3.8 M Urea Triton Polyacrylamide Gel Electrophoresis of *Parechinus angulosus* Sperm and Gastrula Stage Histones

1. Whole histone extract of sperm with 0.25 N HCl.

2. Whole histone extract of gastrula stage embryo with 0.25 N HCl.

3. Perchloric acid (5% w/v) extract of gastrula stage embryo.

4. HCl extraction of gastrula stage embryos after the perchloric acid extraction.

The first set of analytical gels was done by running the H1-fraction on transverse urea gradient gels, one in the presence of 6 mM Triton X-100 and one in the absence of Triton. The gels were prepared as described in Section 5.3.3.1.

Figure 2.2 shows the effect of urea on the separation of the H1-fraction in the absence of Triton. In this gel the three major H1-variants and one faint band migrated closely together and were slightly retarded as the urea concentration increased. The decreased mobility is probably the result of an increase in viscosity due to urea. It is well established that histones do bind Triton to their helical regions and thus assume different mobilities in the presence of the detergent (Zweidler, 1978). The simultaneous presence of urea interfers with this process due to competition with hydrogen bonds and results in highly characteristic behaviour of individual histone fractions in the gel (Zweidler, 1978).

The most dramatic effect was achieved by running a urea gradient gel in the presence of 6 mM Triton X-100 (Figure 2.3). As the urea concentration decreased, the major H1 variants separated into 8 bands. The slowest migrating H1 variant gave rise to at least 5 of the 8 bands that separated in the low urea area of the gradient. Whether this separation was due to primary structure differences or modifications of the variants, was not known. On sequencing the variants and/or studying their modifications, a distinction might be made between these possibilities.

Figures 2.4 and 2.5 demonstrate the effect of Triton X-100 on H1 separation in a polyacrylamide gel. Two transverse Triton gradient gels were prepared, one in the presence of 2.5 M urea and one in the absence of urea (Section 5.3.3.2).

The H1-variant separation was little affected by various concentrations (0-20 mM) of Triton either in the absence of urea or in the presence of 2.5 M urea.

In the presence of Triton and 2.5 M urea the H1-fraction separated into 3 major bands and about 3 minor faint bands while in the absence of urea two of the major bands migrated together.

A transverse polyacrylamide gradient slab gel with the acrylamide gradient from 12% to 18%, with 6 mM Triton X-100 and 2,5 M urea present in the gel did not improve or alter the separation of the H1-variants (Results not shown).

Similarly a transverse gradient gel with a gradient of increasing crosslinking (ratio of acrylamide to N, N' -methylene bis-acrylamide ranging from the 1:150 to 1:25), did not improve or alter the separation of the H1-variants. This gradient gel was done with 6 mM Triton X-100 and 2.5 M urea present in the gel (Results not shown).

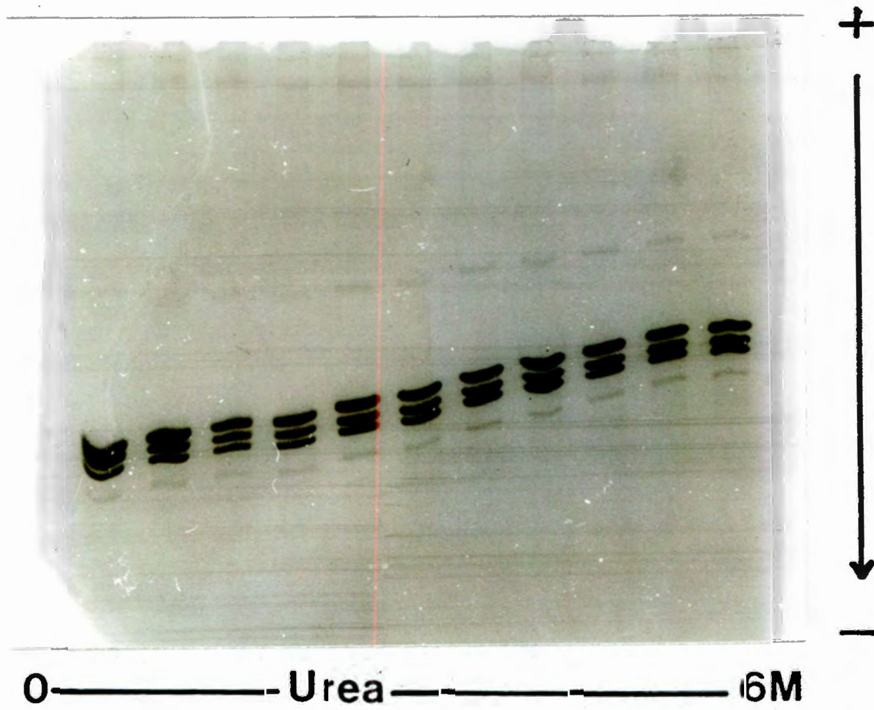


Figure 2.2 Transverse urea gradient (0-6 M urea) polyacrylamide slab gel of the perchloric acid (5% w/v) extracted H1 fraction, with no Triton X-100 present in the gel.

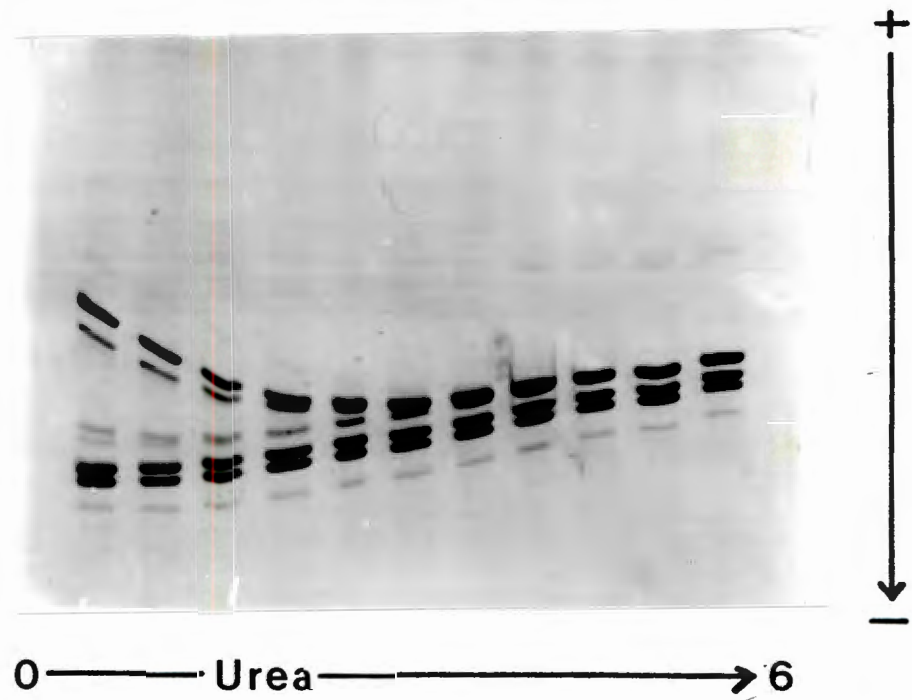


Figure 2.3 Transverse urea gradient (0-6 M urea) polyacrylamide slab gel of the perchloric acid (5% w/v) extracted H1 fraction, with 6 mM Triton X-100 present in the gel.

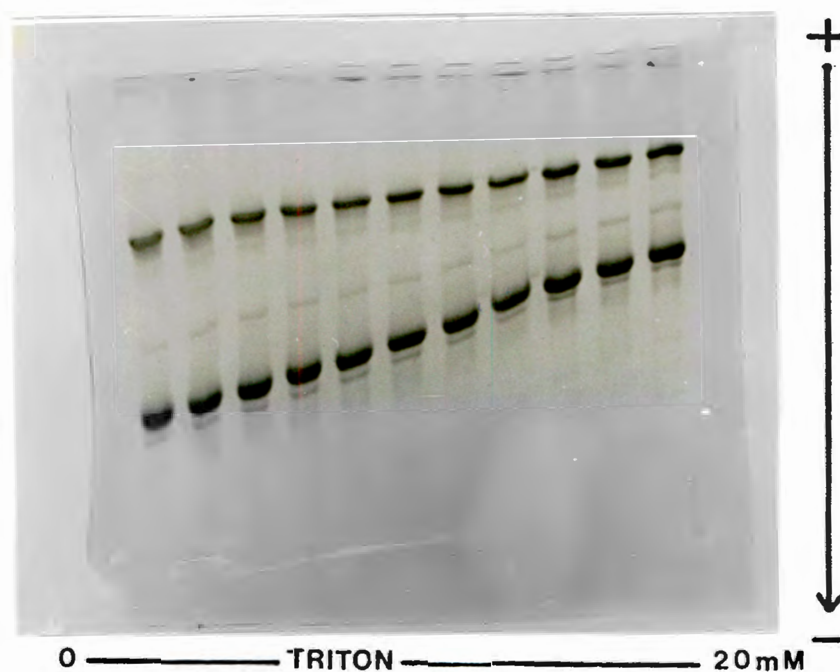


Figure 2.4 Transverse Triton gradient (0-20 mM Triton X-100) polyacrylamide slab gel of the perchloric acid (5% w/v) extracted H1-fraction, with no urea present in the gel.

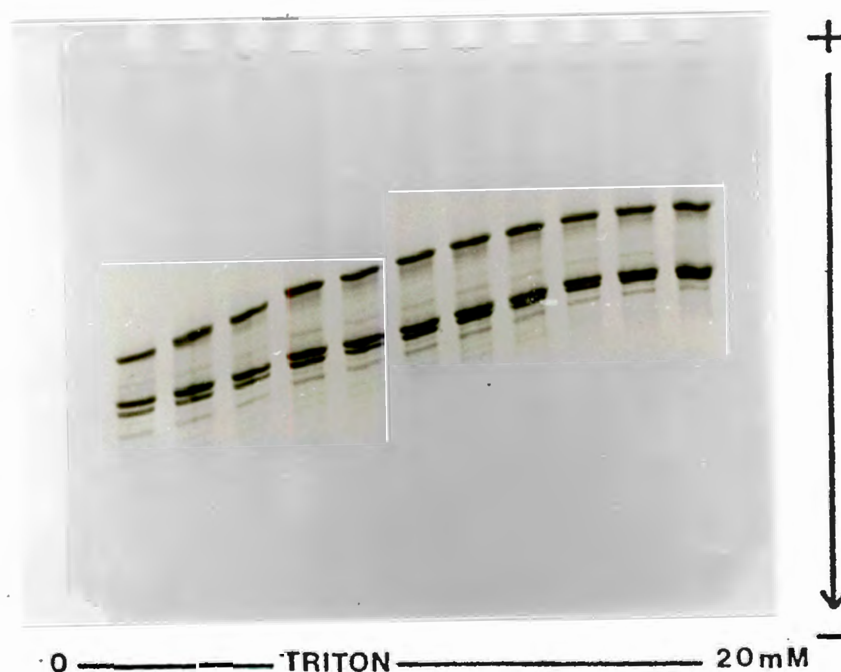


Figure 2.5 Transverse Triton gradient (0-20 mM Triton X-100) polyacrylamide slab gel of the perchloric acid (5% w/v) extracted H1-fraction, with 2.5 M urea present in the gel.

2.1.3. Two Dimensional Gel Electrophoresis of the H1-variants

Two dimensional gel analysis was used to determine the differences in molecular weight of the H1-variants.

Cationic and anionic detergent two dimensional gel systems were used for this purpose.

Both sodium dodecyl sulfate (SDS) and cetyltrimethylammonium bromide (CTAB)- polyacrylamide gels separate proteins on the basis of molecular weight (Weber and Osborn, 1969; Panyim et al., 1977).

The use of Triton acid-urea polyacrylamide gels in the first dimension allowed the optimal separation of histones as well as the histone variants and their modified forms, while SDS- or CTAB- polyacrylamide gel electrophoresis in the second dimension allowed for further separation according to molecular weight.

The first dimensional separation of the H1-variants was done in a 6 mM Triton- 2,5 M-urea polyacrylamide slab gel. A lane of the slab gel (unstained), was cut out and loaded on a CTAB, 7,5% polyacrylamide slab gel (cationic) and ran in the second dimension (Figure 2.6). Similarly an SDS- polyacrylamide slab gel (anionic) system was used to separate the histone variants in the second dimension (Figure 2.7).

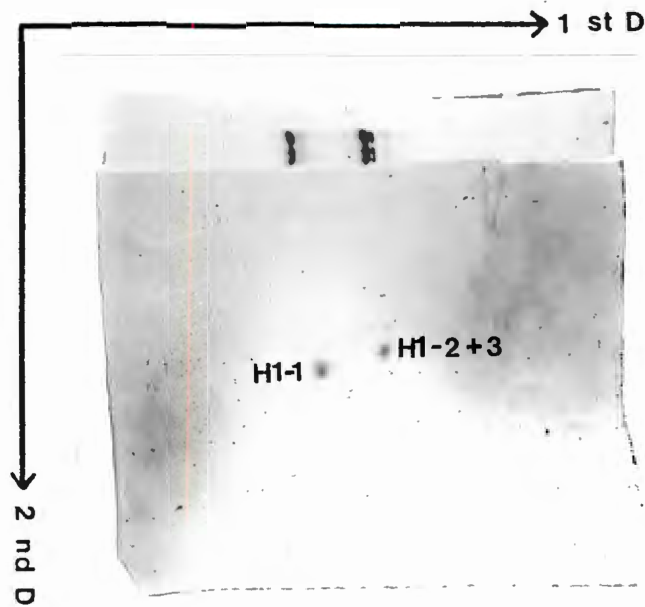


Figure 2.6 Two-dimensional gel of perchloric acid (5% w/v) extracted H1 fraction. The first dimension gel (1st D) was acetic acid/2,5 M urea/6 mM Triton and the second dimension gel (2nd D) was acetic acid/ cetyltrimethylammonium bromide.

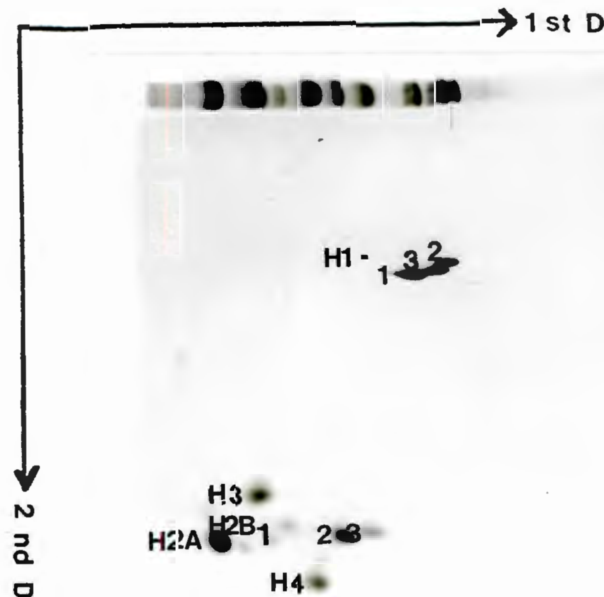


Figure 2.7 Two-dimensional gel of the 0,25 N HCl extract of sea urchin embryo nuclei. The first dimensional (1st D) gel was acetic acid/3,8 M urea/6 mM Triton and the second dimension (2nd D) gel was acetic acid/ 0,1% sodium dodecyl sulfate.

Both two dimensional systems showed differences in migration of the three major H1-variants. The H1-1 migrated slowest in the Triton acid urea gel but moved fastest in the second dimension, while the H1-2 migrated fastest in the Triton-acid urea gel but moved slowest in the second dimension.

This indicated that the H1-1, which appeared first during embryonic development, was slightly smaller than H1-2 and H1-3, which started to be synthesized during blastula stage (see section 3.1).

2.1.4 Column Chromatography of the H1-fraction

2.1.4.1 Carboxymethyl Cellulose (CMC) Chromatography

CMC-chromatography (5.2.3.1) was applied to separate the three major H1-variants, using their differences in charge rather than molecular weight to separate them (Fig. 2.8). Better separation of the H1-variants was achieved by increasing the pH of the sodium acetate buffer from pH 4.5 to pH 5.5.

The H1-fractions were labelled H1-1, H1-2 and H1-3, according to their sequence of elution from the CMC-column (Fig. 2.8). The relative mobilities of the H1-variants in these fractions on Triton-acid-urea gels were different, H1-2 moved faster than H1-3 while H1-1 moved slowest (Fig. 2.9). The chromatographic resolution did not improve in the presence of urea but rather became inferior (Fig. 2.10).

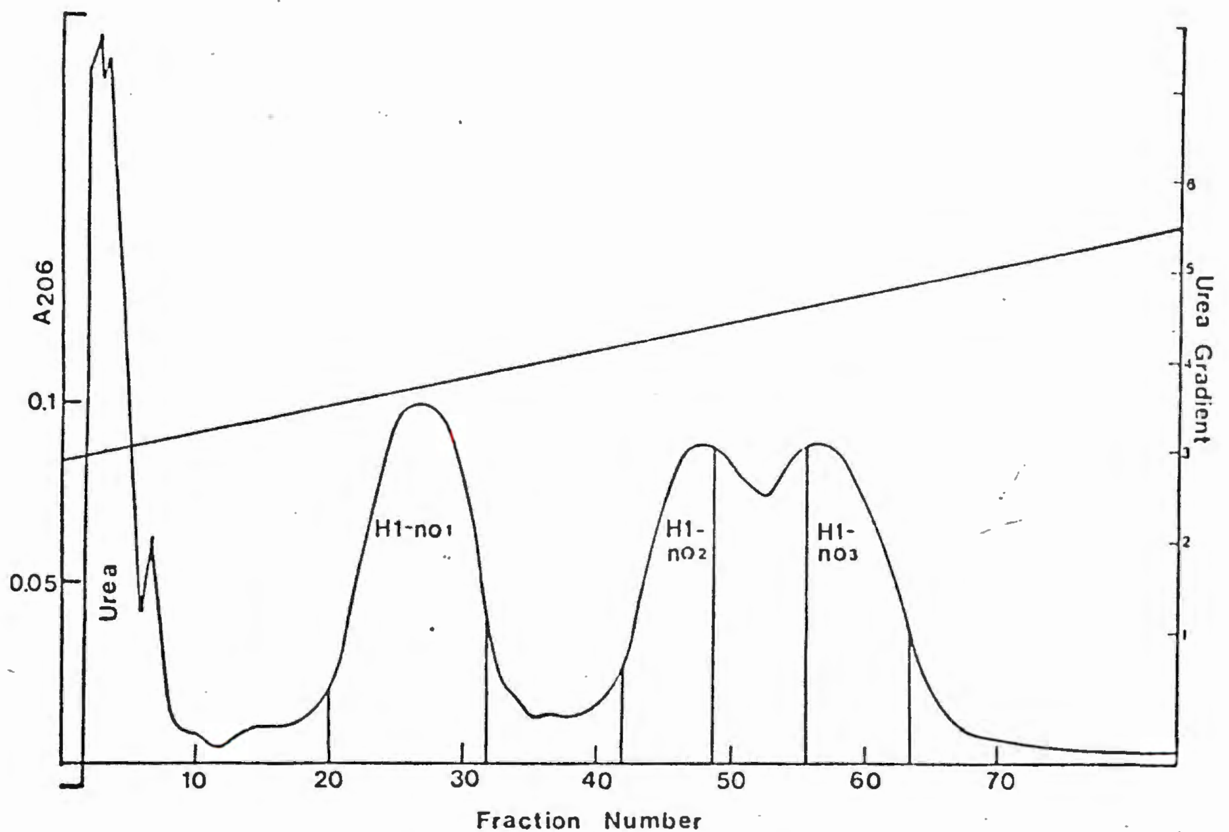


Figure 2.8 Elution Pattern of Perchloric Acid Extracted H1-fraction from a Carboxymethyl Cellulose Column (Whatman CM. 52)
Linear gradient of 300-550 mM sodium chloride in 50 mM sodium acetate/HCl pH 5.5. Column size 9 x 60 mm. Cuvette 3.0 mm. Total volume 200 ml, flow rate 12 ml per hour. 30 mg H1 applied to column which was dissolved in 8 M urea, 50 mM sodium acetate/HCl pH 5,5.

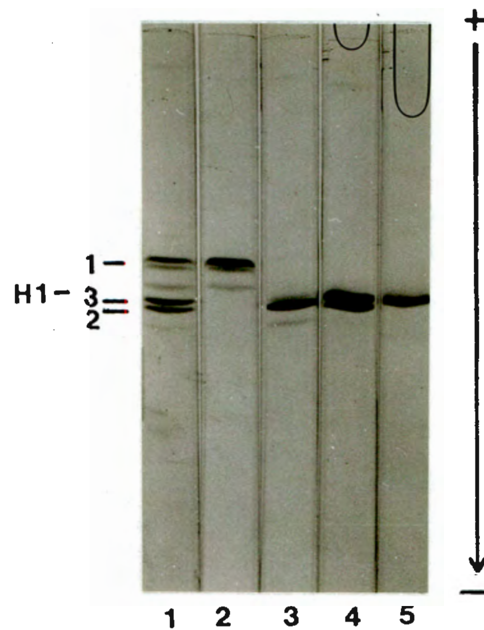


Figure 2.9 Polyacrylamide Gel Electrophoresis of the H1-fractions Eluted from the CMC-column (Fig. 2.8). The polyacrylamide gel consist of 2.5 M urea, 6 mM Triton acetic acid and was run at 3 mM per tube (constant current) for 4½ hours.

1. Perchloric acid extracted H1-fraction
2. H1-1 (peak 2)
3. H1-2 (peak 3)
4. H1-2 + 3 (peak 3 + 4)
5. H1-3 (peak 4)

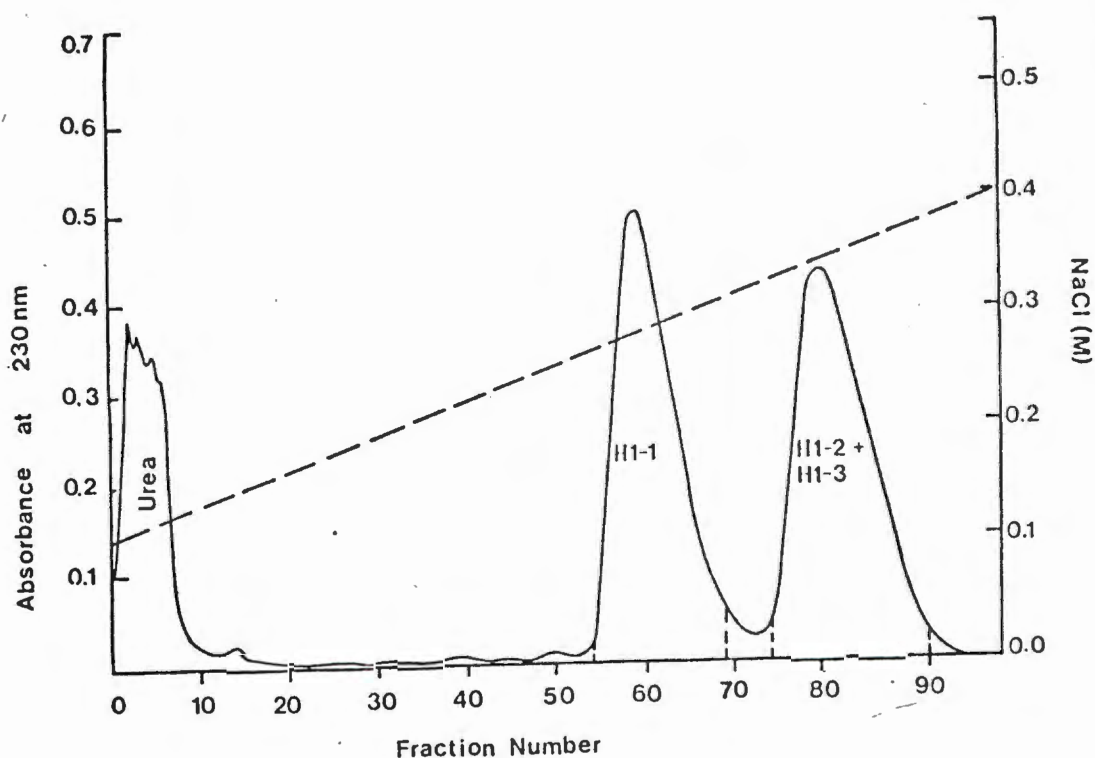


Figure 2.10 Elution Pattern of the Perchloric acid Extracted H1-fraction from a CMC-Column (6 x 90 mm) with a Linear Gradient of 100-400 mM NaCl in 50 mM Na-Acetate Buffer/HCl pH 5.5 and 6 M Urea.

The sample (7 mg) was dissolved in 8 M urea, 50 mM sodium acetate /HCl pH 5.5. Total gradient volume was 200 ml and the flow rate 12 ml/hour.

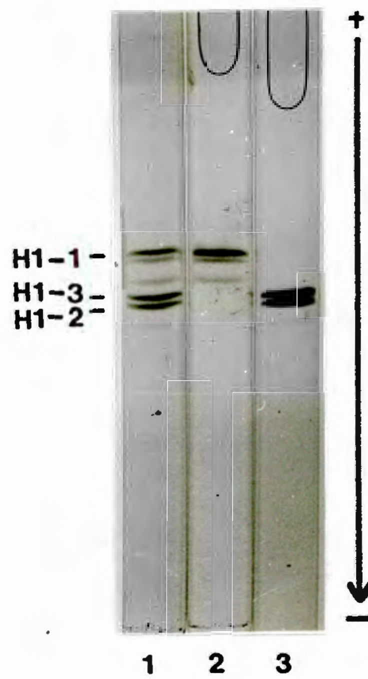


Figure 2.11 Polyacrylamide Gel Electrophoresis of the H1-fractions Eluted from the CMC-Column run with 6 M Urea Present in the Buffers (Fig. 2.10)

The gel was run as described in the legend to Fig. 2.9.

1. Perchloric acid extract
2. H1-1 = peak 2
3. H1-2 + 3 = peak 3

According to the gel electrophoresis two faint bands co-eluted with H1-1 from the CMC-column. These two faint bands migrated to a position between H1-1 and H1-3 (Fig,. 2.9 gel 2). Upon enzymatic cleavage and sequencing, the H1-1 fraction gave rise to a single sequence, therefore these two faint bands that co-migrated with H1-1 from CMC, might be modified or oxidized form of H1-1.

The total H1-fraction was taken and oxidized with hydrogen peroxide (3%) which led to the conversion of methionine to methionine sulfoxide (Neumann, 1972) and another sample of the H1 histones was reduced by the addition of 10 mM DTE in the presence of 10 mM Tris. The reduced and oxidized H1-samples were run on a Triton-acid-urea gel and compared (Fig. 2.12).

In the gel with the oxidized H1-histones (Fig. 2.12 gel 2) the H1-1 band has been reduced to a faint band while the fastest migrating faint band, that co-eluted with H1-1 from the CMC-column, became much darker. This suggested that the faint bands co-migrating with H1-1 on CMC-columns were most probably modified forms of H1-1 in which the methionine became oxidized in the course of the isolation procedures, probably as the result of the perchloric acid used in the extraction.

A faint band co-eluted with H1-2 from the CMC-column. This faint band moved faster than H1-2 on polyacrylamide gel electrophoresis (Fig. 2.9 gel 3). Upon oxidation of the H1-histones, this faint band did not become darker.

H1-3 migrated as a single band on polyacrylamide gel electrophoresis after fractionation on CMC-chromatography (Fig. 2.9 gel 5). On sequencing however, this H1-histone displayed a heterogeneous character (Section 2.2.4).

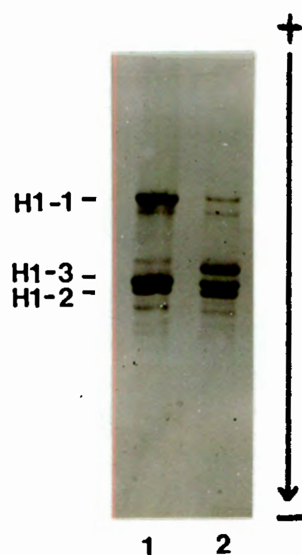


Figure 2.12 Polyacrylamide Gel Electrophoresis of Reduced and Oxidized H1-histones

H1-histones were reduced by incubation with 10 mM Tris, 10 mM DTE at 37°C for 1 hour. A sample of H1-histones was oxidized by incubating the sample in 3% hydrogen peroxide for 1 hour.

1. Reduced H1-histones
2. Oxidized H1-histones

2.1.5 Characterization of the H1-Variants

2.1.5.1 End Group Analysis

Dansylation (5.3.5.1) of the three H1-histone variants resulted in negative results. Upon automatic sequencing of whole H1-hisones of the Beckman Liquid Phase sequencer, no amino terminal fragments were cleaved, indicating the presence of a blocking group on the amino terminal amino acid. This is in contrast the Parechinus angulosus sperm H1 which contains an amino terminal proline residue (Strickland et al., 1980).
Rabbit thymus RTL-2, RTL-3, RTL-4 plus calf thymus CTL-1 H1 histones (R.D. Cole, 1977) and chicken H1 (CEL-5) contain an acetyl-serine blocking group (R.D. Cole, 1977). Trout testis H1 has been found to contain an acetyl-alanine residue at the amino terminus (MacLeod et al., 1977).

Hydrazinolysis (5.3.5.2) of the individual H1-histones followed by treatment with DNS-Cl and identification using polyamide TLC plates with the solvents system as described in section 5.3.5.1, resulted in a positively identified acetyl-DNS-hydrazide.

Having established that the blocking group was acetyl, the method of deblocking through the induction of an N⟶O acyl shift as described by Brandt et al. (1980) was employed. It has been shown by Brandt et al. (1980) that this can be done effectively by treating the protein or peptide with anhydrous heptafluorobutyric acid. Provided the β-carboxyl group of aspartic acid is blocked either via peptide formation with glycine methyl ester or as an ester, the heptafluorobutyric acid treatment leads to specific cleavage through N⟶O shift at seryl and threonyl residues only (Brandt et al., 1980).

Upon incubating the H1-proteins for 40 hr at 55°C with anhydrous heptafluorobutyric acid after treatment for 2 min. with freshly prepared water-free methanolic-HCl (5% w/v) to esterify free carboxyl groups, the proteins were subjected to three sequencing steps on the

Beckman Liquid Phase sequencer. No end group occurred, indicating that the blocked amino acid was neither threonine nor serine. This result was obtained with whole protein H1-1 and H1-2 as well as with the small blocked peptides from H1-1 and H1-2 obtained after cyanogen bromide cleavage. The H1-3 histone similarly did not show an acetyl-seryl or threonyl residue after HFBA treatment and sequencing.

2.1.5.2 Amino Acid Composition

The amino acid composition after acid hydrolysis (5.3.4) of the three *Parechinus angulosus* embryo H1 histones was compared to that of *Parechinus angulosus* gut (Brandt et al., 1979) and sperm (Strickland et al., 1980) H1 and unfractionated calf thymus H1 (Hnilica, 1972) (Table 2.1).

The embryo H1-histones contained more lysine and less arginine than the sperm H1. Also, the embryo H1-histones were less basic than the sperm H1, especially H1-1.

Because of the more acidic nature of the embryo H1-hisones, the variants were analysed for the presence of tryptophan (5.3.4) and their amino acid composition compared to that of some high mobility group proteins, (Table 2.2).

The difference in amino acid composition between embryo H1-histones and HMGs and the lack of tryptophan demonstrated that the proteins which were selectively extracted from sea urchin nuclei with perchloric acid (5%) were indeed H1-histones.

AMINO ACID	Sea Urchin Histone H1 Variants from gastrula Stage 1	2	3	P. angulosus Sperm	Gut	Calf Thymus
Aspartic acid	2.7	2.4	2.2	1.8(4)	4.1	2.0
Threonine	7.9	3.4	4.7	2.0(5)	4.2	5.4
Serine	3.1	4.1	3.3	7.1(19)	4.7	4.7
Glutamic acid	11.0	5.9	3.9	2.3(5)	6.5	3.4
Proline	6.6	8.1	7.7	8.6(20)	7.3	10.1
Glycine	2.9	4.6	4.1	4.4(10)	5.5	6.9
Alanine	20.7	25.6	30.1	29.4(63)	22.0	25.1
Cystine	ND	ND	ND	0	ND	0
Valine	4.1	4.1	2.7	4.0(9)	4.0	4.1
Methionine	0.8	0.7	0.5	1.7(4)	0.9	0.0
Isoleucine	2.6	2.0	2.1	0.8(2)	2.6	0.8
Leucine	3.7	2.9	2.8	2.1(5)	4.2	4.1
Tyrosine	0.4	1.1	2.2	0.8(2)	1.3	0.5
Phenylalanine	1.3	0.7	0.88	0.4(1)	1.1	0.5
Lysine	32.3	34.1	33.0	25.1(69.5)	27.2	28.7
Histidine	0.44	0.6	0.36	0.9(2)	1.0	0.0
Arginine	1.5	1.9	1.8	9.1(26.5)	3.2	1.7
N-terminal	Bl	Bl	Bl	Pro	Bl	Bl
Lys/Arg	21.5	17.9	18.3	2.7	8.5	14.9
Lys+His+Arg / Asp + Glu	2.54	4.4	5.8	8.6	2.96	5.6

Table 2.1 Amino acid composition in molar ratios of the three H1-histones from *Parechinus angulosus* gastrula stage embryos compared to the sperm and gut H1 of the same species, and unfractionated calf thymus H1. Figures in parenthesis are residues determined from sequence analysis.
No corrections have been made for losses and incomplete cleavage during acid hydrolysis. Sperm H1-data from Strickland et al., 1980; Gut H1-data from Brandt et al., 1979; Calf thymus H1-data from Hnilica, 1972.

N.D. = not determined.
B.L. = blocked.

AMINO ACID	Sea Urchin Histone H1 Variants from gastrula Stage 1	2	3	Calf Thymus HMG-1	HMG-2	Trout Testis HMG-T	H6
Aspartic acid	2.7	2.4	2.2	10.7	9.3	11.3	6.7
Threonine	7.9	3.4	4.7	2.5	2.7	3.0	1.6
Serine	3.1	4.1	3.3	5.0	7.4	4.5	5.6
Glutamic acid	11.0	5.9	3.9	18.1	17.5	9.1	6.1
Proline	6.6	8.1	7.7	7.0	8.9	7.6	12.3
Glycine	2.9	4.6	4.1	5.3	6.5	17.4	7.4
Alanine	20.7	25.6	30.1	9.0	8.1	8.3	25.4
Valine	4.1	4.1	2.7	1.9	2.3	3.8	3.4
Methionine	0.8	0.7	0.5	1.5	0.4	1.9	-
Isoleucine	2.6	2.0	2.1	1.8	1.3	1.5	-
Leucine	3.7	2.9	2.8	2.2	2.0	2.6	1.2
Tyrosine	0.4	1.1	2.2	2.9	2.0	2.3	-
Phenylalanine	1.3	0.7	0.88	3.6	3.0	3.4	-
Lysine	32.2	34.1	33.0	21.3	19.4	15.5	23.1
Histidine	0.44	0.6	0.36	1.7	2.0	0.4	-
Arginine	1.5	1.9	1.8	3.9	4.7	5.3	7.2
N-terminal	Bl	Bl	Bl	Pro	Pro	Pro	Pro
Lys/Arg	21.5	17.9	18.3	5.5	4.1	2.9	3.2
$\frac{Lys+His+Arg}{Asp + Glu}$	2.54	4.4	5.8	0.93	0.97	1.04	2.37

Table 2.2 Amino acid composition in molar ratios of the three H1-histones from Parechinus angulosus gastrula stage embryo compared to a variety of high mobility group histones.

No corrections have been made for losses and incomplete cleavage.

HMG-data from Goodwin et al.
N.D. = not determined.
Bl. = blocked.

2.2 PRIMARY STRUCTURE DETERMINATION OF THE EMBRYO H1-HISTONES

2.2.1 Introduction

Structural protein chemistry is based and developed on the principles of sequential N-terminal degradation (Edman, 1950). These principles have led to the development of an automatic protein sequencer (Edman and Begg, 1967), in which peptides are degraded in solution in the "spinning cup" method. An alternative method based on the covalent linkage of the peptide, in this case through homoserine lactone to a solid phase (Laursen, 1971) was useful for the sequencing of small petides generated by cyanogen bromide cleavage. This was done on an automatic solid phase sequencer as developed by Alk et al., 1981).

The number of repetitive degradations which can be achieved is limited in practice due to imcompleteness of the reaction. Due to the blocked nature of the embryo H1-histones the uncleaved proteins could not be sequenced and fragmentation of the proteins was necesssary. Another problem was that peptides contained large portions of lysine residues, and became so hydrophobic after substitution with phenylisothiocyanate in the first step of the normal sequencing program that they washed out of the sequencer cup. Therefore the peptides were reacted with sulfonated phenylisothiocyanate (S-PITC) (Braunitzer et al., 1970) equivalent to their lysine content for ½ hour at 53°C in Quadrol or N,N-dimethylallylamine (DMAA) buffer, in the sequencer cup. The film was then dried and 1 mg blocked histone H4 from sperm of *Parechinus angulosus* was added in the buffer to cover the dried peptide film as a carrier and stabilizer. The yield of the lysine residues and the N-terminal amino acid were reduced due to the inextractability of the sulfonated thiazolinones. The N-terminal amino acids were also identified by dansylation of a separate aliquot.

The three embryo H1-histones which were partially sequenced in this study had similar characteristics and thus certain fragmentation techniques could be used on more than one protein. The procedures for fragmenting each of the embryo H1-histones will be described individually.

2.2.2 Strategy of Sequence Determination

The proteins were first fragmented into a minimum of large peptides and sequenced as far as possible with the avialable techniques, whereupon the large peptides were fragmented into smaller overlapping or adjacent peptides by chemical and enzymatic cleavage.

Predictions as to the type of peptides generated were made. The gene seuqence of *Psammechinus miliaris* embryo H1-gene of the h22- and h19-clone (Schaffer et al., 1978; and results obtained through personal communication with Professor Birnstiel) were helpful in deciding on fragmentation procedures.

As judged by their amino acid composition the H1-histones in this study contained 2 methionine residues each (Table 2.1) Cyanogen bromide cleaves specifically at methionine residues (Gross, 1967; Witkop, 1968). The peptides produced upon cyanogen bromide fragmentation were sequenced as far as possible and used as substrates for further fragmentation.

Trypsin was used to cleave at the carboxyl side of arginine and lysine residues (Smith, 1967). The lysine residues could be reversibly blocked either with maleic anhydride (Butler and Hartley, 1972) or citraconic anhydride (Atassi and Habeeb, 1972), whereupon trypsin cleaved at arginine residues only. Because of the lysine rich character of the H1 histones, the lysine residues were usually blocked before trypsin digestion.

Staphylococcus aureus protease with a cleavage specificity for glutamic acid (Houmard and Drapeau, 1972; Drapeau, 1976) was used in the fragmentation of the large cyanogen bromide peptide of H1-1.

2.2.3 Nomenclature of Peptides

The scheme for the nomenclature of peptides was the same as that described by Strickland et al., 1977.

The first two or three letters indicated the type of cleavage by which the peptide was generated.

These were: CN = cyanogen bromide; MT = tryptic digestion of maleyated protein or peptide; CT = tryptic digestion of citraconylated protein or peptide; TRYP = tryptic digestion; PEP = pepsin digestion and SP = *Staphylococcus aureus* protease. This was followed by a symbol which indicated the relative elution order on column chromatography. A Roman numeral referred to separation by gel filtration on Sephadex (5.4.3.1) while an Arabic numeral indicated chromatography on carboxymethylcellulose (5.4.3.2). For example the peptide CN-3-TRYP-I-I from H1-1 resulted from cyanogen bromide cleavage of the intact protein followed by tyrptic digestion of peptide CN-3, the peptide eluted as the third peptide from a CMC column (Fig. 2.13). Peptide CN-3 was cleaved by trypsin and the fraction CN-3-TRYP-I eluted as the first fraction from a P-10 column (Fig. 2.15 A). This fraction was rerun on a P-2 column to be eluted as a pure fraction, CN-3-TRYP-I-I, eluting as the first fraction from the P-2 column (Fig. 2.15 B). (Also see Table 2.3).

Peptides from the CMC-columns were desalted depending on size, on Sephadex G25, G15 or G10 with 10 mM HCl as the eluant. Steps on desalting columns were not included in the coding of the peptide, unless some fractionation was achieved.

End group analysis (dansylation) of a peptide at each purification step was done in order to determine the homogeneity of the peptide. If only one amino acid was present or only one band appeared upon polyacrylamide gel electrophoresis, the peptide preparation was presumed to be pure and subjected to amino acid analysis and finally to sequence analysis.

2.2.4 Partial Sequence of H1-1

2.2.4.1 Generation and Purification of Peptides

2.2.4.1.1 Cyanogen Bromide Cleavage

The whole protein was cleaved with cyanogen bromide (5.4.1) and the peptides fractionated on a CMC-column. The peptides were recovered from the CMC-column (6 x 60 mm) using 50 mM Na-acetate/HCl buffer pH 5.5 and a 0-400 mM NaCl gradient (total volume was 200 ml). Figure 2.13 shows the typical elution profile of the cyanogen bromide-cleaved H1-1 histone protein. (See also Table 2.3 for amino acid composition of peptides.) The total amino acid composition of embryo H1-1 indicated the presence of two methionine residues (Table 2.3). Therefore, cleavage should have yielded three peptides, but upon fractionation on a CMC column (9 x 60 mm) five fractions were produced (fig. 2.13). This was due to incomplete cleavage at the methionine residues. Peptide CN-3 was the blocked-amino terminal peptide resulting from cleavage at the first methionyl-residue, while CN-4 was the blocked amino terminal peptide produced by cleavage at the second methionine. The amino acid composition (in mole %) of the peptides from embryo H1-1 did not correspond well with the amino acid composition of the peptides as determined by sequence analysis (Table 2.3). This indicated contamination of the peptides by other peptides from H1-1. These contaminants were not present in sufficient amounts to be detected upon sequencing.

AMINO ACID	H1-1 ~ 238 Residues a	CN-1 ~ 31 Residues	CN-3 ~23 Residues BLOCKED b	CN-4 ~ 54 Residues	CN-5 ~ 186 Residues a	CN-5 -MT-III -III b	CN-5 -MT-I- III b	CN-5 -SP-4 a	CN-5 -SP-11 b	CN-5 -SP-12 b	CN-3 -TRYP- I-I b	CN-3 -TRYP- II-II b	CN-5 -SP-2 a
ASPARTIC ACID	2.7 (6.1)	9.8 (3)	10.4 (2)	4.6	1.6 (2.9)	2.2 (1)	0.3 (0.2)	-	-	-	-	15.6 (1.6)	-
THREONINE	7.9(17.6)	12.1 (5)	10.5 (2)	8.5	7.5(14.1)	2.9 (1)	13.5(10.1)	-	12.3 (8.6)	12.9(11.12)		17.1 (1.7)	-
SERINE	3.1 (7.4)	7.1 (2)	6.0 (1)	4.6	3.1 (5.8)	3.6 (0)	-	-	-	-	-	12.3 (1.3)	-
GLUTAMIC ACID	11.0(25.8)	19.0 (5)	7.6 (1)	6.2	11.6(21.5)	18.2 (7)	7.6 (5.7)	13.9 (1)	1.0 (0.7)	5.2 (3.9)	-	0.2(0)	30.5(3)
PROLINE	6.6(16.3)	0	12.1 (3)	13.8	4.6 (8.5)	0.8 (0)	8.1 (6.0)	-	7.7 (5.3)	7.7 (6.6)	29.7 (2.9)	0.2(0)	-
GLYCINE	2.9 (6.8)	4.1 (1)	2.9 (0)	0.2	3.3 (6.1)	6.2 (0)	0.4 (0.3)	4.8 (0)	-	-	-	0.2(0)	-
ALANINE	20.7(47.1)	7.5 (3)	20.4 (4)	22.9	22.6(42.0)	20.8 (7)	26.9(20.1)	36.8 (5)	14.1 (9.8)	21.4(17.2)	37.4 (3.6)	11.9 (1.2)	21.2 (2)
VALINE	4.1 (9.7)	6.9 (1.5)	6.4 (1)	4.8	3.9 (7.3)	4.7 (1)	3.6 (2.7)	-	2.9 (2.1)	3.4 (2.9)	-	8.5 (0.9)	-
METHIONINE	0.8 (2)	0 (1)	0.1 (1)	-	0 0	-	-	-	-	-	0.2 (1.0)	0.4 (0)	-
ISOLEUCINE	2.6 (6.1)	6.6 (2.5)	6.0 (1)	4.5	1.4 (2.6)	2.3 (0)	-	-	1.4 (1.0)	-	4.5 (0.4)	1.5 (1)	-
LEUCINE	3.7 (8.6)	6.9 (2)	2.3 (0)	0.3	3.9 (7.3)	7.5 (2)	1.3 (1.0)	4.8 (0)	-	1.1 (1.0)	-	-	8.0 (1)
TYROSINE	0.4(0.96)	3.0 (0)	0.3 (0)	-	0.1 (0)	-	-	-	-	-	-	-	-
PHENYL-ALANINE	1.3 (2.9)	3.0 (1)	0.2 (0)	-	1.3 (2.4)	2.3 (0)	-	-	-	-	-	-	-
LYSINE	32.2(75.3)	9.2 (3)	18.9 (4)	24.8	33.6(62.4)	26.6 (8)	38.3(28.6)	41.8 (5)	60.4(41.9)	48.2(40.8)	17.9 (1.7)	32.0 (3.4)	32.4 (2)
HISTIDINE	0.44(1.01)	1.6 (0)	5.6 (1)	4.7	-	-	-	-	-	-	10.3 (1.0)	-	-
ARGININE	1.5 (3.4)	3.2 (1)	0.5 (0)	-	1.4 (2.6)	1.8 (1)	-	-	-	-	-	-	7.8 (1)

Table 2.3 Amino Acid Composition in mole % of P. angulosus Embryo H1-1 and of Peptides used in Sequence Analysis
Figures in parentheses are the number of residues calculated from the amino acid hydrolysis results (a) or determined from the sequence analysis (b).

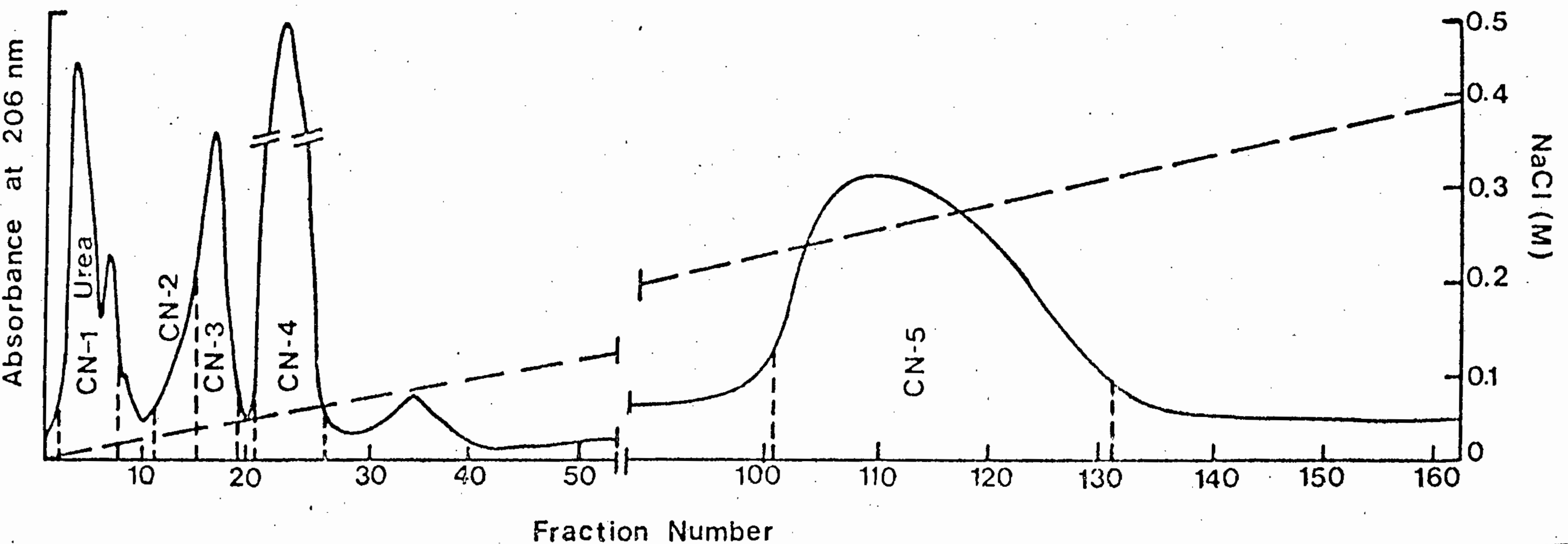


Figure 2.13 Fractionation of a CNBr-digest of *P. angulosus* Hl-1.

Column: 6 x 60 mm Carboxymethylcellulose with 50mM Na-acetate/HCl buffer pH 5,5 as eluant.
Flow rate: 12 ml/hour, cuvette: 3 mm, linear gradient: 0-400 mM NaCl (total volume 200 ml). The dotted lines indicate the fraction cuts.

2.2.4.1.2 Staphylococcus aureus Protease Digestion

CN-5 generated from cyanogen bromide cleavage of the whole H1-1 protein was digested with *S. aureus* protease as described in section 5.4.2.2. Digestion was done in 50 mM ammonium acetate buffer pH 4.0 at 37°C for 18 hours. The cleaved peptides were recovered from a CMC-column (6 x 60 mm), using 50 mM Na acetate/HCl buffer pH 5.5 as eluant and a linear gradient of 0-400 mM NaCl (total volume 300 ml) (Fig. 2.14). A total of thirteen fractions was recovered from the CMC-column. The peptides produced by *S. aureus* protease all had a lysine end group according to dansyl end group analysis. Therefore the larger peptides were electrophoresed on a 2,5 M urea Triton gel for 1½ hours at 15 mA (constant current). This gave a clear indication as to the homogeneity of the fractions. Peptides CN-5-SP-2, CN-5-SP-4, CN-5-SP-11 and CN-5-SP-12 were to sequenced. The amino acid composition of the sequenced peptides is listed in Table 2.3.

2.2.4.1.3 Trypsin Digestion

Trypsin was used to digest the blocked amino terminal peptide CN-3, produced by cyanogen bromide cleavage of the whole protein (2.2.4.1.1). CN-3 was not maleyated since the peptide contained only four lysine residues and no arginine residues (Table 2.3). Two fractions were recovered from a G10 column (Fig. 2.15A), which were rerun on a P-2 column (Fig. 2.15 B & C). Fractions CN-3-TRYP-II-II and CN-3-TRYP-I-I were sequenced. By subtracting the amino acids sequenced of peptides CN-3-TRYP-I-I from the amino acid composition of CN-3, the amino acid composition of the blocked amino terminal end could be determined. The amino acid composition of CN-3-TRYP-II-II (Table 2.3) indicated that it was contaminated by the uncleaved CN-3.

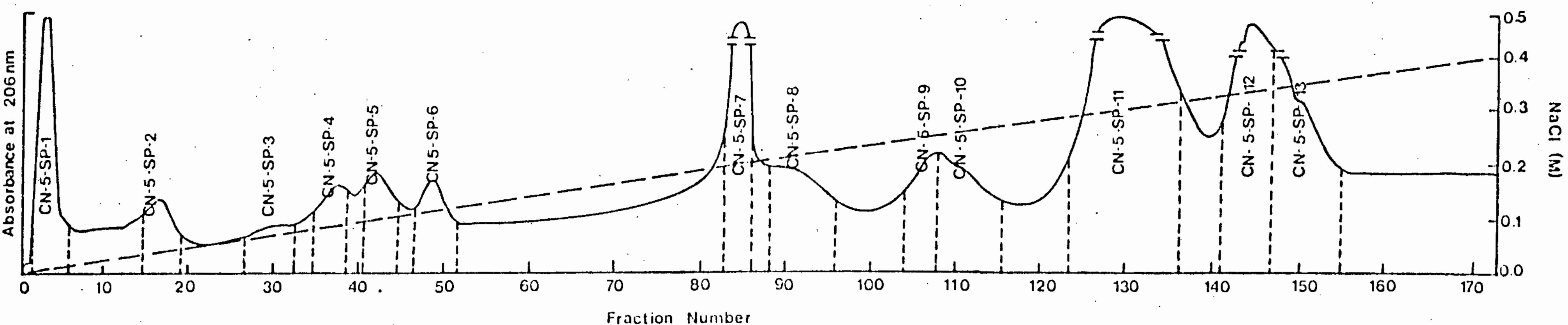


Figure 2.14 Fractionation of S. aureus protease digest of CN-5 (Fig. 2.13).

Column: 6 x 60 mm CMC, eluant: 50 mM Na-acetate/HCl pH 5,5, linear gradient: 0-400 mM NaCl (total volume 300 ml), flow rate: 12 ml/hour, cuvette: 3 mm. The dotted lines indicate the fraction cuts.

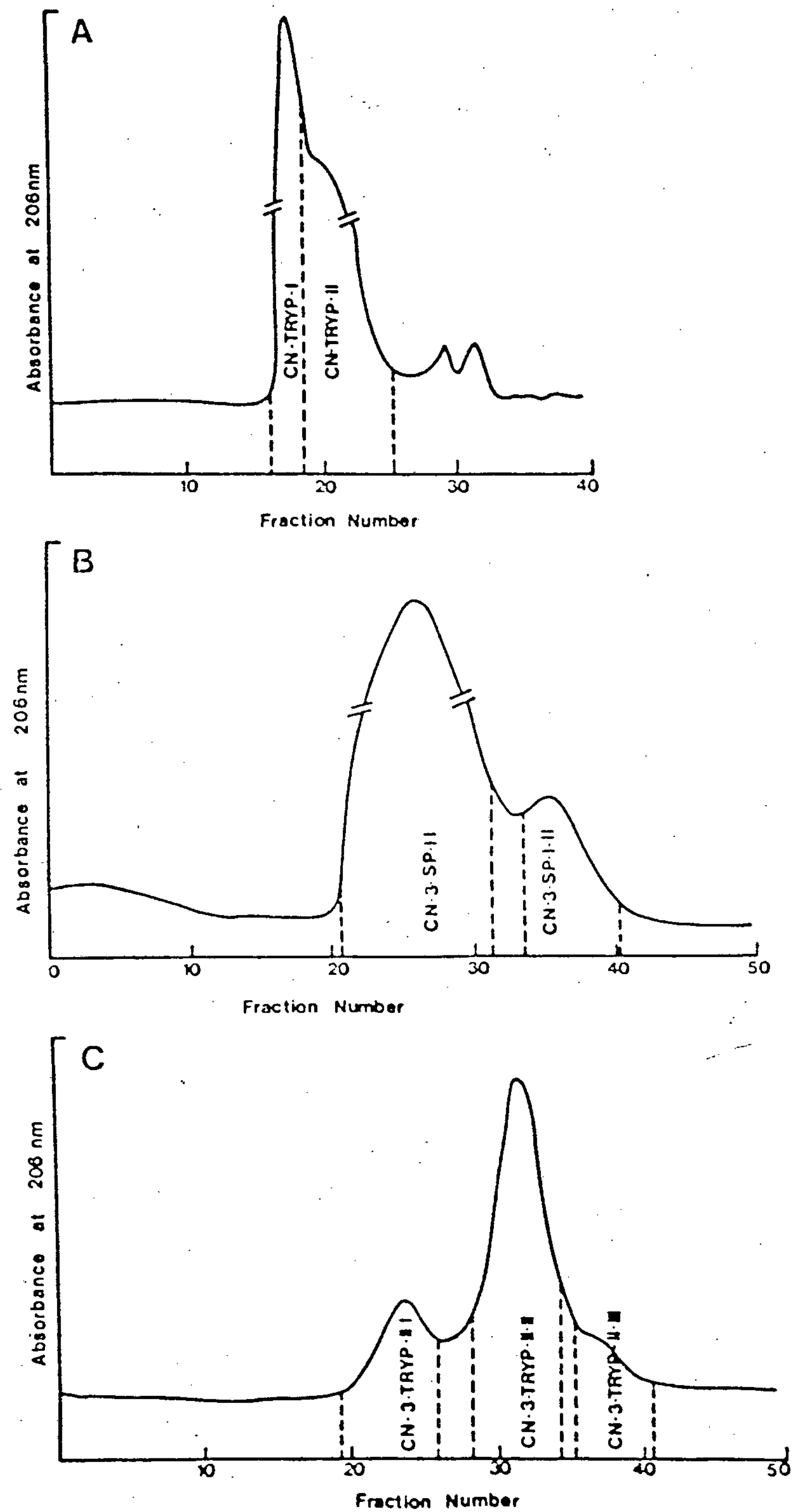


Figure 2.15 Fractionation of a tryptic digestion of CN-3 of P. angulosus embryo H1-1

A. Column: 26 x 100 mm Sephadex G-10 medium, eluant: 10 mM HCl, cuvette: 3 mm, 0,2 mg CN-3 (Figure 2.13) digested with trypsin, Fraction size: 2,8 ml.

B. Column: 26 x 100 mm Biogel P-2 medium, eluant: 10 mM HCl, cuvette: 3 mm, sample: fraction CN-3-TRYP-I from A (above), Fraction size: 2,5 ml.

C. Column: 26 x 100 mm Biogel P-2 medium, eluant: 10 mM HCl, cuvette: 3 mm, sample: fraction CN-3-TRYP-II from A (above), fraction size: 2,5 ml.

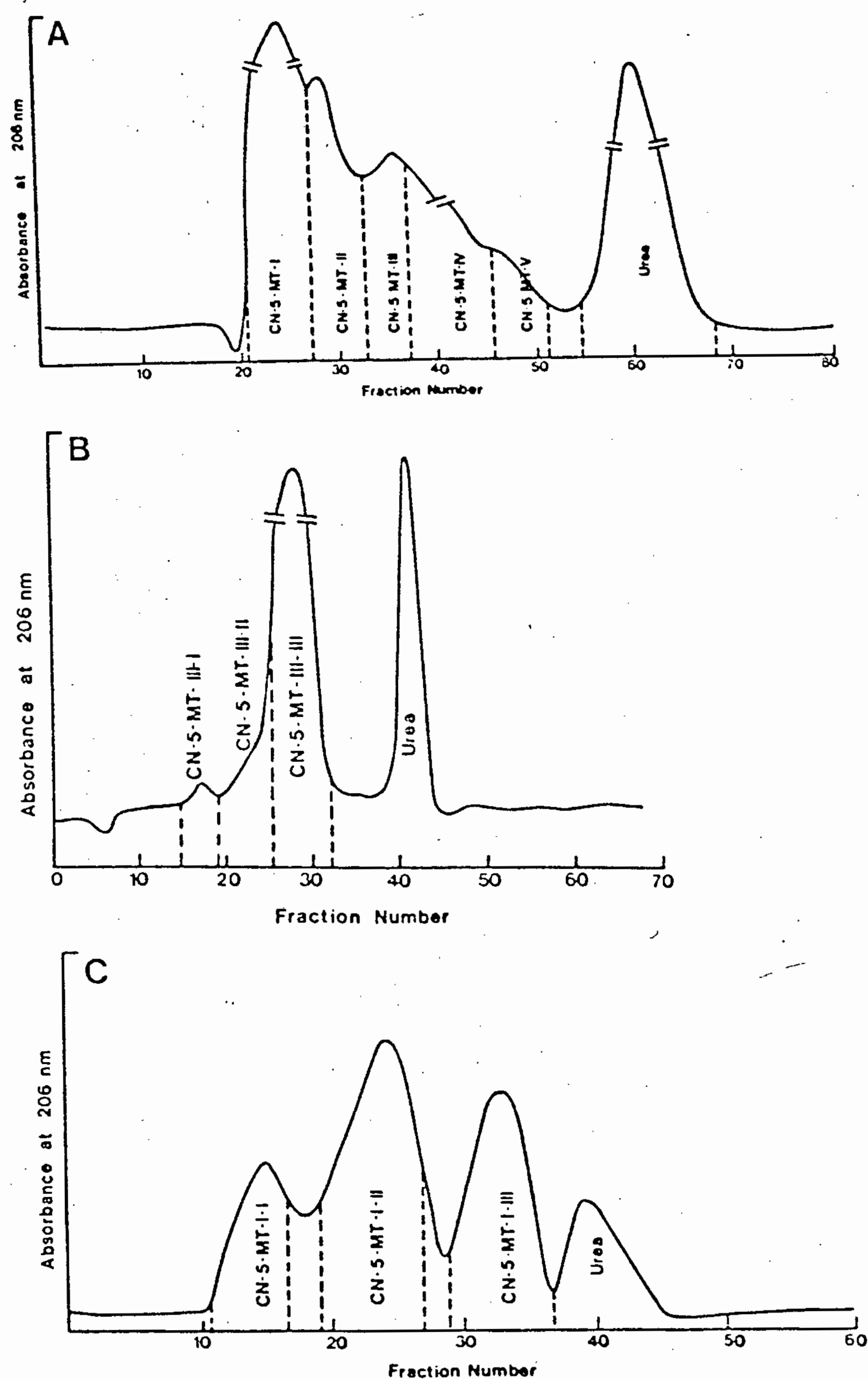


Figure 2.16 Fractionation of a tryptic digestion of maleylated CN-5 of *P. angulosus* embryo H1-1.

A. Column: 26 x 100 mm Sephadex G50 medium, eluant: 100 mM NH_4 HCO_3 pH 8,5 cuvette 3 mm;, sample: 39 mg CN-5 (Figure 2.13) maleylated and digested with trypsin, fraction size: 2,6 ml.

B. Column: 26 x 100 mm Sephadex G50 medium, eluant: 100 mM NH_4 HCO_3 pH 8,5, cuvette: 3mm, sample: CN-5-MT-III from A (above), fraction size 2,5 ml.

C. Column: 100 mm Sephadex G100 medium, eluant: 10 mM HCl, cuvette: 3 mm, sample: demaleylated CN-5-MT-I from A (above), fraction size: 2,6 ml.

Trypsin was also used to digest maleyated CN-5 which represented the carboxyl terminal peptide recovered after cyanogen bromide cleavage of the whole protein (2.2.4.1.1).

Peptide CN-5-MT-III-III was recovered from a G50-column and rerun on the G50-column for further purification (Fig. 2.15 A & B). CN-5-MT-I-III was recovered from the G-50 column and rerun on a G100-column (Fig. 2.15 A & C) (see Table 2.3 for amino acid composition).

Peptide CN-5-MT-III-III was the result of cleavage at the carboxyl-side of phenylalanine. This cleavage at phenylalanine might have been the result of contamination of the trypsin with a chymotryptic type of activity or trypsin might have a specificity for a phenylalanyl-lysine-peptide bond.

2.2.4.2 Alignment of Peptides and Deduction of Partial Sequence of H1-1

Parechinus angulosus embryo histone H1-1 was cleaved by chemical and enzymatic means into a total of ten peptides (Fig. 2.19) which were partially or completely sequenced. Alignment was done by initially comparing the sequenced peptide structure and composition to that of the gene sequences of *Psammechinus miliaris* embryo h19 and h22-clones (Schaffner et al., 1978; Birnstiel - personal communications).

Peptide CN-5, the large carboxyl terminal peptide, generated from cyanogen bromide cleavage of the whole H1-protein (2.2.4.1.1), was sequenced on the liquid phase sequencer for 57 steps. The sequenced 57 steps represented residues 55-114 (Fig. 2.18 B). Peptide CN-5 was maleylated and subjected to tryptic cleavage. Possible chymotryptic activity present in the trypsin preparation caused cleavage at phenylalanyl-residue 85. This peptide, CN-5-MT-III-III was sequenced for 16 steps. These sequenced steps represented residues 87-102 (Fig.

2.18 F). The carboxyl terminal lysine-rich peptide obtained upon tryptic digestion, CN-5-MT-I-III (2.2.4.1.3), was sequenced for 15 steps, i.e. from residue 114-130 (Fig. 2.18 E).

Peptide CN-5 was digested with S. aureus protease into numerous peptides. By the criteria of sequence analysis a number of these peptides were found to be pure. Four of them were positioned in the unsequenced part of the large carboxyl terminal end (Fig. 2.18 G-J). Peptide CN-5-SP-2 confirmed the sequence for residues 107-112 (Fig. 2.18 G). Residues 115-124 were identified by sequence analysis of CN-5-SP-4 (Fig. 2.18H) and this overlapped with the sequenced part of peptide CN-5-MT-I-III (Fig. 2.18 E). The sequence analysis of peptide CN-5-SP-12 represented residues 124-152 (Figure 2.18 J). Residues 172-200 were identified by sequencer analysis of CN-5-SP-II (Fig. 2.18 I). The primary structure of the amino terminal end of histone H1-1 was determined by using CN-3 as substrate for trypsin. Assignments of residues 6-21 were made via sequence analysis of CN-3-TRYP-II-II and CN-3-TRYP-I-I (Fig. 2.18 C & D). The amino acid composition of the first 5 amino acids of the blocked amino end was calculated by subtraction (Fig. 2.19).

Peptide CN-1 was hooked up via the carboxyl terminal homoserine to a solid phase resin (4.5.2) and subjected to automatic sequential degradation on the solid phase sequencer. Twenty nine steps were determined and the last two amino acids were calculated by subtraction. CN-1 represented residues 23-53 (Fig. 2.18 A). The amino acid compositions of the uncleaved protein and of the overlapping peptides (Table 2.3) corresponded to the composition found by sequence analysis (Fig. 2.18). The partial amino acid sequence of Parechinus angulosus embryo histone H1-1 obtained from these data is given in Figure 2.19. Out of the total of approximately 238 residues, 170 have been unequivocally positioned.

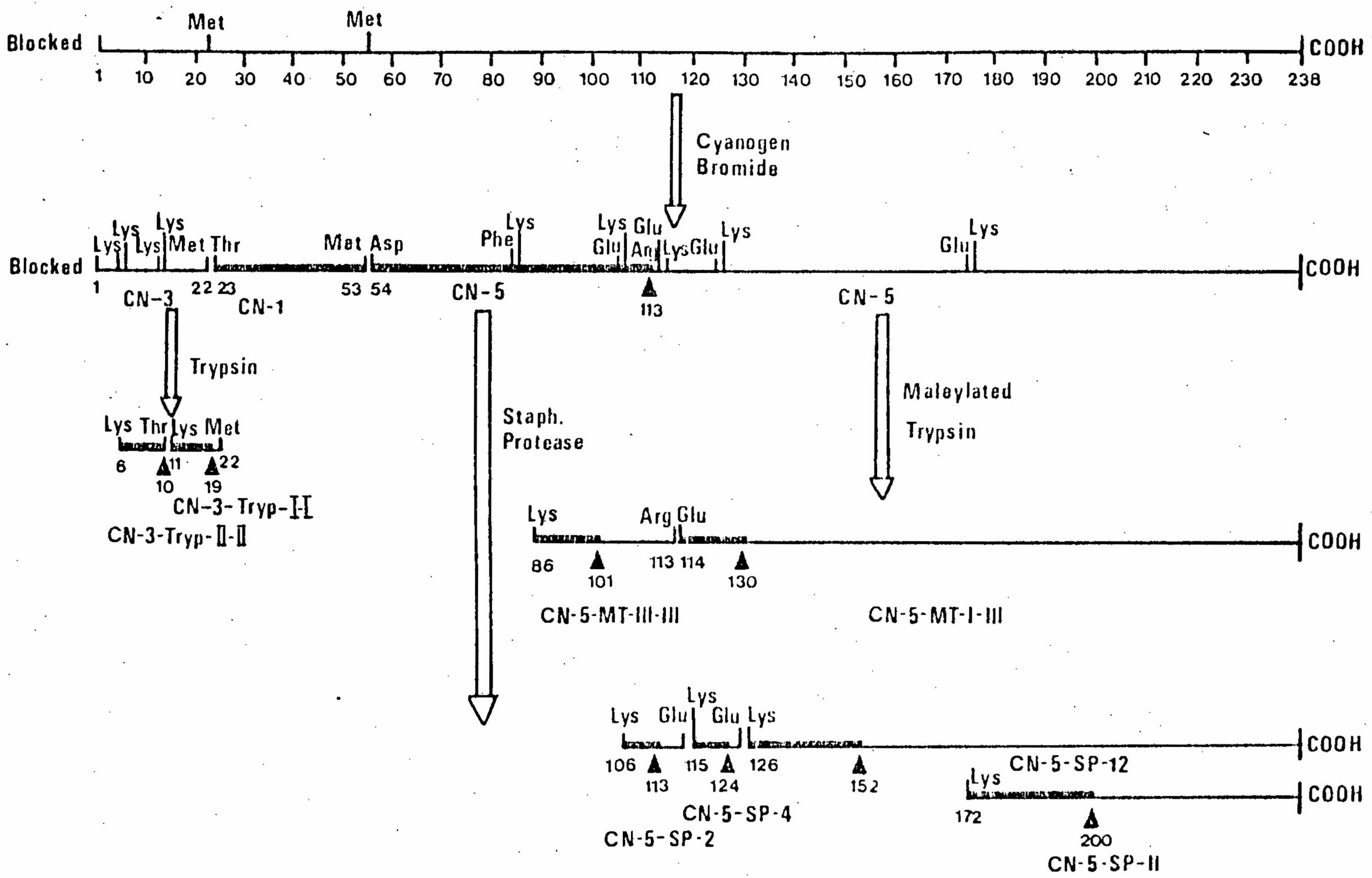


Figure 2.17 Alignment of peptides generated from P. angulosus embryo H1-1. Arrow indicates final residue sequenced in that peptide and the thick line indicates the sections that have been sequenced.

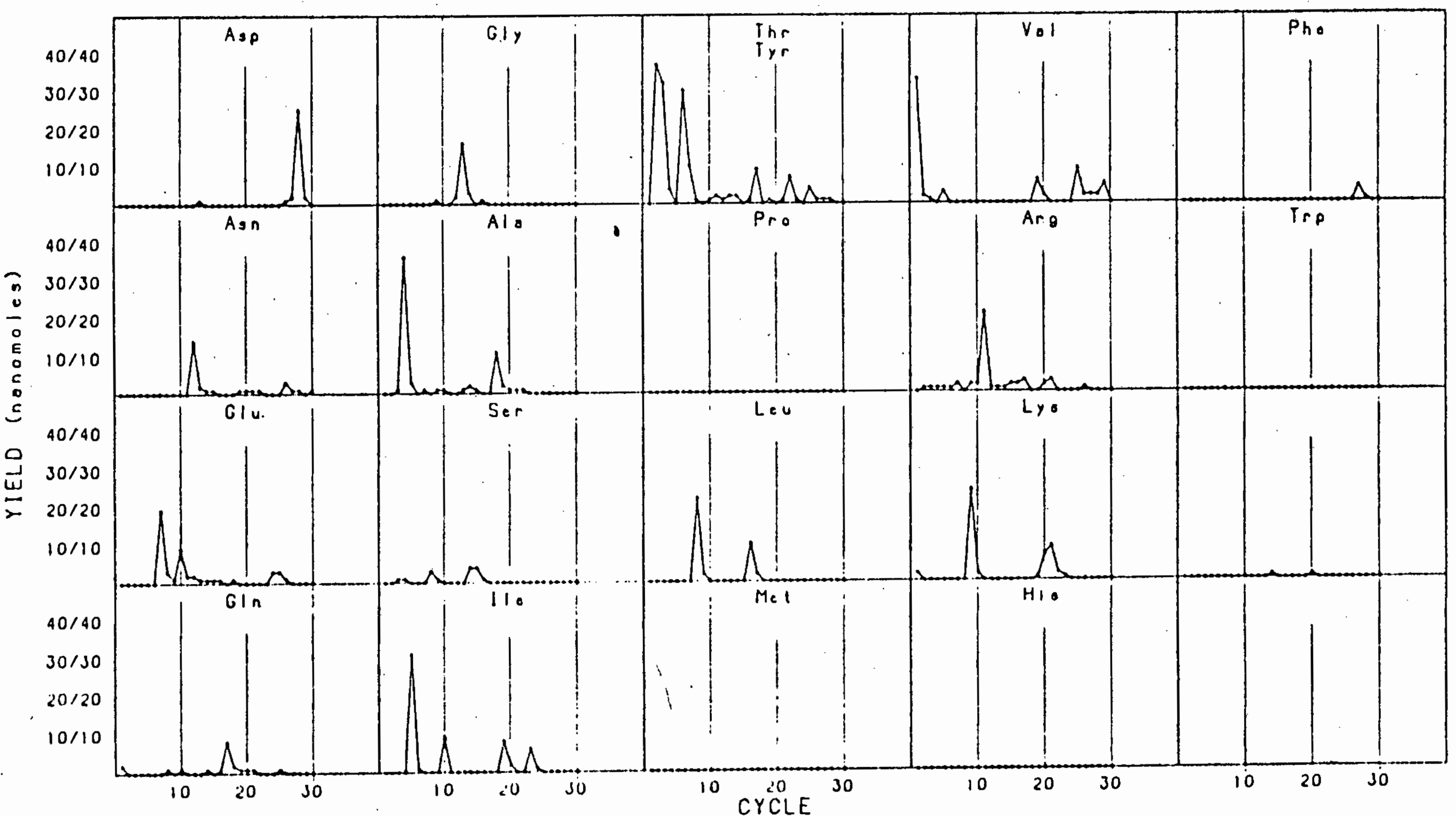
Figure 2.18 Automatic Sequence Analysis of Parechinus angulosus Histone H1-1 Peptides

The amount of peptide subjected to degradation varied between 20 and 200 nmoles. Amounts of amino acid derivative below 0.5 nmoles have been recorded as 0. Phenylthiohydantoins were identified by high-pressure liquid chromatography using gradient and isocratic elution in a methanol system (4.5.3.2). (●——●) yield in nmoles; scale changes are indicated by a break in the connecting line between two steps. (+) yield due to the presence of a second peptide identified by subtracting the known sequence of the first peptide or determined by varying amounts of the two peptides present in the sequencing mixture. (X) yield due to a third peptide. The appearance of a contaminant co-eluting with Pth-Val in the DMAA-peptide program led to uncertainties in the assignment of valine as the level of the contaminant progressively increased, masking the valine signal. All peptides sequenced in the Liquid Phase Sequencer were reacted with sulfonated phenylisothiocyanate (S-PITC) equivalent to their lysine content and 1 mg histone H4 (from P. angulosus sperm) was added as a carrier to the peptide in the cup. (A) Yield of phenylthiohydantoins recovered by degradation of CN-1 on the solid phase sequencer. (B-J) Yields of phenylthiohydantoins recorded by degradation of CN-5, CN-3-TRYP-II-II, CN-3-TRYP-I-I, CN-5-MT-I-III, CN-5-MT-III-III, CN-5-SP-2, CN-5-SP-4, CN-5-SP-11 and CN-5-SP-12 on the liquid phase sequencer.

(A) SEA URCHIN EMBRYO H1-1,CN-1

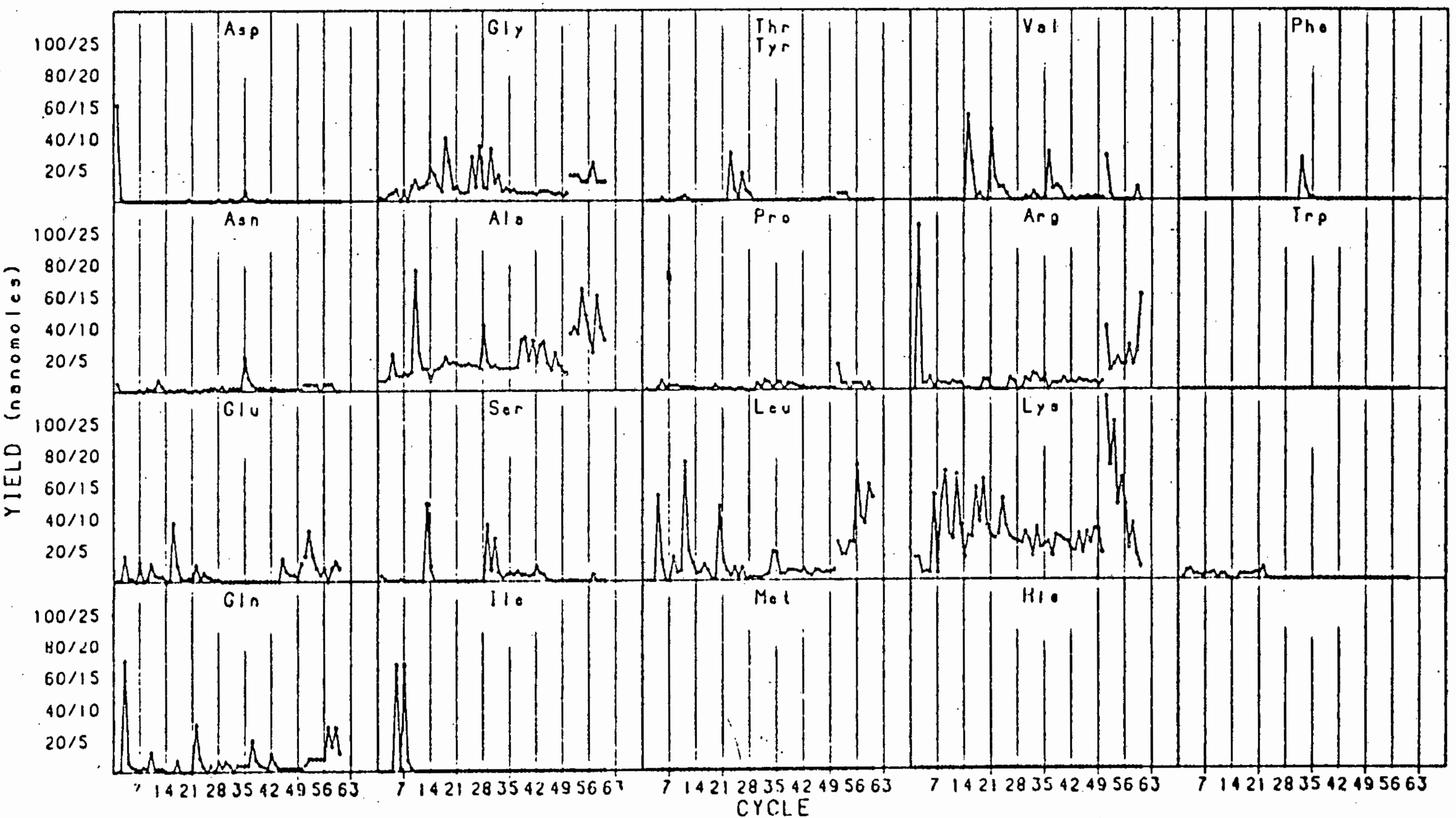
VAL-THR-THR-ALA-ILE(5)-THR-GLU-LEU-LYS-GLU(10)-ARG-ASN-GLY-SER-SER(15)-LEU-GLN-ALA-ILE-LYS(20)-

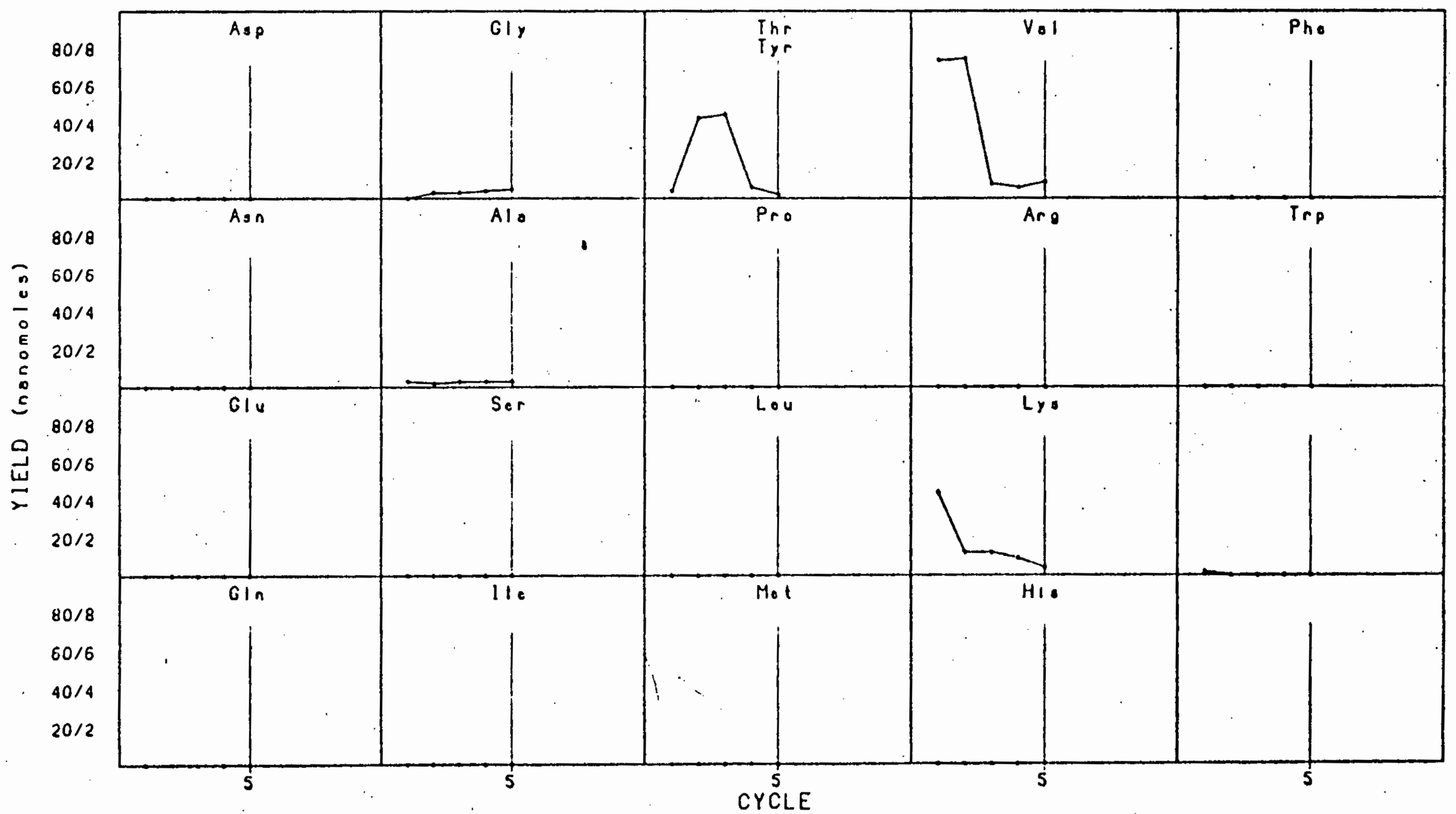
LYS-TYR-ILE-GLY-THR(25)-ASN-(PHE)-ASP-VAL-GLN(30)-(MET)



(B) P.ANGULOSUS EMBRYO H1-1, CN-5

ASP-ARG-GLN-LEU-ILE(5)-LYS-ILE-LYS-LYS-ALA(10)-LEU-LYS-SER-GLY-VAL(15)-GLU-LYS-GLY-LYS-LEU(20)-
VAL-GLN-THR-LYS-GLY(25)-THR-GLY-ALA-SER-GLY(30)-SER-PHE-LYS-LEU-ASN(35)-VAL-GLN-ALA-ALA-LYS(40)-
ALA-GLN-ALA-ALA-GLU(45)-LYS-ALA-LYS-LYS-GLU(50)-LYS-GLU-LYS-ALA-LYS(55)-LEU-GLN-ALA-GLN-ARG(60)-

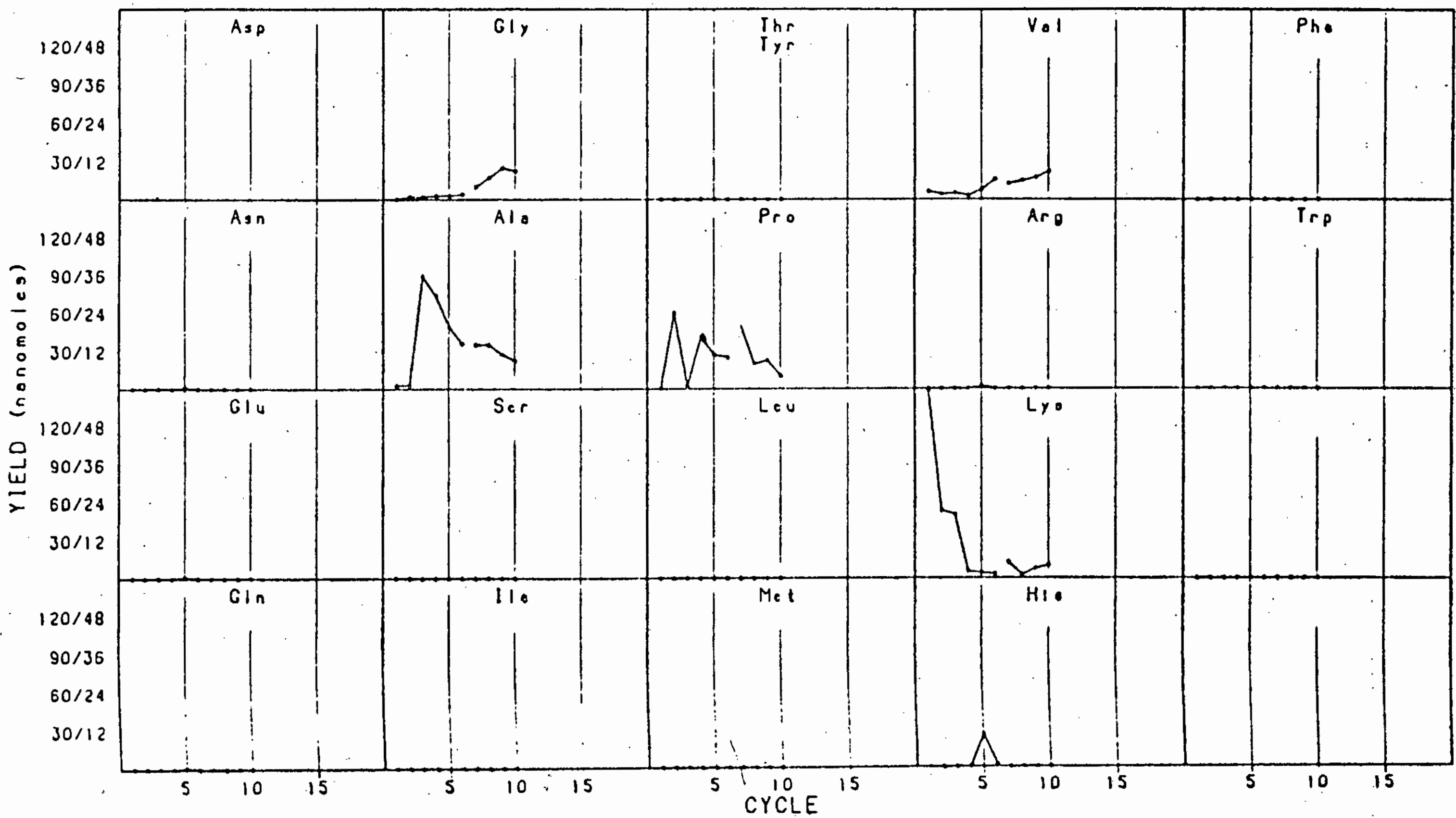




(C) P.ANGULOSUS EMBRYO H1-1 CN-3-TRYP-II-II
4
(LYS)-VAL-THR-THR-(LYS)
4
(LYS)-LYS-VAL-THR-THR
Asp
Gly
Thr
Tyr
Val
Phe
Asn
Ala
Pro
Arg
Trp
Glu
Ser
Leu
Lys
Gln
Ile
Met
His
80/8
60/6
40/4
20/2
YIELD (nanomoles)
5
CYCLE

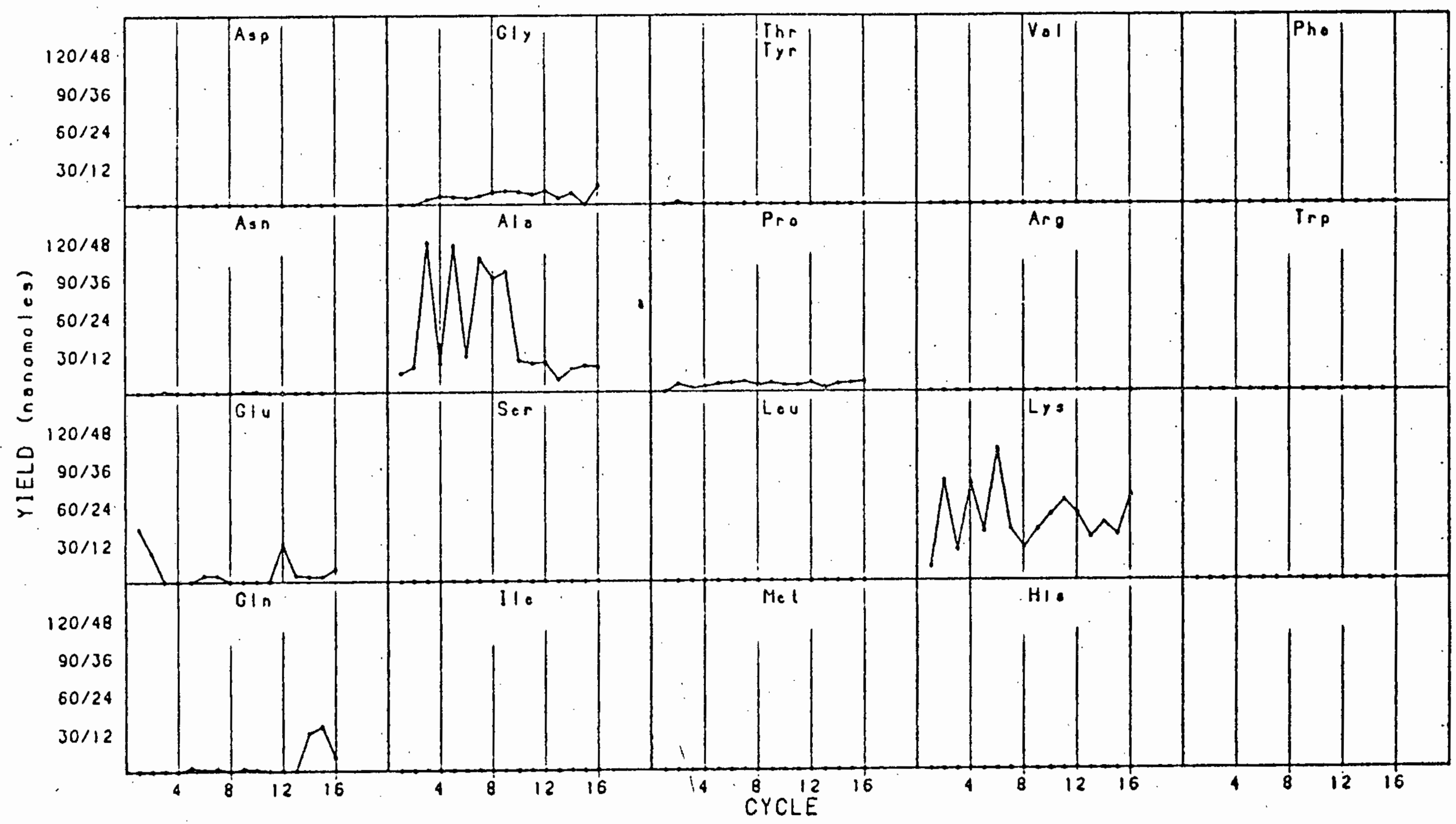
(D) P. ANGULOSUS EMBRYO HI-1, CN-3-TRYP-I-I

(LYS)-LYS-PRO-ALA-ALA-HIS-PRO-PRO-ALA-(ALA-GLU-MET)



(E) P. ANGULOSUS EMBRYO HI-1.CN-5-MT-I-III

(ARG) GLU-LYS-ALA-LYS-ALA(5)-LYS-ALA-ALA-ALA-LYS(10)-LYS-GLU-(LYS)-GLN-GLN(15)-LYS

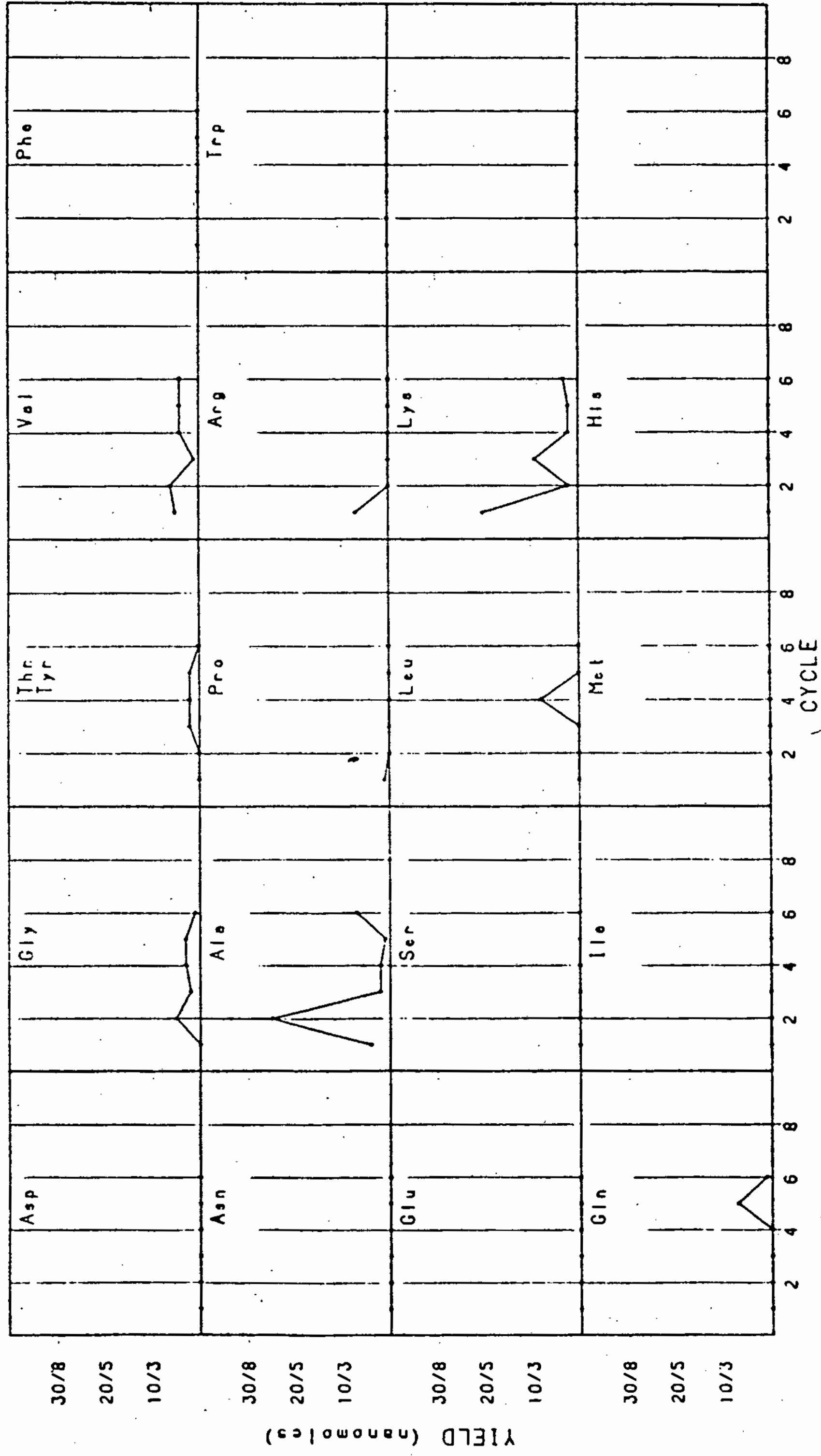


(F). P.ANGULOSUS EMBRYO H1-1, CN-5-MT-III-III

(PHE)-LYS-LEU-ASN-VAL-GLN-ALA-ALA-LYS-ALA-GLN-ALA-ALA-GLU-LYS-ALA-LYS

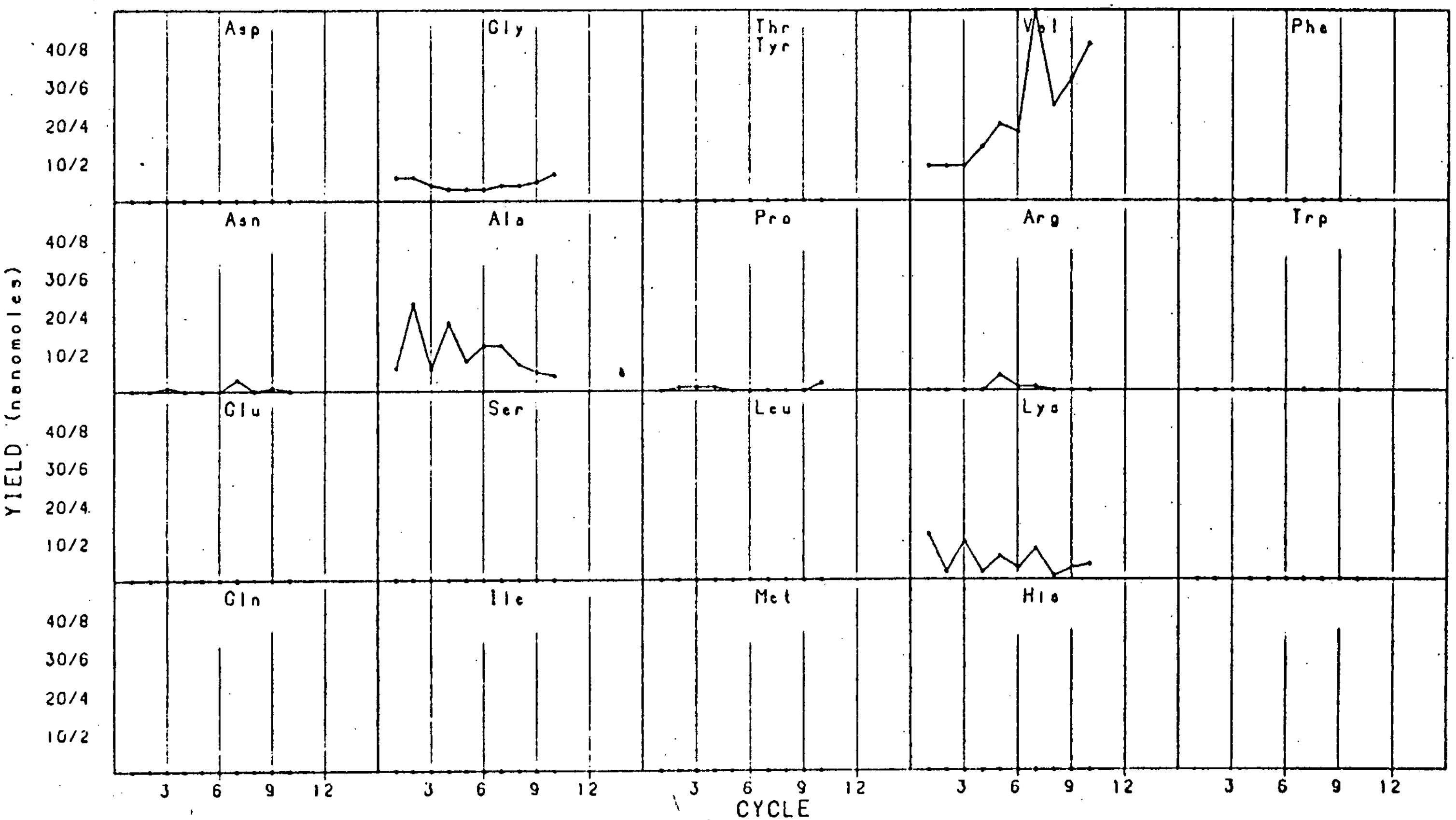
(G) P. ANGULOSUS EMBRYO H1-1, CN-5-SP-2

(GLU)-LYS-ALA-LYS-LEU-GLN-ALA (5 above GLN)



(H) P. ANGULOSUS EMBRYO H1-1, CN-5-SP-4

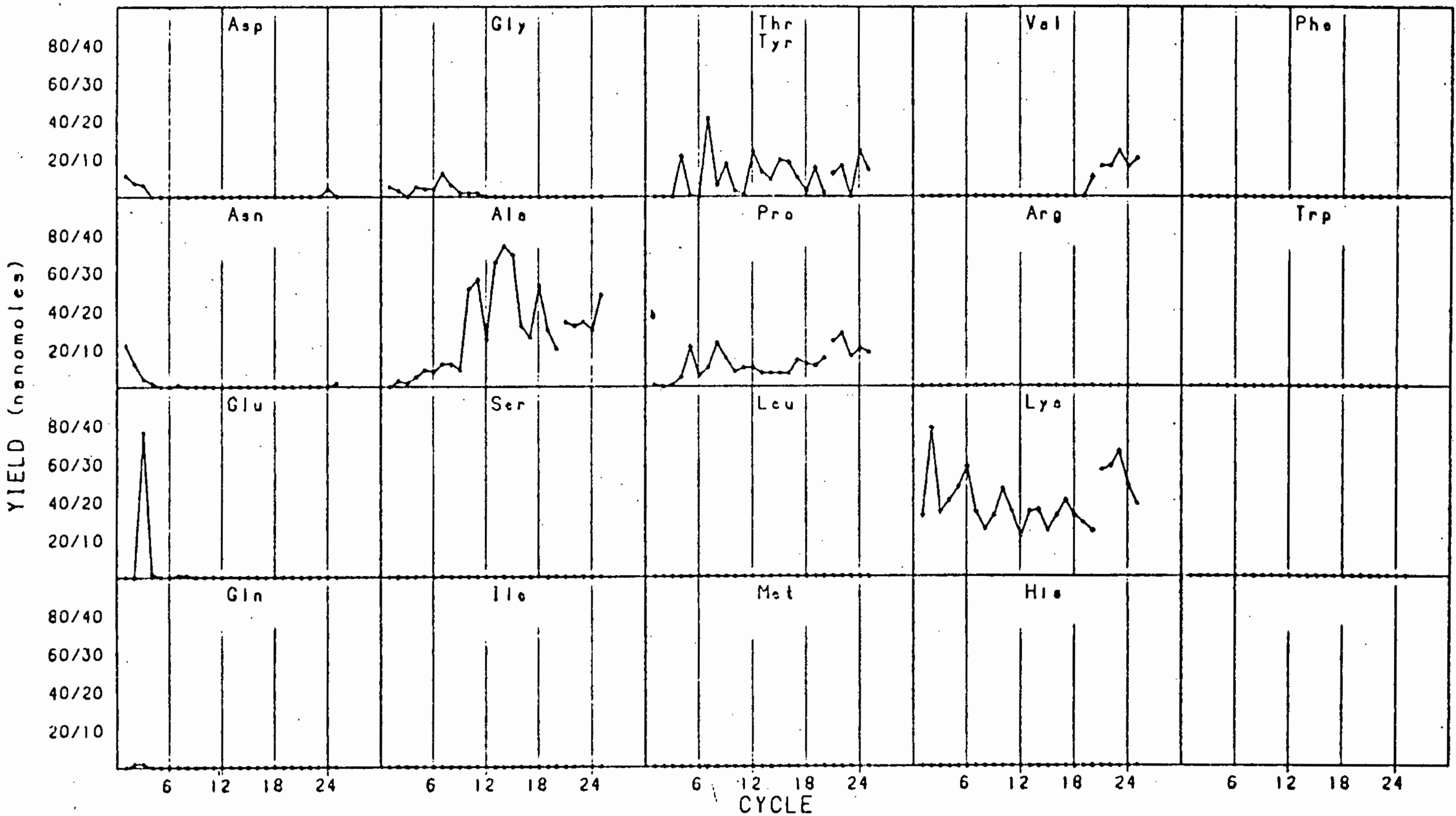
(GLU)-LYS-ALA-LYS-ALA-LYS-ALA-ALA-ALA-LYS-LYS (5 and 10 printed above the 5th and 10th residues)



(I) P. ANGULOSUS EMBRYO H1-1, CN-5-SP-11

(GLU)-LYS-LYS-GLU-LYS-LYS[5]-LYS-THR-PRO-LYS-LYS[10]-ALA-THR-ALA-ALA-THR[15]-LYS-LYS-ALA-THR-PRO[20]-LYS-THR-VAL-THR

(GLU)-LYS-LYS-LYS-THR-PRO[5]-LYS-LYS-ALA-THR-ALA[10]-ALA-THR-LYS-LYS-ALA[15]-THR-PRO-LYS-THR-VAL[20]-THR-(LYS-LYS-PRO-ALA[25])



(J) P. ANGULOSUS EMBRYO H1-1. CN-5SP-12

(GLU)-LYS-GLN-GLN-LYS-ALA[5]-THR-ALA-ALA-LYS-LYS[10]-ALA-LYS-ALA-ALA-PRO[15]-LYS-LYS-LYS-PRO-ALA[20]-LYS-LYS-ALA-VAL-(LYS[25]-LYS-ALA)

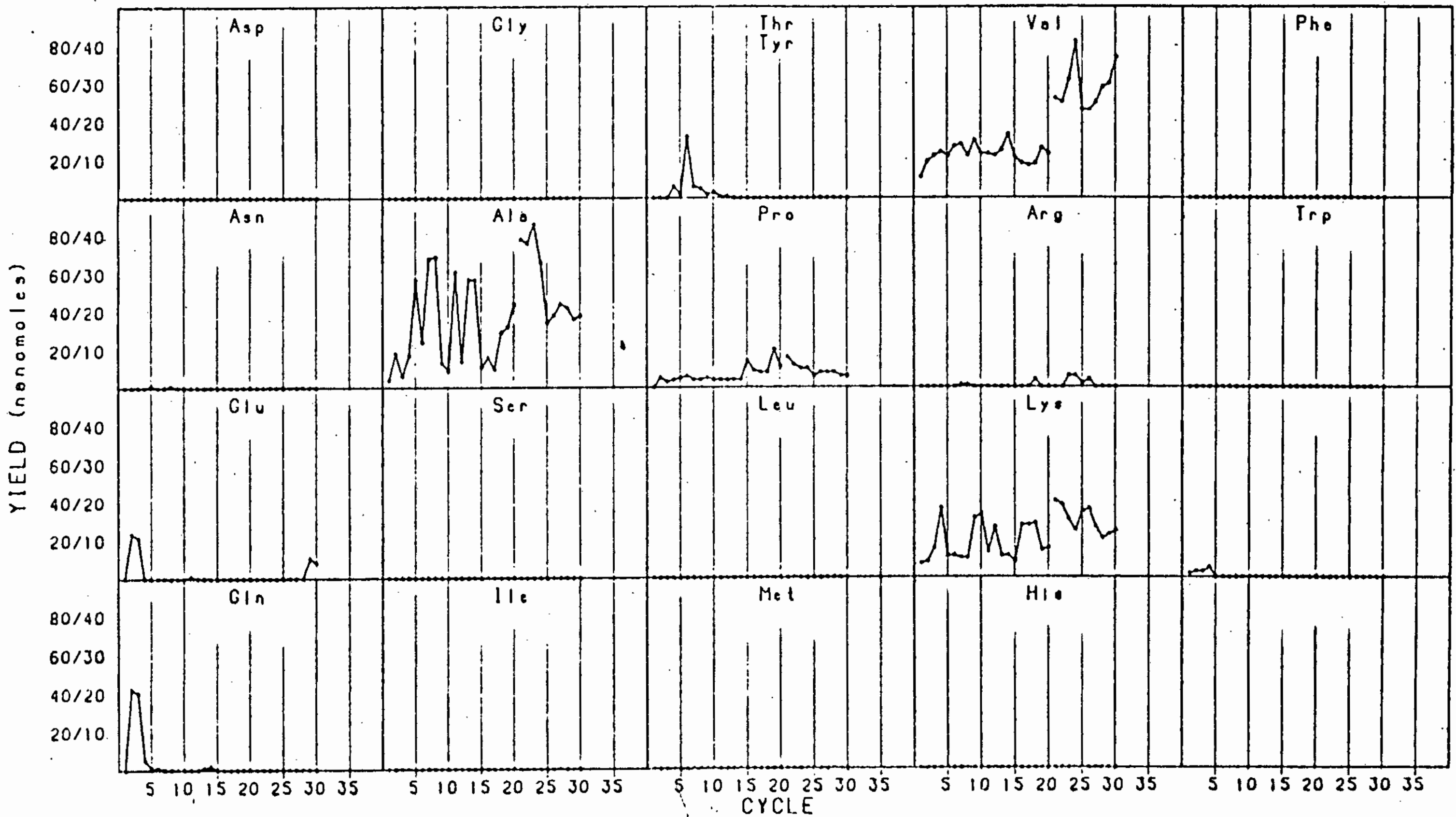


Figure 2.19 Partial Amino Acid sequence of *P. angulosus* embryo histone H1-1. Solid lines indicate the extent of the amino acids sequenced while the interrupted line indicates the distance to the end of that peptide. Unsequenced part of the histone was filled in by a dotted line.

Types of Cleavage: CN = Cyanogen Bromide — TRYP = Trypsin

SP = *Staphylococcus aureus* protease — MT = Maleylated Trypsin

6 10

Blocked NH_2-end-(Ala, Asp_2, -Ser-Ile) - Lys- Lys - Val - Thr - Thr -

|—— CN-3-TRYP-I-I —— -|

15 20

Lys - Lys - Pro - Ala - Ala - His - Pro - Pro - Ala - (Ala - Glu) - Met -

|—— CN - 3 - TRYP - I - I —— - - - -|

25 30 35

Val - Thr - Thr - Ala - Ile - Thr - Glu - Leu - Lys - Glu - Arg - Asn - Gly

|—— CN-1 ——

40 45

Ser - Ser - Leu - Gln - Ala - Ile - Lys - Lys - Tyr - Ile - Gly - Thr - Asn -

—— CN-1 ——

50 55 60

(Phe) - Asp - Val - Gln - Met - Asp - Arg - Gln - Leu - Ile - Lys - Ile

—— CN-1 —— - - -| |—— CN-5 ——

65 70

Lys - Lys - Ala - Leu - Lys - Ser - Gly - Val - Glu - Lys - Gly - Lys -

—— CN-5 ——

75 80 85

Leu - Val - Gln - Thr - Lys - Gly - Thr - Gly - Ala - Ser - Gly - Ser - Phe - Lys

—— CN-5 ——

|—— CN-5-MT III-III

90 95

Leu - Asn - Val - Gln - Ala - Ala - Lys - Ala - Gln - Ala - Ala - Glu - Lys -

—— CN-5 ——

—— CN-5-MT-III-III ——

100 105 110

Ala - Lys - Lys - Glu - Lys - Glu - Lys - Ala - Lys - Leu - Gln - Ala

—— CN-5 ——

—— - - - - - - - - - - - - CN-5-MT-III-III - - - - - - - - - -

|—— CN-5-SP-2 ——

115 120

Gln - Arg - Glu - Lys - Ala - Lys - Ala - Lys - Ala - Ala - Ala - Lys -

—— - - - - - - - - - - CN-5 - - - - - - - - - - - - - - - - - -

- - - - -| -CN-5-MT-III-III

- - CN-5-SP-2 -|

|—— CN-5-MT-I-III ——

|—— CN-5-SP-4 ——

125 130 135
Lys - Glu - Lys - Gln - Gln - Lys - Ala - Thr - Ala - Ala - Lys - Lys -

CN-5
CN-5-MT-I-III
CN-5-SP-4
CN-5-SP-12

140 145
Ala - Lys - Ala - Ala - Pro - Lys - Lys - Lys - Pro - Ala - Lys - Lys -

CN-5
CN-5-MT-I-III
CN-5-SP-12

150
Ala - Val - (Lys - Lys - Ala) ....

CN-5
CN-5-MT-I-III
CN-5-SP-12

....

CN-5
CN-5-MT-I-III
CN-5-SP-12

172 180
Glu - Lys - Lys - Glu - Lys - Lys - Lys - Thr - Pro - Lys - Lys - Ala - Thr

CN-5
CN-5-MT-I-III
CN-5-SP-12
CN-5-SP-11

185 190 195
Ala - Ala - Thr - Lys - Lys - Ala - Thr - Pro - Lys - Thr - Val - Thr -

CN-5
CN-5-MT-I-III
CN-5-SP-12
CN-5-SP-11

200
(Lys - Lys - Pro - Ala) .... 238 COOH

CN-5
CN-5-MT-I-III
CN-5-SP-12
CN-5-SP-11

2.2.5 Partial Sequence of Parechinus angulosus Embryo H1-2

2.2.5.1 Generation and Purification of Peptides

2.2.5.1.1 Cyanogen Bromide Cleavage

The intact protein was cleaved with cyanogen bromide (5.4.1) and the peptides fractionated on a CMC column (9 x 60 mm) using 50 mM Na acetate/HCl buffer pH 5.5 and a 0-600 mM NaCl gradient (total volume 400 ml). Figure 2.20 shows the elution profile of the cyanogen bromide cleaved protein. This elution profile was not what would be expected if the protein had only two methionine residues. Six major peaks eluted from the CMC-column instead of the expected three. This indicated heterogeneity.

However, protein H1-2 eluted as a single peak upon CMC fractionation of the perchloric acid extract from sea urchin embryo nuclei (Fig. 2.10) and ran as a single main band plus a faint faster migrating band on Triton-acid-urea polyacrylamide gels (Fig. 2.11). Nevertheless, the cyanogen bormide fractionation results together with the amino acid analysis and sequence analysis of the CNBr-peptides clearly indicated that there were at least two proteins present in the H1-2 fraction. The two proteins present in the H1-2 fraction had very similar amino acid compositions, overall charge and molecular weights according to their behaviour in urea-Triton X-100 (Fig. 2.11) and SDS gels (Fig. 2.9) and their behaviour on ion exchange (Fig. 2.10) and gel exclusion chromatography.

The amino acid composition of the cyanogen bromide fractions (Table 2.4) showed that the composition of CN-1 was very similar to that of CN-2; and the amino acid composition of CN-4 compared very well with that of CN-5 and similarly the amino acid composition of CN-7 resembled that of CN-8 (Table 2.4).

Peptides CN-4 and CN-5 both had blocked amino end, showing negative results when subjected to dansylation and sequencing.

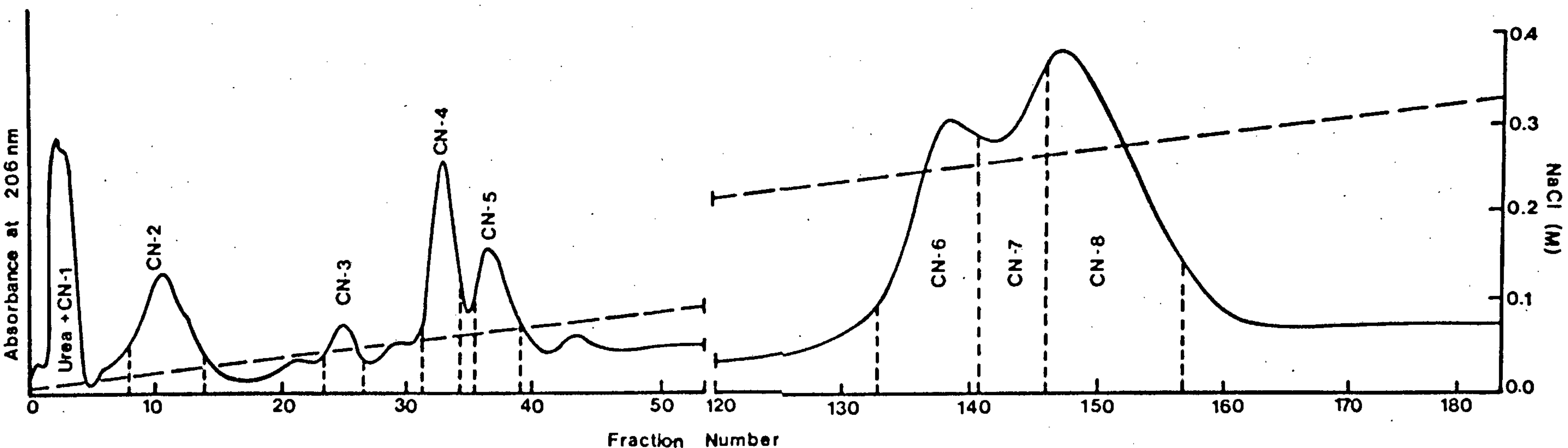


Figure 2.20 Fractionation of a cyanogen bromide digest of *P. angulosus* embryo H1-1.

Column: 9 x 60 mm carboxymethylcellulose with 50 mM Na acetate/HCl buffer pH 5,5 as eluant, flow rate: 12 ml/hour, cuvette: 3 mm, linear gradient: 0-600 mM NaCl (total volume 400 ml). Dotted lines indicate the fraction cuts.

2.2.5.2 Alignment of Peptides and Deduction of Partial Sequence

P. angulosus embryo histone H1-2 was cleaved only by chemical means, namely with cyanogen bromide. The fractionation of H1-2 after cyanogen bromide cleavage yielded a series of peptides (Fig. 2.20) due to the heterogeneity of H1-2. According to the amino acid analysis (Table 2.4) of these peptides, there were at least two proteins present in the H1-2 fraction with very similar amino acid compositions.

Peptides CN-4 and CN-5 had similar amino acid compositions (Table 2.4) and both had a blocked amino terminal end. Thus CN-4 and CN-5 were regarded as the amino terminal peptides of two proteins.

The two small peptides CN-1 and CN-2 were the two peptides which by omology to the histone H1-1 fitted between the two methionine residues of the two respective proteins of the H1-2 fraction. Both CN-1 and CN-2 had a valine amino terminal end group and similar amino acid compositions (Table 2.4).

Peptides CN-6, CN-7 and CN-8 by composition were the large carboxyl-terminal peptides generated by cyanogen bromide cleavage. These peptides CN-6, CN-7 and CN-8 on Triton-2 M urea polyacrylamide gel displayed the heterogenous character of histone H1-2 fraction (Figure 2.21).

CN-6 resolved into a series of peptides on polyacrylamide gel electrophoresis (Fig. 2.21 lanes 3 & 4). End group analysis showed mainly valine (4.3.5.1) as the amino terminal amino acid. Sequence analysis of CN-6 (Fig. 2.22 C) demonstrated that CN-6 was the result of cleavage at the first methionine residue but no cleavage at the second methionine in the proteins, probably due to oxidation of that methionine residue.

The polyacrylamide gel of CN-7 (Fig. 2.21 lanes 5 & 6) showed that this fraction contained a slow migrating band that corresponded to uncleaved H1-2 plus a band that migrated faster than the uncleaved H1-2. This band was unique to fraction CN-7. Fraction CN-7 also contained the two bands dominant in fraction CN-8.

AMINO ACID	H1-2 ~ 246 Residues	CN-1 ~ 28 Residues	CN-2 ~ 31 Residues	CN-4 ~ 23 Residues	CN-5 ~ 31 Residues	CN-6 ~ 205 Residues	CN-7 ~ 175 Residues	CN-8 ~ 201 Residues
Aspartic Acid	2.4 (5.9)	6.6 (1.7)	6.9 (1.9)	5.2 (1.1)	5.3 (1.6)	2.07 (4.2)	1.1 (2.0)	0.86 (1.8)
Threonine	3.4 (8.5)	3.9 (1.0)	3.1 (0.9)	0.7 -	3.3 (0.97)	3.7 (7.6)	2.3 (4.0)	3.1 (6.2)
Serine	4.1 (9.7)	7.8 (2.0)	9.3 (3.0)	7.7 (1.7)	8.7 (2.6)	3.5 (7.1)	2.3 (4.1)	2.5 (5.1)
Glutamic Acid	5.9 (14.1)	10.7 (2.7)	10.0 (3.2)	10.1 (2.2)	5.5 (1.5)	5.5 (11.3)	3.6 (6.4)	5.3 (10.8)
Proline	8.1 (17.1)	2.1 (0.5)	-	14.7 (3.2)	16.9 (4.9)	5.4 (11.1)	4.6 (8.0)	5.6 (11.4)
Glycine	4.6 (11.1)	7.4 (1.9)	10.9 (3.3)	1.6 (0.3)	2.9 (0.9)	4.7 (9.7)	3.3 (5.8)	4.4 (8.9)
Alanine	25.6 (62.6)	13.4 (3.4)	14.2 (4.6)	26.8 (5.9)	24.6 (7.2)	28.1 (57.4)	21.4 (37.6)	30.1 (60.9)
Valine	4.1 (10.1)	6.8 (1.7)	8.9 (2.4)	5.2 (1.1)	3.5 (1.0)	3.9 (7.9)	2.8 (4.9)	4.2 (8.5)
Methionine	0.7 (1.7)	0.7 (0.2)	(1.0)	(1.0)	(1.0)	-	-	-
Isoleucine	2.0 (4.8)	8.5 (2.2)	9.5 (3.15)	-	1.9 (0.6)	1.9 (3.8)	1.1 (1.9)	0.75 (1.5)
Leucine	2.9 (7.2)	3.7 (0.9)	3.9 (1.2)	-	1.2 (0.4)	3.3 (6.8)	2.2 (3.9)	2.6 (5.4)
Tyrosine	1.1 (2.5)	4.1 (1.1)	6.5 (1.9)	-	-	0.8 (1.6)	-	-
Phenylalanine	0.7 (1.5)	-	-	-	-	1.0 (2.1)	0.7 (1.2)	1.2 (3.0)
Lysine	34.1 (80.9)	16.5 (4.2)	10.4 (3.7)	20.9 (5.4)	19.7 (5.8)	34.6 (70.8)	53.3 (93.3)	38.5 (77.9)
Histidine	0.6 (1.5)	-	-	3.8 (0.8)	3.4 (0.99)	-	-	-
Arginine	1.9 (4.6)	7.3 (1.8)	5.6 (1.8)	4.5 (1.0)	3.4 (1.0)	1.5 (3.1)	1.2 (2.1)	0.7 (1.3)

Table 2.4: Amino Acid composition in mole % of P. angulosus embryo histone H1-2 and of the peptides produced by cyanogen bromide cleavage. Figures in parenthesis are the number of residues calculated from the amino acid hydrolysis results.

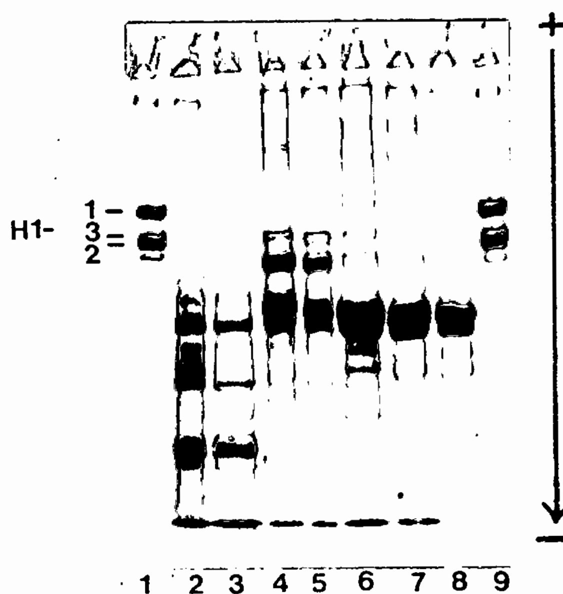


Figure 2.21 Polyacrylamide Gel Electrophoresis of the Carboxyl-terminal Cyanogen Bromide Fractions of P. angulosus Embryo H1-2

The fractions were recovered from a CMC-column (Fig. 2.20). The gel consisted of 2.0 M urea 6 mm Triton X-100 and acetic acid buffer and was run for 2 hours at a constant current of 15 mA.

Lane 1 = Perchloric acid extracted H1 fraction
2 - 3 = 10 μg and 5 μg of CN-6
4 - 5 = 10 μg and 5 μg of CN-7
6 - 8 = 10 μg and 5 μg and 2 μg of CN-8
9 = H1- standard mixture

Sequence analysis of CN-8 (Fig. 2.22 D) showed a single sequence for the first 47 steps sequenced, while the gel (Fig. 2.21 lanes 7-9) showed that the bulk of CN-8 consisted of two closely co-migrating bands.

It was possible to align the peptides but not to assign them to the sub-fractions. Figure 2.23 merely indicates how the peptides generated by cyanogen bromide cleavage could have been derived from the H1-2 histone fraction. The peptides produced by cyanogen bromide cleavage of H1-2 were the result of cleavage at the two methionine residues present in the two H1 proteins that co-migrated in fraction H1-2 from CMC (Fig. 2.10).

CN-4 and CN-5 were the blocked amino terminal peptides and were respectively 23 and 31 amino acids long, as calculated from the amino acid analysis results (Table 2.4). Treatment of CN-4 with anhydrous heptafluorobutyric acid and subjection of the peptide to automatic sequencing, did not give positive results with the Edamn degradation as might have been expected if an N$\longrightarrow$O acyl shift at the first amino acid had occurred. This indicated that the acetyl-blocked amino terminal amino acid was neither threonine nor serine (Brandt et al., 1980).

After attachment to a solid phase glass support (5.5.2) by the homoserine lactone method (Laursen, 1971) peptide CN-2 was subjected to 18 automatic N-terminal degradation steps on the Solid Phase Sequencer (Fig. 2.22 B).

Peptide CN-1 was sequenced for 16 steps on the Liquid Phase Sequencer, using the peptide program (Fig. 2.22 A). The primary structure of the homologous CN-2 compared well with that of CN-1 for the parts sequenced, except for positions 6 and 10 (boxed in, Fig. 2.24). Peptide CN-2 contained a glycine in the 6th position sequenced, while peptide CN-1 had a threonine. In position 10 peptide CN-2 had an alanine while peptide CN-1 displayed a glutamic acid (Fig. 2.24).

Peptide CN-8 was subjected to automatic N-terminal degradation on the Liquid Phase Sequencer, using the protein program, for 48 steps (Fig. 2.22 D).

Sequence analyses of peptide CN-6 (Fig. 2.22 C) demonstrated that this fraction was the result of cleavage at the first methionine residue only (Fig. 2.24) of both proteins present in fraction H1-2.

Figure 2.22 Automatic Sequence Analyses of *P. angulosus* H1-2 Cyanogen Bromide Cleaved Peptides.

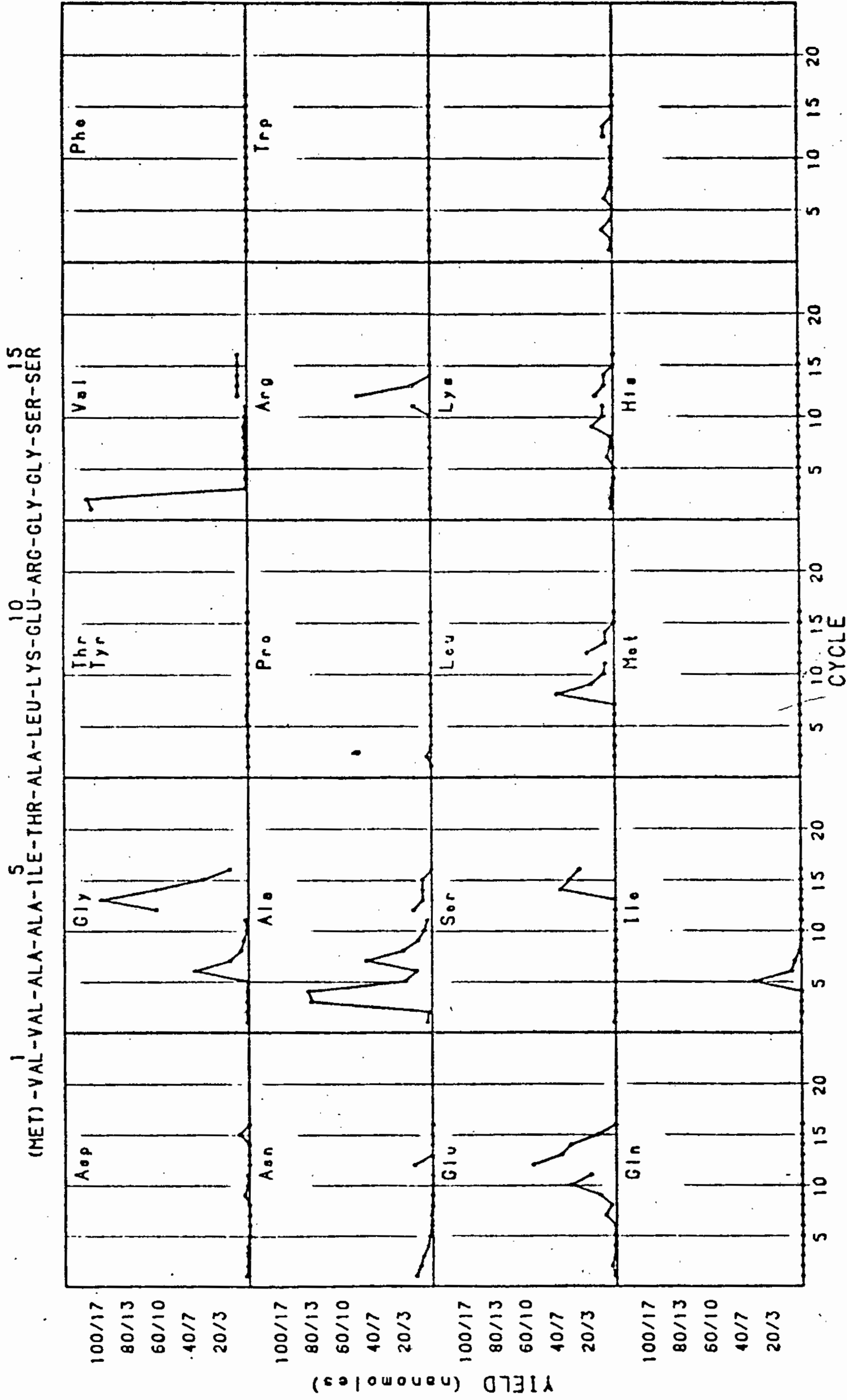
The amount of peptide subjected to degradation varied between 30-300 nmoles. Amounts of amino acid derivatives below 0.5 nmoles were recorded as zero. Phenylthiohydantoins were identified by high pressure liquid chromatography using gradient and isocratic elution in a methanol system (5.5.3.2). (●———●) yield in nmoles; scale changes are indicated by a break in the connecting line between two steps. The appearance of a contaminant co-eluting with Pth-Val in the DMAA-peptide program led to uncertainties in the assignment of valine as the level of the contaminant progressively increased, masking the valine signal. All peptides sequenced on the Liquid Phase Sequencer were reacted with sulfonated phenylisothiocyanate (S-PITC), equivalent to their lysine content, plus 1 mg histone H4 (from *P. angulosus* sperm) added as a carrier to the peptide in the cup.

(A) Yields of phenylthiohydantoins recovered by degrading CN-1 for 15 steps on the Liquid Phase Sequencer.

(B) Phenylthiohydantoin yields recovered by sequencing CN-2 on the Solid Phase Sequencer for 19 steps.

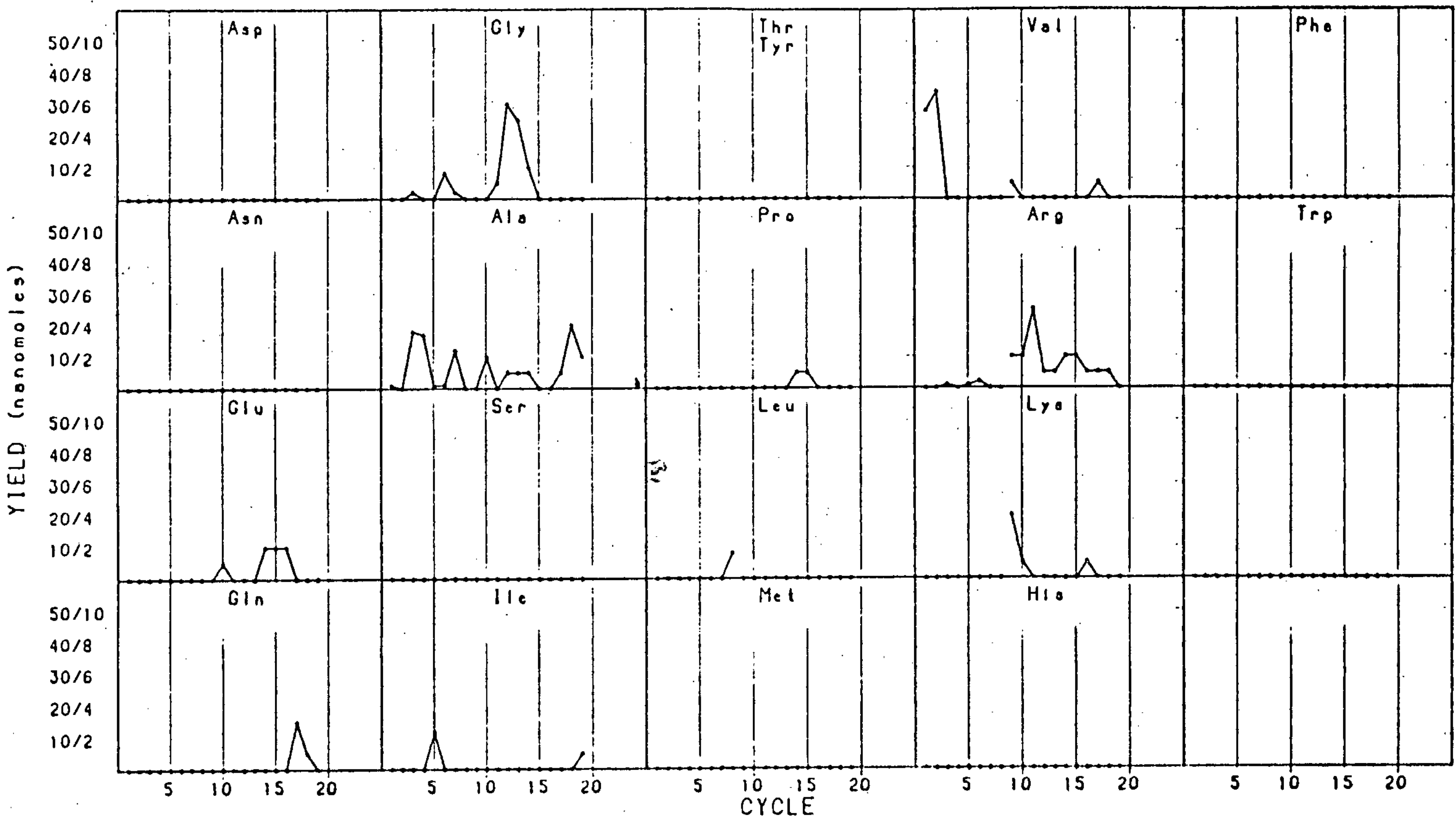
(C) Yields of phenylthiohydantoins recovered by degrading the peptide CN-6 for 15 steps on the Liquid Phase Sequencer.

(D) Yields of the phenylthiohydantoins recovered by sequencing peptide CN-8 on the Liquid Phase Sequencer.


(A) P. ANGULOSUS EMBRYO H1-2, CN-1
1 5 10 15
(MET)-VAL-VAL-ALA-ALA-ALA-ILE-THR-ALA-LEU-LEU-LYS-GLU-ARG-GLY-GLY-SER-SER
Asp
Gly
Thr
Tyr
Val
Phe
Asn
Ala
Pro
Arg
Trp
Glu
Ser
Leu
Lys
Gln
Ile
Met
His
YIELD (nanomoles)
100/17
80/13
60/10
40/7
20/3
5
10
15
20
CYCLE

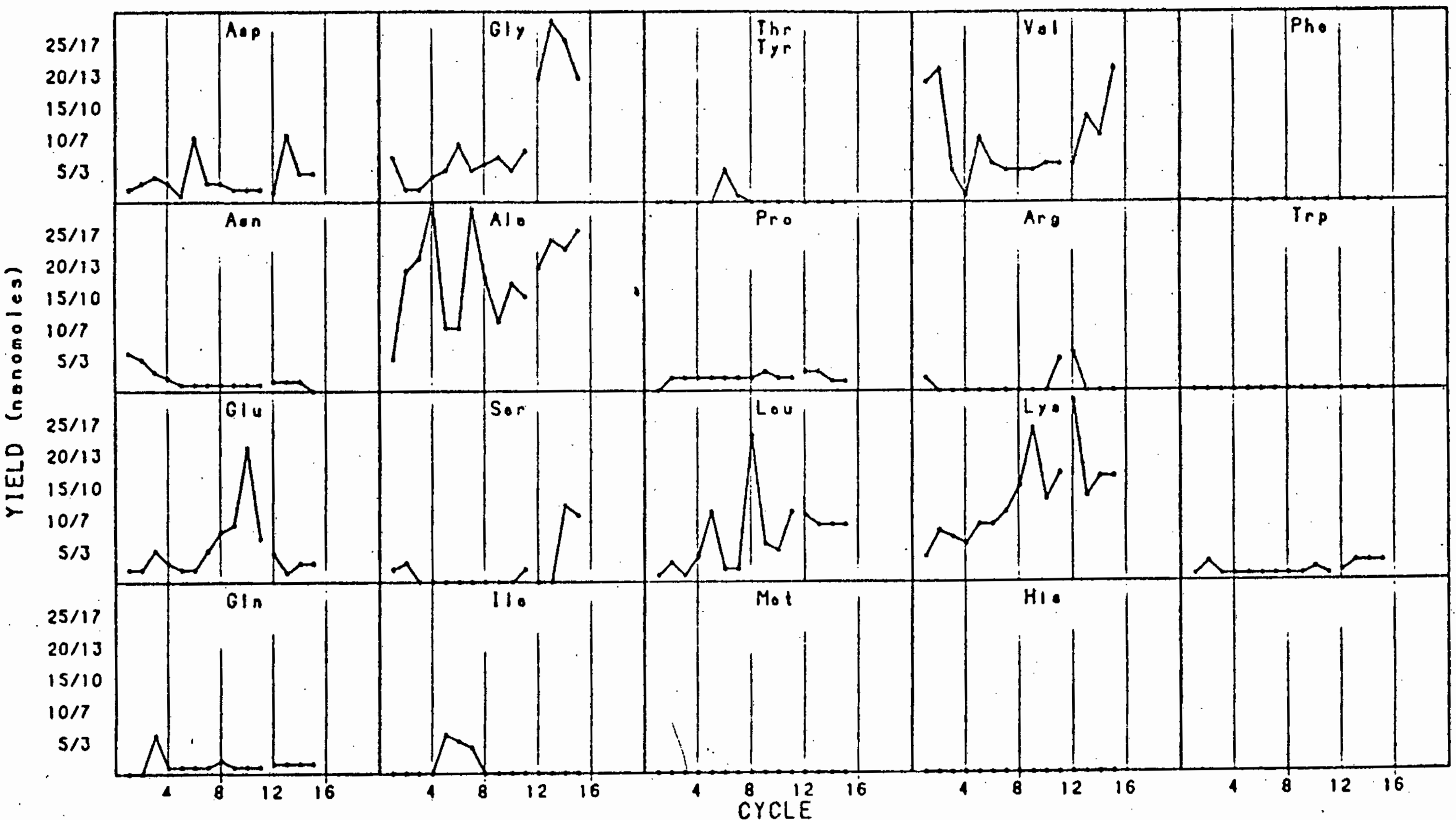
(B) P.ANGULOSUS EMBRYO H1-2.CN-2

(MET) VAL-VAL-ALA-ALA-ILE-GLY-ALA-LEU-LYS-ALA-ARG-GLY-GLY-(GLU-GLU-GLU-GLN-ALA-ILE)



(C) P. ANGULOSUS EMBRYO H1-2, CN-6

1 5 10 15
(MET)-VAL-VAL-ALA-ALA-ILE-GLY-ALA-LEU-LYS-ALA-ARG-GLY-GLY-(SER-SER)



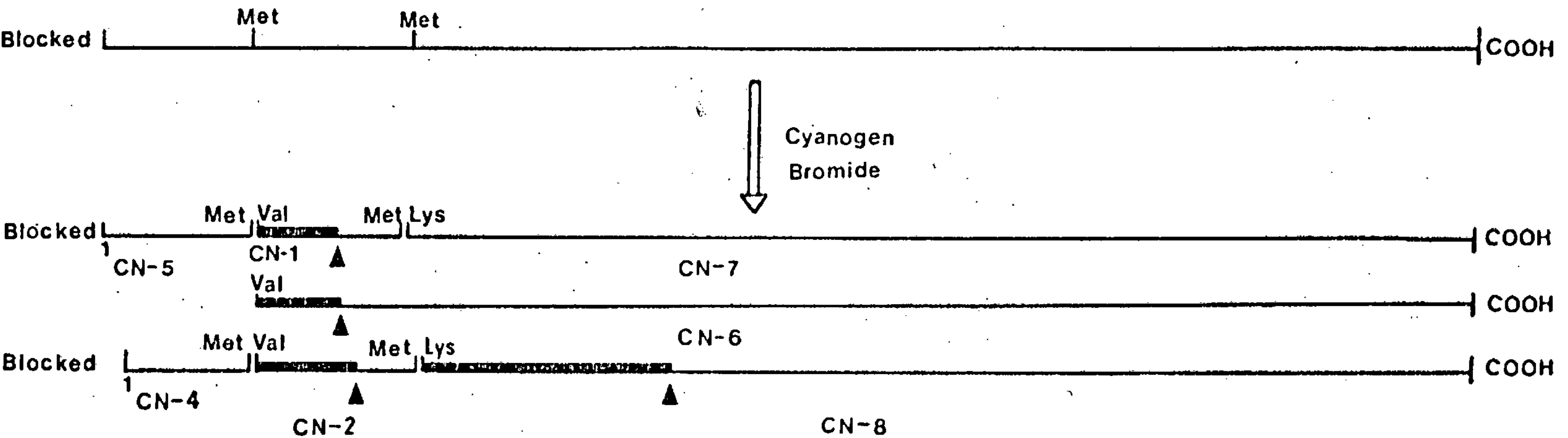


Figure 2.23 Tentative alignment of peptides generated by cyanogen bromide cleavage of *P. angulosus* embryo H1-2.

The arrow indicates the final residue sequenced in that peptide and the thick line indicates the sections that have been sequenced.

2.2.6 Partial Sequence of Parechinus angulosus Embryo H1-3

2.2.6.1 Generation of Peptides

2.2.6.1.1 Cyanogen Bromide Cleavage

Parechinus angulosus embryo histone fraction H1-3 ran as a single band on Triton-urea gel electrophoresis (Fig. 2.11) after separation from the other H1-fractions on carboxy methylcellulose chromatography (Fig. 2.10).

Histone fraction H1-3 contained 2 methionine residues (Table 2.1) and cyanogen bromide cleavage yielded 3 peptide fractions upon CMC-chromatography (Fig. 2.25). The amino acid composition of these fractions is listed in Table 2.5.

2.2.6.2 Partial Primary Structure Determination of H1-3

Fraction CN-3 eluted as an asymetrical peak from the CMC-column (Fig. 2.25) and Triton-urea polyacrylamide gel electrophoresis of peptide CN-3 resulted in a complex series of bands (Fig. 2.26). Fraction CN-3 was rerun on Sephadex G100 (Fig. 2.27) and eluted as an asymetrical peak which was divided into five fractions. These fractions were run on a Triton, 2,5 M urea polyacrylamide gel (Fig. 2.28) and displayed a slight separation of peptides.

Polyacrylamide gel electrophoresis of these fractions revealed that the main fraction, CN-3-III (Fig. 2.28), ran as two close migrating bands in approximately equal amounts (Fig. 2.28). Fraction CN-3-II (Fig. 2.27) displayed on gel electrophoesis a small amount of uncleaved H1-3, a double band of incompletely cleaved H1-3 protein plus the two bands that represent the major fractions of CN-3-III (Fig. 2.28).

AMINO ACID	H1-3 ~ 242 Residues	CN-1 ~ 31 Residues	CN-2 ~ 34 Residues	CN-3 ~ 162 Residues
Aspartic Acid	2.2 (5.3)	0.83 (0.2)	-	1.96 (3.1)
Threonine	4.7 (10.0)	3.8 (1.1)	2.9 (1.1)	3.46 (5.5)
Serine	3.3 (8.4)	8.5 (2.6)	9.8 (3.3)	2.5 (3.9)
Glutamic Acid	3.9 (9.3)	1.7 (0.5)	-	3.4 (5.5)
Proline	7.7 (19.5)	14.4 (4.3)	19.9 (6.8)	3.01 (4.8)
Glycine	4.1 (9.6)	2.5 (0.7)	-	3.5 (5.6)
Alanine	30.1 (68.5)	30.8 (9.2)	32.8 (11.3)	25.5 (40.7)
Valine	2.7 (6.7)	-	-	2.24 (3.6)
Methionine	0.5 (1.5)	0.9 (1.0)	2.6 (0.94)	-
Isoleucine	2.1 (5.2)	-	-	1.9 (3.1)
Leucine	2.8 (6.3)	1.06 (0.3)	-	2.5 (4.0)
Tyrosine	0.8 (1.9)	-	-	0.6 (1.0)
Phenylalanine	0.88 (1.9)	-	-	0.77 (1.2)
Lysine	33.0 (82.9)	32.7 (9.7)	22.4 (7.7)	46.8 (74.8)
Histidine	0.4 (0.9)	2.9 (0.9)	4.2 (1.4)	-
Arginine	1.8 (4.2)	-	4.75 (1.7)	1.75 (2.8)

Table 2.5: Amino Acid Composition in mole % of *P. angulosus* embryo H1-3 and of the peptides produced by cyanogen bromide cleavage of H1-3. Figures in parenthesis are the number of residues calculated from the amino acid hydrolysis results.

Cyanogen bromide cleavage of histone H1-3 resulted in the production of 3 fractions upon CMC-fractionation (Fig. 2.23), as would be expected if histone fraction H1-3 was a single protein containing two methionine residues. However by gel electrophoresis a high degree of heterogeneity was demonstrated. Peptide CN-3 clearly displayed a heterogeneous character when subjected to separation on gel electrophoresis (Figure 2.26-28). Peptide CN-3 contained uncleaved H1-3 protein, two bands of incompletely-cleaved H1-3 protein and two bands representing the carboxyl terminal cyanogen bromide peptides. Sequence analysis of peptide CN-3 for 36 steps revealed the presence of three sequences (Fig. 2.29 A) on the Liquid Phase Sequencer. The dominant sequence present in peptide CN-3 was that of a peptide cleaved at the first methionine. This sequence compared well with that of peptide CN-6 of the H1-2 protein (Fig. 2.22 C). The other two sequences could not be assigned and positioned with certainty. A partial purification of the dominant peptide was achieved on rechromatography.

Fraction CN-3 (Fig. 2.25) was divided in two by rerunning fraction CN-3 on the CMC column and collecting the first half(CN-3A) of the peak separate from the second half (CN-3B). Fraction CN-3B was sequenced (Fig. 2.29 B) for 18 steps on the Liquid Phase Sequencer. The sequence corresponded to that of peptide CN-6 of H1-2 which was the peptide produced by cleavage at the first methionine and no cleavage at the second methionine.

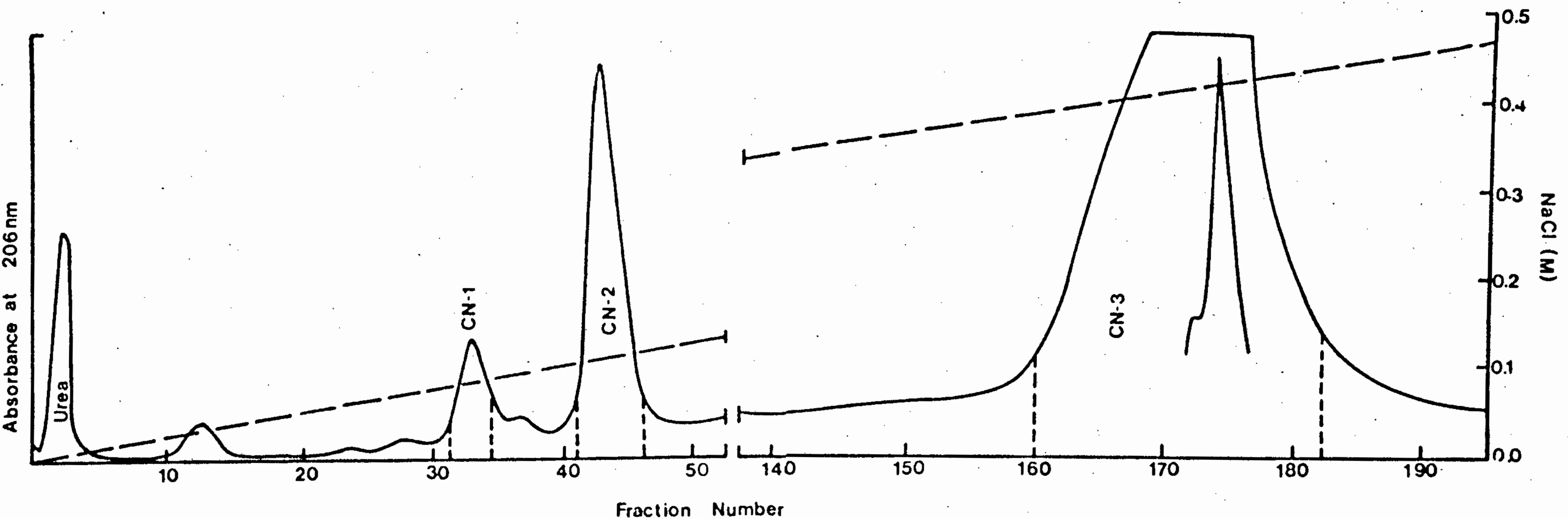


Figure 2.25 Fractionation of a cyanogen bromide digest of *P. angulosus* embryo HI-3.
Column: 6 x 60 mm carboxymethylcellulose with 50 mM Na-acetate/-HCl buffer pH 5,5 as eluant, flow rate: 12 ml/hour, linear gradient: 0-400 mM (total volume 400 ml). Dotted lines indicate the fraction cuts.

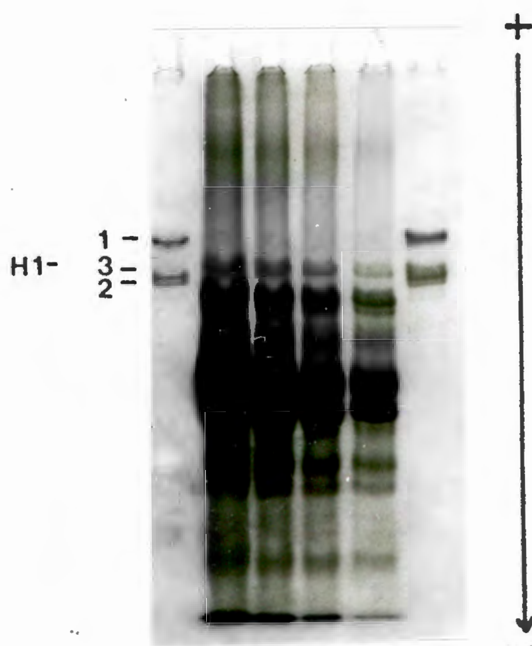


Figure 2.26 Polyacrylamide Gel Electrophoresis of the Carboxyl-terminal Cyanogen Bromide Fraction of P. angulosus Embryo H1-3.

The peptide fraction CN-3 was recovered from a CMC-column (Fig. 2.25). The gel consisted of 6 mM Triton, 2 M urea and was run for 2 hours at a constant current of 15 mA. Various amounts of peptide CN-3 were applied to the gel.

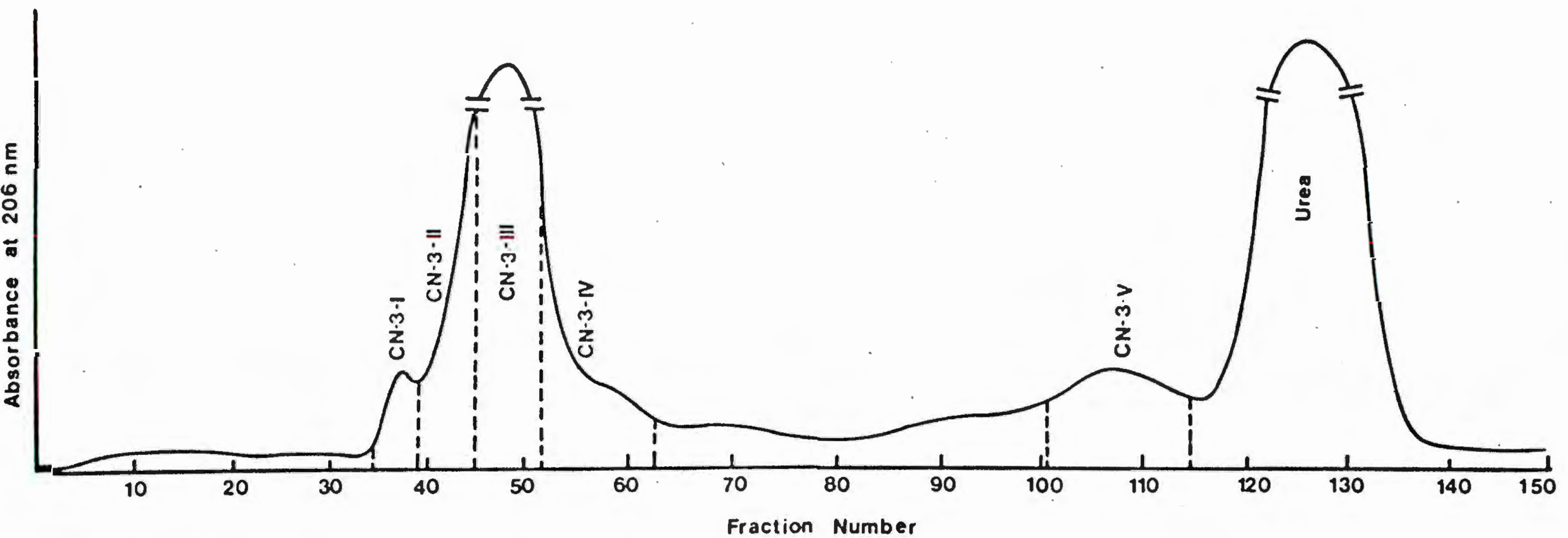


Figure 2.27 Fractionation of fraction CN-3 on gel exclusion chromatography.

Column: 26 x 100 mm Sephadex G-100 medium, eluant: 10 mM HCl, cuvette 3 mm, fraction size: 2,5 ml. The dotted lines indicate the fraction cuts.

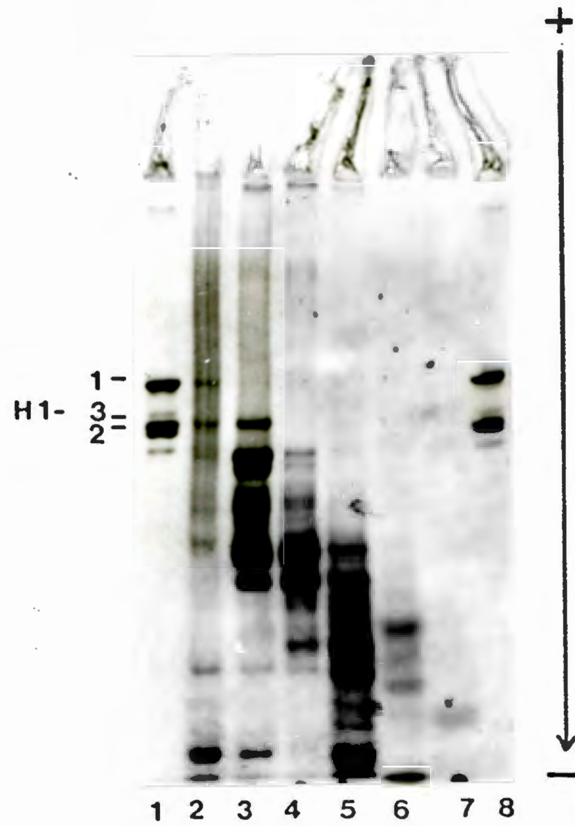


Figure 2.28 Polyacrylamide Gel Electrophoresis of the Fractions Recovered upon Fractionation of P. angulosus Embryo H1-3 Peptide CN-3 on Sephadex G100 (Fig. 2.25).

The gel consisted of 6 mM Triton, 2,5 M urea and was run at a constant current of 15 mA for 2½ hours.

Lane 1 & 8 = Perchloric acid extract
2 = CN-3-I
3 = CN-3-II
4 = CN-3-III
5 = CN-3-IV
6 = CN-3-V
7 = Urea

Figure 2.29 Automatic Sequence Analysis of *P. angulosus* Embryo H1-3 Cyanogen bromide Cleaved Peptide Fraction CN-3.

The amount of peptide subjected to degradation was 60-120 nmoles. Amounts of amino acid derivatives below 0,5 nmoles havebeen recorded as zero. Phenylthiohydantoins were identified by High Pressure Liquid Chromatography using gradient and isocratic elution in a methanol system (5.5.3.2). (●——●) yield in nmoles; scale changes are indicated by a break in the connecting line between two steps.

Peptide CN-3 was reacted with sulfonated phenylisothiocyanate (S-PITC) equivalent to its lysine content and 1 mg histone H4 (from *P. angulosus* sperm) added as a carrier to the peptide in the cup. Peptide H1-3, CN-3 was sequenced on the Liquid Phase Sequencer. (+) yield due to the presence of a second peptide identified by subtracting the known sequence of the first peptide or determined by varying amounts of the two peptides present in the sequencing mixture. (X) yield due to a third peptide.

(A) Yield of phenylthiohydantoins recovered by degrading CN-3 on the Liquid Phase sequencer.

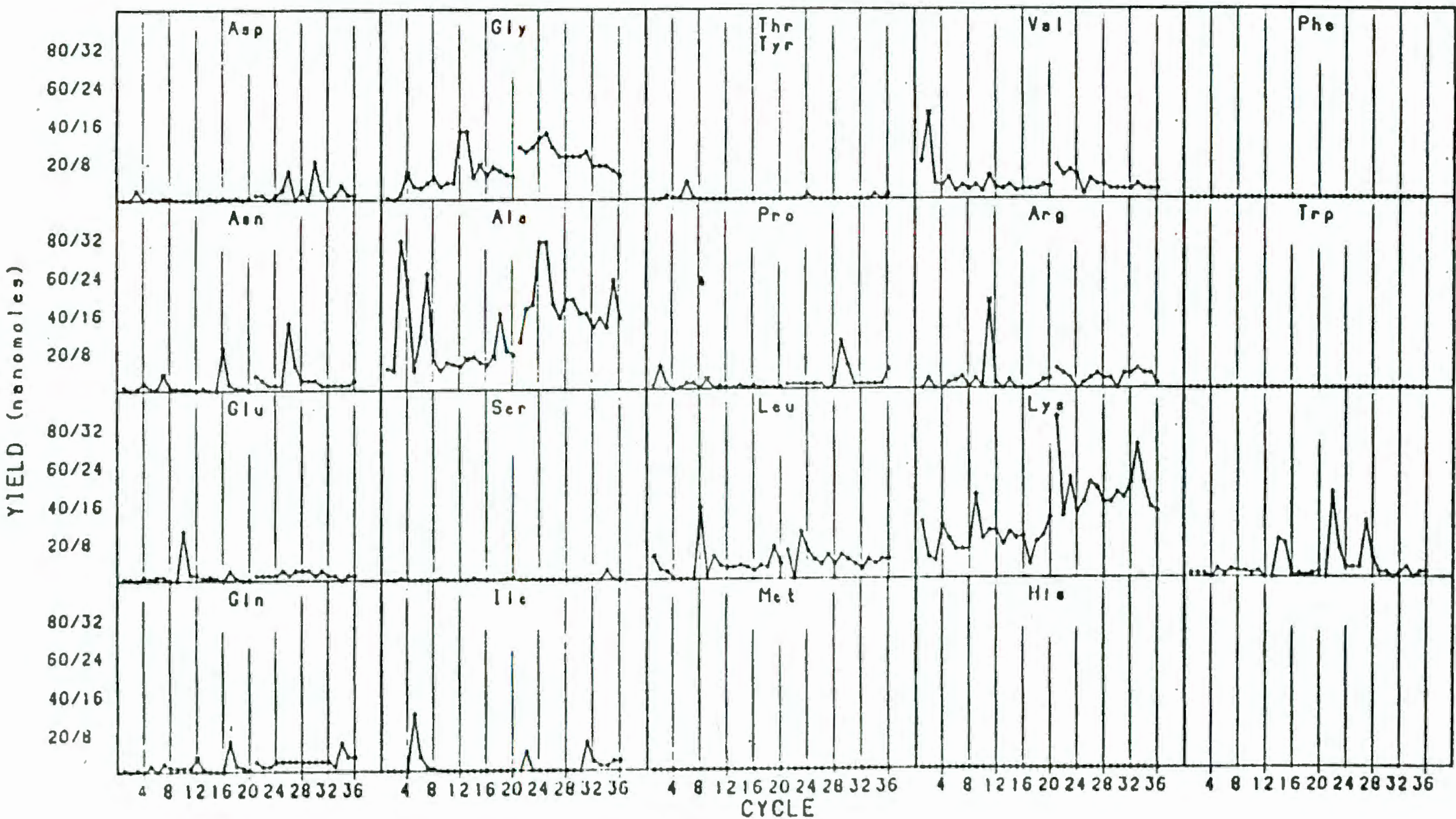
(B) Yield of phenylthiohydantoins recovered by degrading CN-3B on the Liquid Phase Sequencer.

(A) P.ANGULOSUS EMBRYO H1-3, CN-3

5 10 15 20
LYS-VAL-ALA-ALA-ILE-ALA-ALA-LEU-LYS-GLU-ARG-GLY-GLY-TYR-TYR-ASN-GLN-ALA-LEU-LYS-

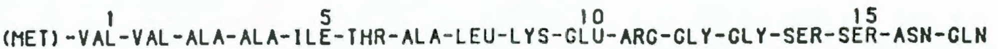
VAL-PRO-ALA-LYS-LYS-THR-ALA-LEU-LYS-LEU-VAL-GLY-GLY-LYS-LYS-LYS-GLY-LYS-LYS-LYS-
LEU-VAL-ALA-GLY-VAL-( )-ASN-GLY-PRO-ALA-ARG-GLN-ALA-ALA-GLY

25 30 35
LYS-TYR-LYS-ALA-ALA-ASN-TYR-ALA-PRO-ASP-ILE-LYS-LYS-GLN-ALA-PRO



(B) P.ANGULOSUS EMBRYO H1-3. CN-3B

1 5 10 15
(MET)-VAL-VAL-ALA-ALA-ILE-THR-ALA-LEU-LYS-GLU-ARG-GLY-GLY-SER-SER-ASN-GLN



PART 3

THE SYNTHETIC PROGRAM AND SOME MODIFICATIONS OF P. ANGULOSUS EMBRYO HISTONES

3.1 The Histone Programme During Embryonic Development of the Sea Urchin Parechinus angulosus

3.1.1. Schedule of Histone H1-synthesis and Incorporation into Chromatin During the Early Development of P. angulosus

It has been known for some time that the number of H1-proteins increases during embryonic development of the sea urchin, from one component at the morula stage to three components at the gastrula stage (Hill et al., 1971; Cohen et al., 1973; Seale and Aronson, 1973). It was also shown that the morula type of H1 ceased to be synthesized at the blastula stage, although it persisted in the chromatin long afterward (Ruderman and Gross, 1974) even through metamorphosis (Poccia and Hinegardner, 1975). This has been demonstrated in the sea urchin species Strongylocentrotus purpuratus (Seale and Aronson, 1973); Lytechinus pictus (Arceci et al, 1976; Poccia and Hinegardner, 1975); Arbacia punctulata (Ruderman and Gross, 1974); Arbacia lixula (Ruiz-Carrillo and Palau, 1973) and Parechinus angulosus (Brandt et al., 1979).

Newrock et al. (1977) demonstrated in S. purpuratus that there might be three histone-like proteins that are synthesized and incorporated into chromatin even earlier than the morula (or α) subtypes. One of these resembled histone H1. These histone-like proteins were called cleavage stage proteins. Having established a considerable degree of heterogeneity of the embryonic histone H-1 in the the previous section it was desirable to establish whether or not their synthesis was programmed. The period of synthesis of the histone variants was

determined by following the time course of the incorporation of 3H lysine into the histones. This was a convenient label for the study of H1 histone synthesis, since H1-histones are lysine rich.

^{3}H Lysine was added to growing cultures at a concentration of 200 µCi (80 Ci/mmol) per 100 ml growing culture, which contained approximately 4 x 10^6 embryos (5.6.1).

All of the lysine label was incorporated within one hour after adding it to the growing culture (Fig. 3.1). Therefore the ^{3}H-lysine incorporated represented the proteins that were synthesized in the hour after the addition of the label.

The embryo cultures were subsequently allowed to grow for 24 hours before the histones were prepared by acid extraction of nuclei. After this 24 hour period all the histone variants which had been synthesized from the time of fertilization were present, but not all of them were labelled.

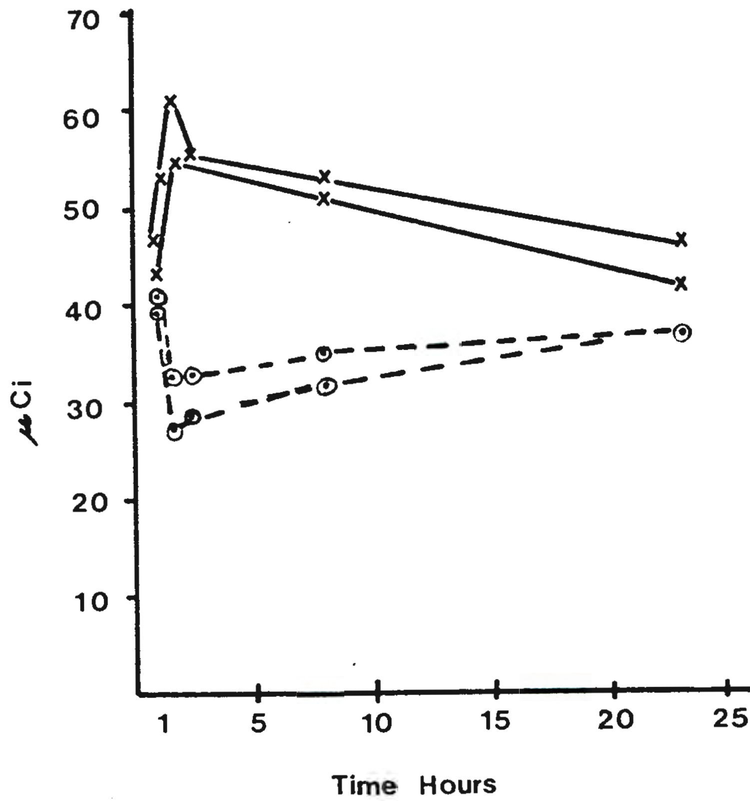


Figure 3.1 Uptake of ^{3}H-Lysine

Lysine in SDS soluble proteins (X——X) and in sea water (θ——θ) of a synchronized sea urchin embryo culture (this graph was reproduced with permission from the work done on sea urchin embryos by M. Strickland).

The acid extracted histone variants were displayed on Triton-acid-urea gels using the two urea concentrations that allowed the optimal separation of the variants. These were respectively 3,8 M urea-Triton gels for the separation of the histone H2B into its three variants and H1 into its 3 variants (Fig. 3.2) while the 8,5 M urea-Triton gel system allowed the separation of H2A into its three variants (Fig. 3.3).

For the detection of the labelled histone variants the Coomassie Brilliant Blue stained gels were prepared for fluorography by soaking them in 1 M sodium salicylate for ½ hour and drying them under a vacuum between Whatman 3 M paper and clear film (5.6.5). The dried gel was exposed to X-ray film at -180°C until a suitable exposure was achieved, usually after 7 days for 2000 cpm/slab gel lane.

Figures 3.2 and 3.3 demonstrate that between 9 and 12 hours after fertilization a new set of histones was synthesized while the earlier set of histones ceased to be synthesized. The nine hour old embryos were at the late blastula stage. The time of switch over from the early set of histones to the later set of histones, occurred apparently earlier in the embryonic development of P. angulosus than in the embryos of the species S. purpuratus (Newrock et al., 1977). By 15 hours after fertilization there was no detectable trace of synthesis of the early histones.

In a comprehensive label study (Figs. 2.2 and 2.3) of the synthesis of the histone variants we have not been able to show synthesis of cleavage stage proteins. The absence of cleavage stage histones may have been due to limitations of the Triton-acid-urea gel systems, which did not clearly separate cleavage stage histones and the early set of histones. In addition, cleavage stage proteins may have been present in such small amounts that they were not detectable.

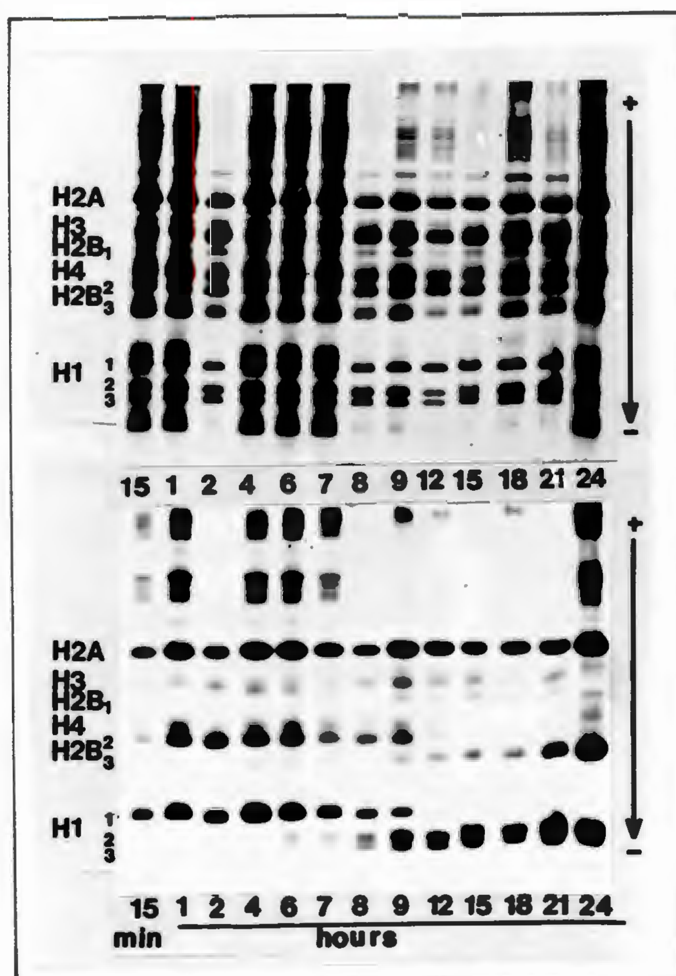


Figure 3.2 Histones prepared by acid extraction from 24 hour old embryos displayed on Triton-acid-3,8 M - urea gels.

The proteins were labelled with ^{3}H-lysine and the label was added at the times indicated. The gel consisted of 6 mM Triton, 3,8 M urea and was prepared and run as described in section 5.3.1. Histones were extracted from nuclei preparations except for the 15 mins, 1, 4, 6, 7 and 24 hour labelled cultures. Those histones were extracted from washed chromatin.
Top: Comassie Brilliant Blue stained gel.
Bottom: Fluorogram.
(This figure was reproduced with permission from the sea urchin embryo work done by M. Strickland).

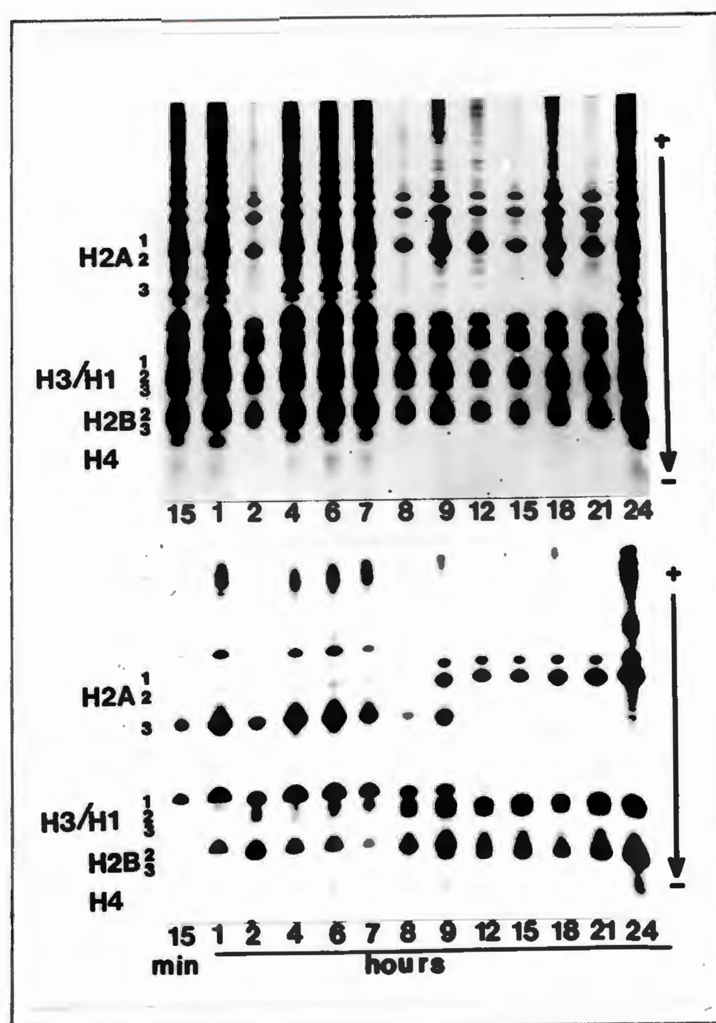


Figure 3.3 Acid extracted histones of 24 hour old embryos run on 6 mM Triton, 8,5 M urea gels.

Proteins were labelled with ^{3}H-lysine and the label was added at the times indicated. Histones were extracted from nuclei preparations except for the 15 min, 1, 4, 6, 7 and 24 hour labelled cultures. Those histones were extracted from washed chromatin.
Top: Coomassie Brilliant Blue stained gel.
Bottom: Fluorogram.
(This figure was reproduced with permission from the sea urchin embryo work done by M. Strickland).

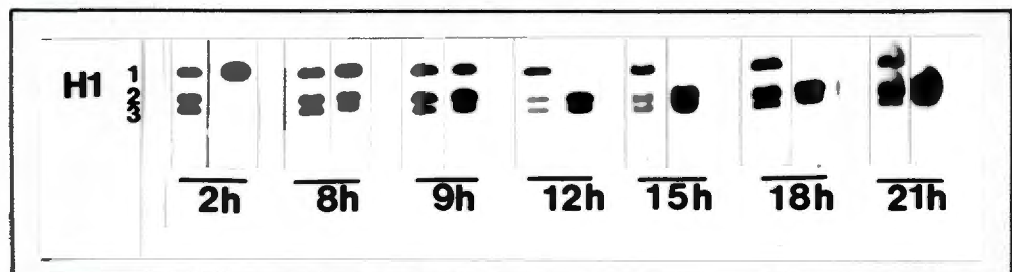


Figure 3.4 Synthesis of H1-histone variants during the development of the embryo *P. angulosus.*

The polyacrylamide gel contains 6 mM Triton and 3,8 M urea. Proteins were labelled with ^{3}H lysine added at the times indicated while the histones were harvested from 24 hour old embryos.
Left lane = protein stain
Right lane = fluorogram

In the developmental schedule of the histone variants associating with the chromatin of Parechinus angulosus embryo, two stages were perceived. During the first stage the chromatin became populated with the early variants, which were H1-1, $H2A_3$ and $H2B_2$ (Figs. 3.2 & 3.3). The early variants were present shortly after fertilization. There were no traces of any cleavage stage proteins. Stage two started with the synthesis of the second set of histone variants. The second set of histone variants started to be synthesized at 8 hours after fertilization. By the 12 hour stage the second set of histone variants were the dominant histones being synthesized (Figs. 3.2 & 3.3). With the onset of synthesis of the second set of histones, the early set ceased to be synthesized although they were retained in the chromatin long afterwards (the embryos were harvested at the 24 hour stage). The second set of histone variants consisted of $H2A_2$ and $H2A_1$, $H2B_1$ and $H2B_3$, H1-2 and H1-3. Histones H3 and H4 began to be synthesized shortly after fertilization and continued throughout the observed period.

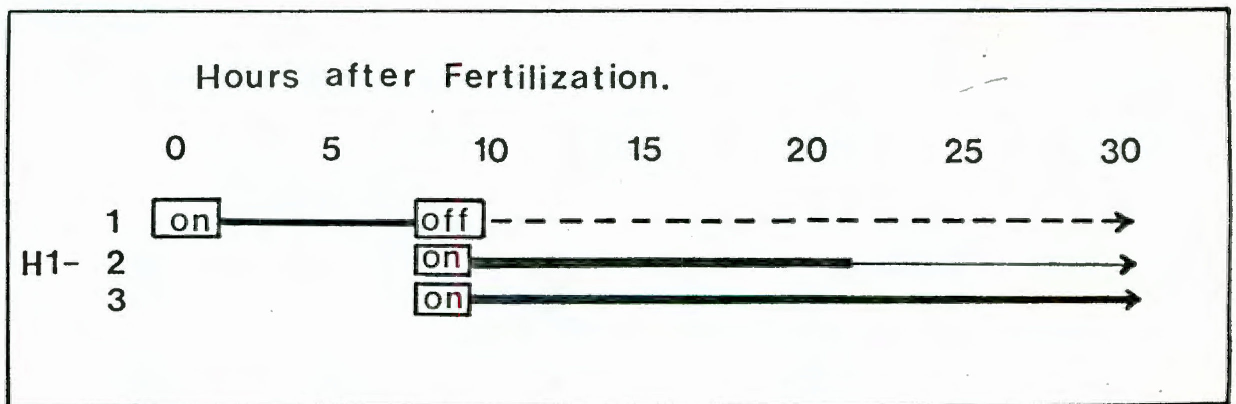


Figure 3.5 Schedule of synthesis and incoporation into chromatin of the H1-variants during the early development of Parechinus angulosus as detected by incorporation of ^{3}H-lysine.

Heavy line indicates an abundant component; narrow lines indicate a minor component; solid line shows period during which a component was synthesized; dashed line shows the period during which a protein was no longer synthesized, but was still maintained in the chromatin.

3.2 Histone Modifications Occurring During an Early Cell Cyle of the Embryo of Parechinus angulosus

3.2.1 Introduction

Diversity in chromatin structure is generated as development proceeds (Mahowald, 1968), and may be related to the functional diversification of chromatin leading to cell differentiation.

Many investigators have proposed that DNA activities might be controlled by modulating the structure of chromatin through reversible modifications of the histone proteins. Since histones are so closely associated with DNA and have a high affinity for DNA, they would be good candidates for influencing the structural organization of chromatin. During the embryonic development, the histone complement associated with the DNA initially contained only an early set of histones but later contained a second set in addition to the early set. The second set of histones differ in primary structure from the early set and become the dominant histones associating with the DNA (Hill et al., 1971; Zweidler and Cohen, 1972; Cohen et al., 1973; Seale and Aronson, 1973; Cole, 1975; Newrock et al., 1977; Brandt et al., 1979; von Holt et al., 1979).

This increase in histone diversity during development and chromosomal protein modification may be correlated with changes in chromatin structure and function.

Similarly, the progression of the cell cycle is characterized by changes in the structure and function of chromatin (Mitchison, 1971).

The changes occurring during the cell cycles are thought to be regulated mainly by chromosomal protein modifications such as phosphorylation (Bradbury et al., 1973, 1974; Lake and Saltzman, 1972; Gurley et al., 1974, 1978; Inglis et al., 1976), acetylation (Sung and Dixon, 1970; Ruiz-Carrillo et al., 1975; Jackson et al., 1975; Sealy

and Chalkley, 1978), methylation (Lee et al., 1973; Thomas et al., 1975) and poly(ADP-ribosyl)ation (Ueda et al., 1975; Tanuma et al., 1977).

Quantitative and qualitative modifications of the histones and their variants, during a cell cycle result in a complex pattern. This pattern of histone modification is a function of the different stages of the cell cycle and the different stages of embryonic development. The first stage of this study was to identify the histones and their complement of variants that become modified during an early cell cycle. Stage two was an examination of whether the histones that became modified, became differentially modified during the cell cycle.

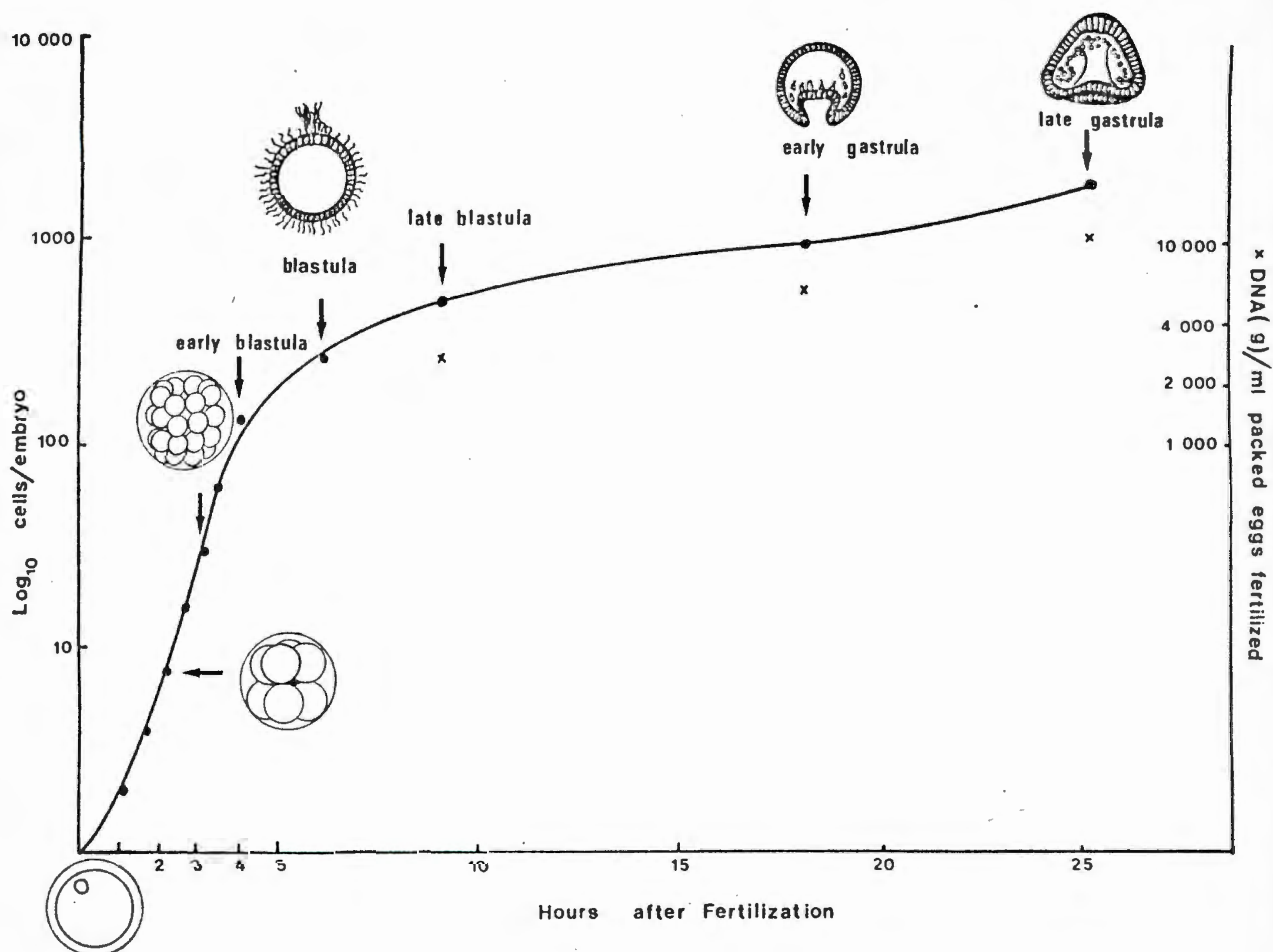


Figure 3.6 The developmental stages, cell number and DNA content of the synchronized embryo culture of P. angulosus.

The embryos were grown under standardized conditions and the developmental stages were determined by examination of the culture under a microscope (this graph was reproduced with permission from the sea urchin study done by Mrs. F. Davids of this department).

The phosphorylation and acetylation of the histone variants were investigated during an early cell cycle of the sea urchin embryo. The cell cycle chosen was that occurring during the period when the first set of histone variants was switched off and the synthesis of the second set of histone variants was initiated. The period of histone synthesis switch over, from the first set of histone variants to the second set, occurred between 7 and 12 hours after fertilization (see section 3.1). Figure 3.6 demonstrates the cell number and DNA content of the synchronized sea urchin embryo from fertilization to 25 hours after fertilization. Between 7 and 12 hours the embryo went through the ninth cell cycle (Fig. 3.6), multiplying from 256 cells to 512 cells. To determine the exact duration of the ninth cell cycle, the growing embryo culture was labelled with ^{14}C -thymidine (5.6.2.1). ^{14}C thymidine was added to a growing culture at a constant rate of 0.01 μCi/25 ml culture/minute. The accumulative incorporation of ^{14}C thymidine was examined during the period 3 hours to 10 hours after fertilization. The period of DNA synthesis was represented by an increase in ^{14}C-thymidine incorporation and the period of mitosis by a levelling off, of ^{14}C thymidine incorporation. Figure 3.7 demonstrates the accumulative incorporation of ^{14}C thymidine into the DNA of the developing embryo. From this it was deduced that the ninth cell cycle started at 5 hours 20 minutes after fertilization and continued to 9 hours after fertilization.

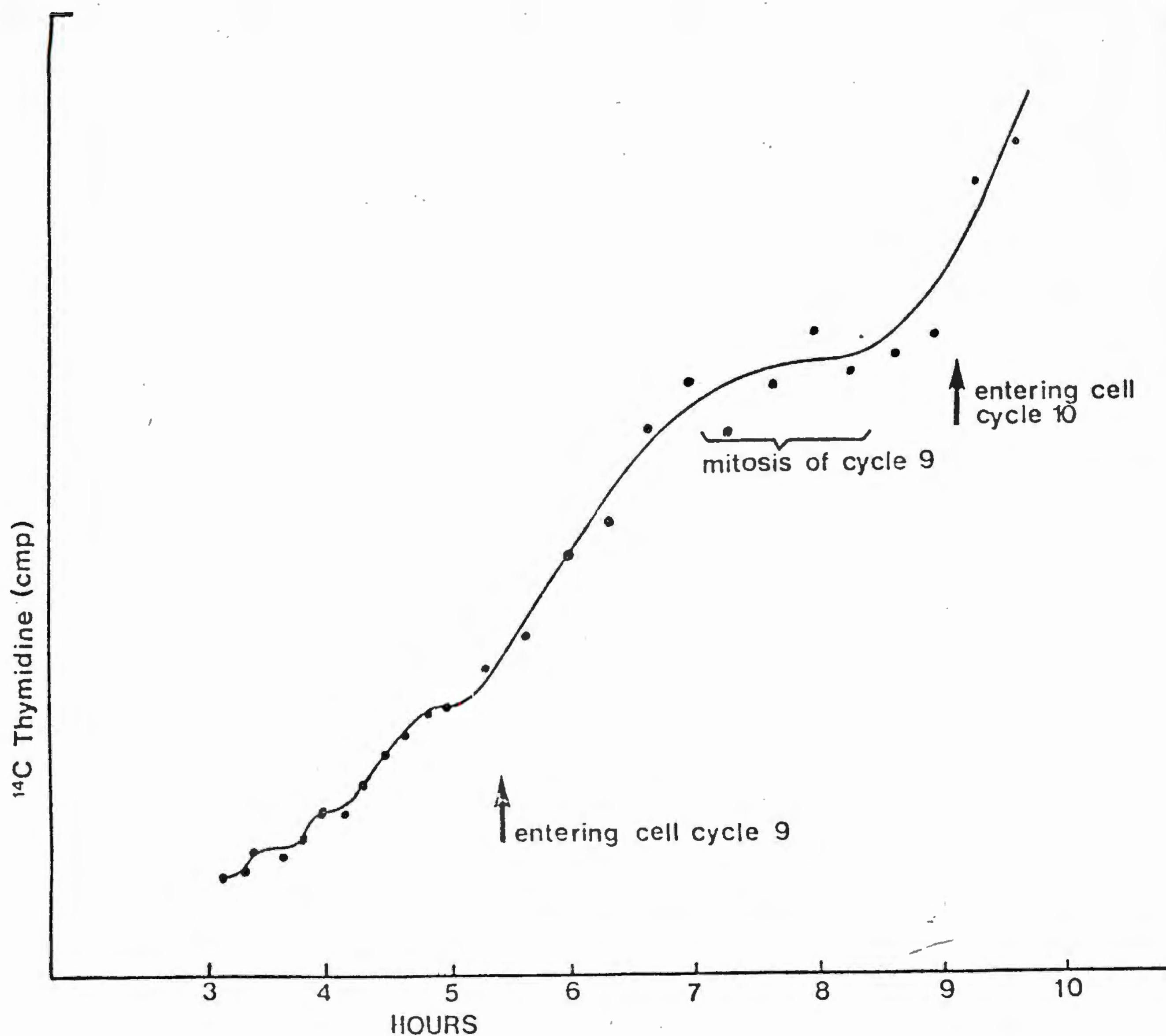


Figure 3.7 Time course of ^{14}C -thymidine incorporation into DNA of *P. angulosus* embryos.

The embryos were incubated at 20°C in sea water, containing Streptomycin Sulfate (50 µg/ml) and Penicillin G (100 µg/ml), at a concentration of 4 x 10^6 eggs/ml. ^{14}C- Thymidine (58 mCi/mmol) was continuously pumped into the culture at a rate of 0.01 µCi/25 ml culture/minute. A 200 µl sample was taken at the times indicated, washed three times with cold 5% TCA (w/v) plus 1 mM cold Thymidine. The precipitate was washed, collected and the radioactivity measured in a Liquid Scintillation counter.

3.2.2. Phosphorylation of the Histones During the Ninth Cell Cycle

There is evidence that gene activation, DNA replication and mitotic condensation of chromatin all require phosphorylation of H1, probably at different intramolecular sites (Langan, 1969; Lake et al., 1972; Bradbury et al., 1974; Hohmann et al., 1976). In mammalian cells, H1 subtypes differ from one another in the number of phosphorylation sites (Langan et al., 1971; Ajiro et al., 1976), providing a possible means for regional control of these chromatin functions.

In this study I concentrated on determining which histone variants became phosphorylated and whether there was a difference between the phosphorylation of the histone variants of the first set and second set and, also whether there was a change in the degree of histone phosphorylation during the cell cycle.

The ninth cell cycle was chosen for the study of histone modifications for the following reasons:

1. The ninth cell cycle covered the period during which the first set of histone synthesis ceased and synthesis of the second set of histones commenced. Though the synthesis of the first set of histones had stopped at this stage, it was still the dominant set of histones present in the chromatin.
2. The duration of the ninth cell cycle was about 3 hours 40 minutes, which was a short and convenient time for label studies with a sufficient degree of synchronization (see Figs. 3.6 & 3.7).

The phosphorylation of histones during the ninth cell cycle was examined by labelling separate growing embryo cultures for a short period at different stages of the cell cycle.

Nuclei were extracted from the embryo cultures immediately after the period of labelling, and were stored at -180°C until the histones were extracted at a later stage (5.6.4). The embryo cultures (6×10^5 embryos/15 ml) were labelled for 20 minute periods with 150 µl [^{32}P]

orthophosphate (1 mCi/ml) during the period 5½ to 10½ hours after fertilization. The labelled histones were analyzed by electrophoretic separation of the histones on polyacrylamide gels and exposure of the gels to X-ray film (Kodak X-omat RP film).

To have a complete picture of all the histone variants, electrophoretic separation was done by using acid-urea-Triton gels with two different urea concnetrations namely 3,8 M urea and 8,5 M urea (5.3.1).

A gel consisting of 3,8 M urea, 6 mM Triton X-100 separated the H2B and H1 histones into its variants while the H2A histone variants ran as a single band. The 3,8 M urea gel system also allowed the separation of H3 and H4 without any histone variants co-migrating with them (Fig. 3.2).

The second gel system, comprising 8,5 M urea, 6 mM Triton X-100 (5.3.1), allowed for the separation of the H2A histone into its three variants (Fig. 3.3). Histone H3 co-migrated with the H1-variants and histone H2B did not separate into all its variants in this particular gel system. Histone H4 migrated as a single fast migrating band in 8,5 M urea Triton polyacrylamide.

For the analysis of labelled histone variants it was therefore necessary to examine the extracted histones in both polyacrylamide urea gel systems.

Extraction of [^{32}P] labelled histones was done in the presence of 50 mM sodium bisulfite, a phosphatase inhibitor. Gurley et al. (1975) have shown that 50 mM sodium bisulfite prevented histone dephosphorylation during the extraction procedure of Johns (1964). Figure 3.8 exhibits the difference in [^{32}P] label associated with the histones during the histone extraction in the presence (Fig. 3.8, bottom) and absence (Fig. 3.8, top) of 60 mM sodium bisulfite.

Figures 3.9 and 3.10 respectively show the 3,8 M and 8,5 M urea polyacrylamide gels (top) of the histone variants extracted at the various times, with the fluorogram of the respective gel (bottom).

Figure 3.10 shows that the [^{32}P] label was mainly associated with histones in the H3/H1-region and in the H2A region. In the H2A region (Fig. 3.10), there were three labelled bands moving slightly faster than $H2A_3$. These three labelled bands in the region of H2A are probably phosphorylated histone $H2A_3$ that separated according to the amount of phosphate groups bound to it. The stained gel shows that $H2A_3$ was the dominant H2A histone variant present (Fig. 3.10, top). Histone variants $H2A_1$ and $H2A_2$ were faint bands initially but gradually became more pronounced during the cell cycle. Two bands in the region of $H2A_1$ and $H2A_2$ became phosphorylated, moving slightly slower than their unphosphorylated putative parent molecules. These bands were phosphorylated throughout the cell cycle.

Figure 3.9 shows that phosphate label has not been incorporated into H2B and H4, even in this overexposed fluorogram.

A great deal of phosphate label was located in the H3/H1 region of the gel (Fig. 3.10). From Figure 3.9 it is clear that histone variant H1-1 became heavily phosphorylated. No phosphorylation of histone variants H1-2 and H1-3 could be detected. It thus seems that the early H1-variant (H1-1) became preferentially labelled at that stage. This was different to the H2A-variants. Various degrees of phosphorylation of the early H2A variant (i.e. $H2A_3$) and also phosphorlation of a band in the region of the late H2A histone variants, i.e. $H2A_1$ and $H2A_2$, (Fig. 3.10) became apparent.

Separation of the labelled histones in the 3,8 M urea gel system (Fig. 3.9) shows two labelled bands in the region above H2A. The faster band migrated nearly the same distance as the H2A.

No phosphate label became associated with either H2B or H4 as can be seen in the separation of histones in the 8,5 M urea gel (Fig. 3.10). In the 3,8 M urea gel (Fig. 3.9), labelled bands were present in the

region of $H2B_1$ and H4. Since $H2B_1$ and H4 did not show any phosphate label in the 8,5 M urea system (Fig. 3.10), these labelled bands migrating in the region of $H2B_1$ and H4 in the 3,8 urea gel (Fig. 3.9) cannot be identified with the latter two. Whether these two bands represent phosphorylated subfraction of $H2A_3$, the Triton binding of which may well be affected by changes in urea concentration (Fig. 3.10), or whether these represent the phosphorylated form(s) of an unidentified iso-histone similar to the histone D2 subcomponent of _Drosophila melanogaster_ (Palmer et al., 1980), remains unclear.

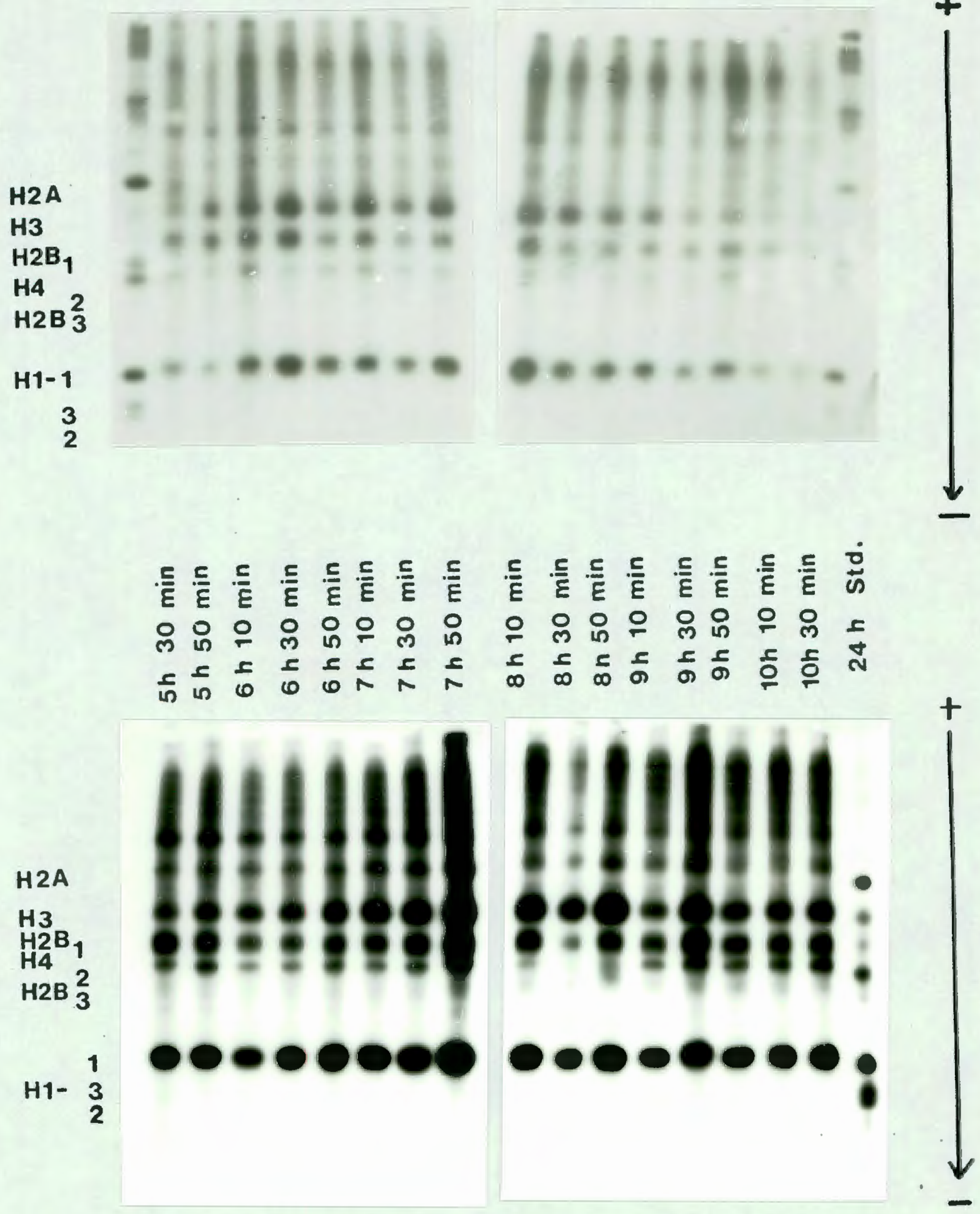


Figure 3.8 Effect of sodium bisulfite as a phosphatase inhibitor.

Parallel histone extraction of [^{32}P] labelled sea urchin embryo cultures were done. The fluorogram on the bottom displays histones extracted in the presence of 50 mM sodium bisulfite and the fluorogram on the top displays histones extracted without sodium bisulfite present in the extraction medium. The histones were displayed on 3,8 M urea, 6 mM Triton polyacrylamide gels. Both gels were exposed to the X-ray plates during the same period and for the same duration of time. Embryo cultures were labelled as described in section 5.6.4.

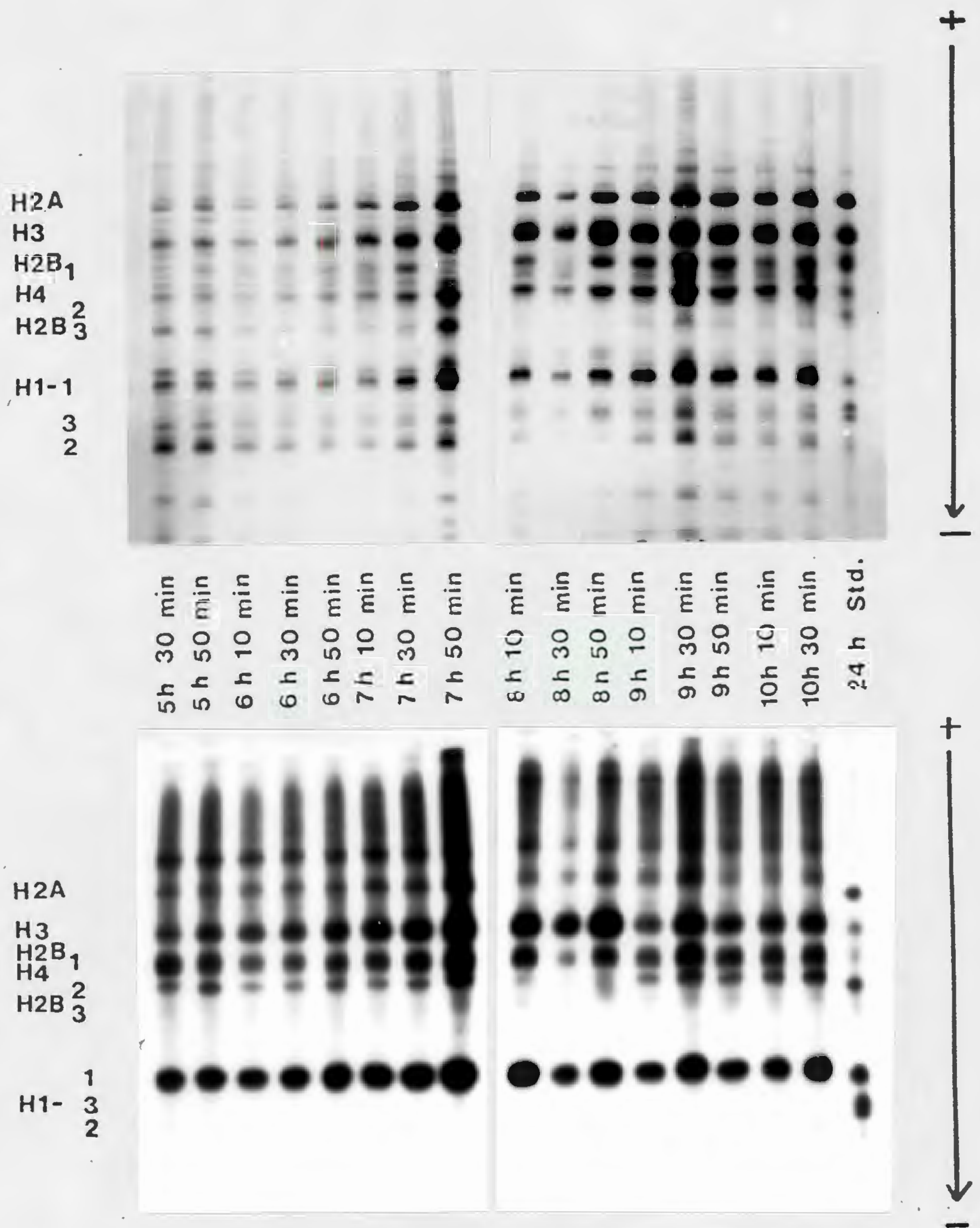


Figure 3.9 Separation of [^{32}P] labelled histones in a 3,8 M urea, 6 mM Triton gel.

[^{32}P] Orthophosphate (1 mCi/ml) was added to embryo cultures (150 µl/6 x 10^5 embryos suspended in 15 ml) at the times indicated. The embryos were grown for 20 minutes after the addition of the label, whereupon the nuclei were extracted (5.2.1). Histones were extracted from the nuclear preparation with 50 mM sodium bisulfite present in the extraction solvents.

Coomassie Brilliant Blue stain gel = top
Fluorogram = bottom
Standard histones were ^{3}H lysine labelled histones.

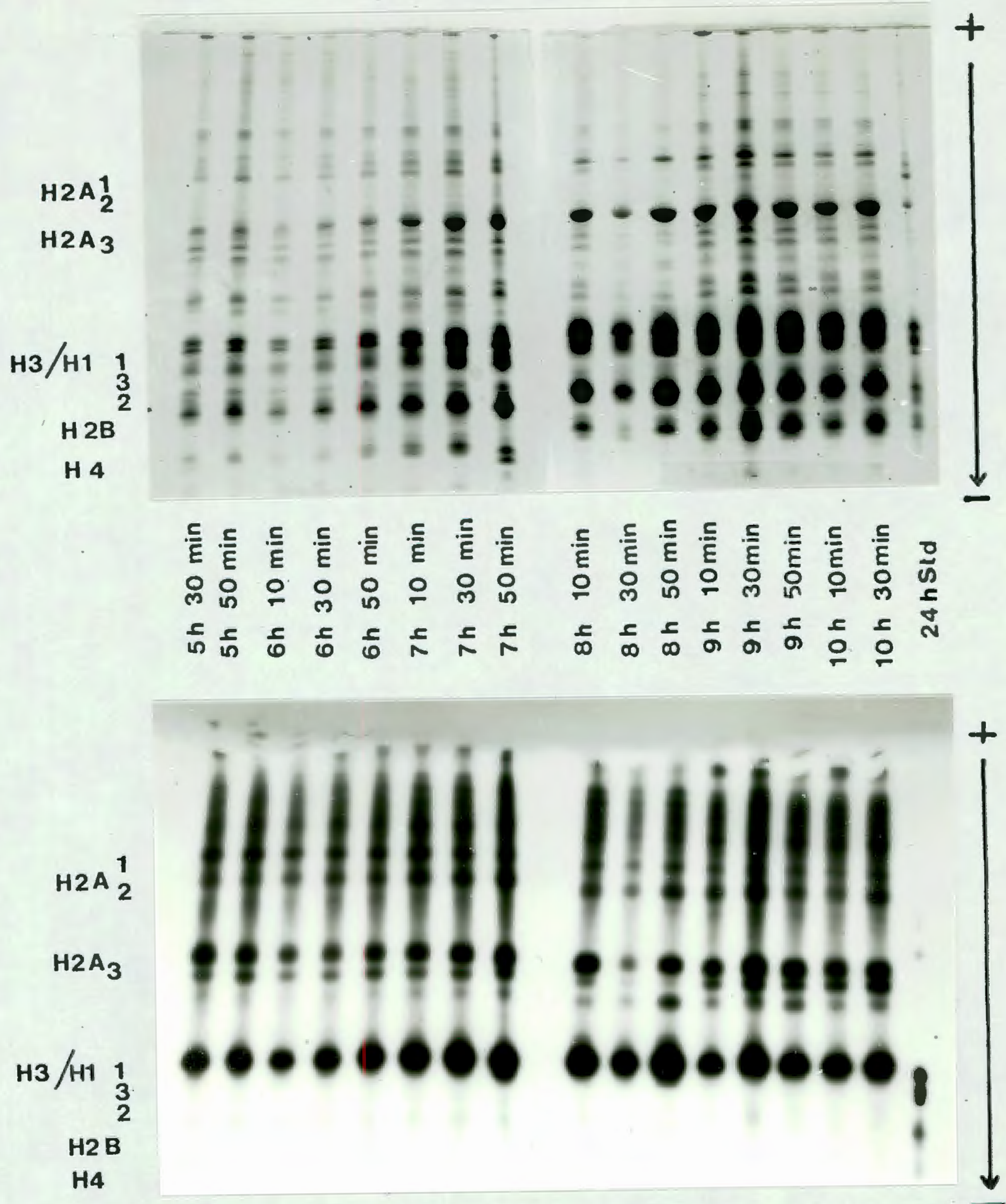


Figure 3.10 Separation of [^{32}P] labelled histones in 8,5 M urea, 6 mM Triton gel.

[^{32}P] Orthophosphate (1 mCi/ml) was added to embryo cultures (150 µl/6 x 10^5 embryos suspended in 15 ml) at the times indicated. The embryos were grown for 20 minutes after the addition of the label, whereupon the nuclei were extracted. Histones were extracted from the nuclei preparations with 50 mM sodium bisulfite present in the extraction solvents.

Coomassie Brilliant Blue stained gel = top
Fluorogram = bottom
Standard histones were ^{3}H-lysine labelled histones.

The described phosphorylation pattern appeared to be confirmed when the protein fractions were aligned with the fluorograms, in Figure 3.11 and 3.12 for 3,8 M urea and 8,5 M urea respectively.

[^{3}H] Lysine labelled standard histones were run in separate lanes of the gels for proper alignment.

The inspection of the fluorograms from the various intervals of the cell cycle, indicated the following:
Histone H1-1 was heavily phosphorylated throughout the ninth cell cycle (Fig. 3.9). None of the other histones H-1, though present, underwent phosphorylation. It was not possible to tell, (Fig. 3.9) whether the H1-1 became more phosphorylated at certain stages of the cell cycle than at other stages. The fluorogram was too dark for densitometry scans.

The two slow migrating bands in the region of $H2A_1$ and $H2A_2$ (Fig. 3.9), stayed equally phosphorylated throughout the period 5½ to 10½ hours after fertilization.

In the region of $H2A_3$ (Fig. 3.9) there were initially two dominant bands present and a faint fast migrating band. The faint fast migrating band in the $H2A_3$ region (Fig. 3.9) became more prominent at about 7½ hours while the middle band became fainter. At 9½ hours the middle band became more intense again while the faster migrating band became less phosphorylated at 10 hours 10 minutes. This period corresponded to mitoses of cell cycle 9 and the entry into cell cycle 10 (Fig. 3.7). The histones H2B and H4 were not phosphorylated at any stage, whereas histone H3 was evenly phosphorylated throughout the cycle.

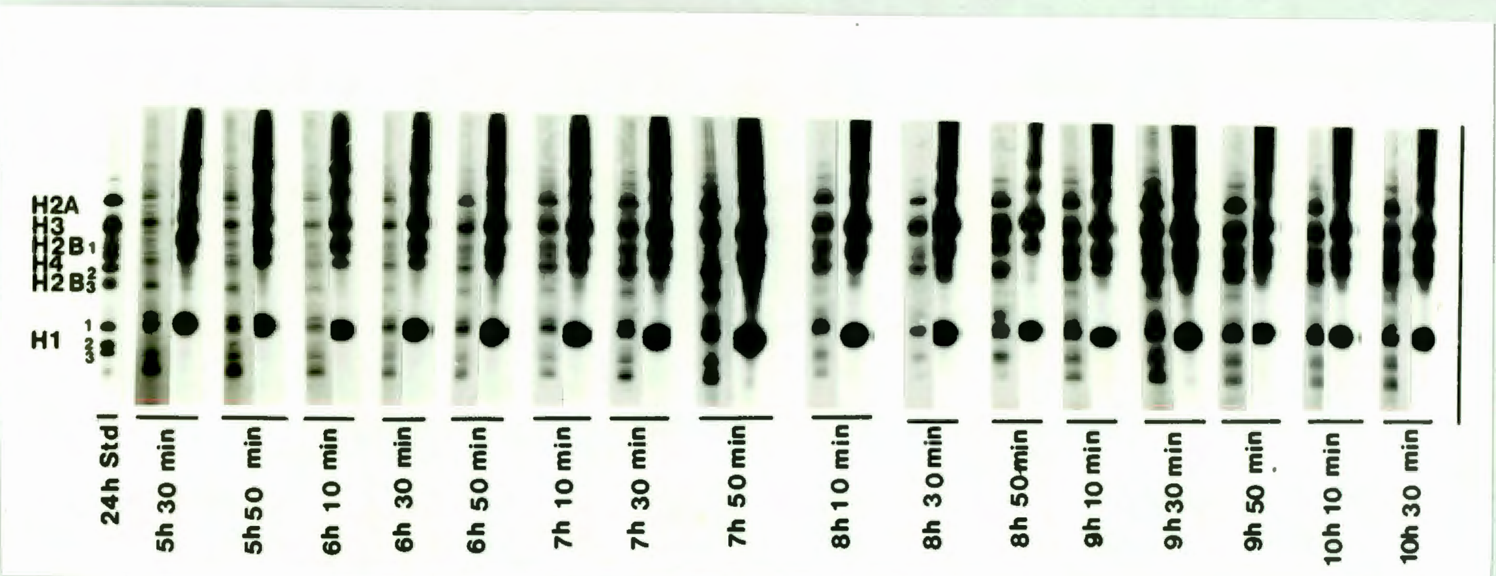


Figure 3.11: Comparison between the stained histone bands and [^{32}P] labelled histone bands in the 3,8 M urea-Triton-polyacrylamide gel system.

Coomassie Brilliant Blue stained proteins = left;
Fluorogram = right;
Standard histone proteins = ^{3}H-lysine labelled

The times are when the [^{32}P] orthophosphate was added to the growing culture (section 5.6.4.)

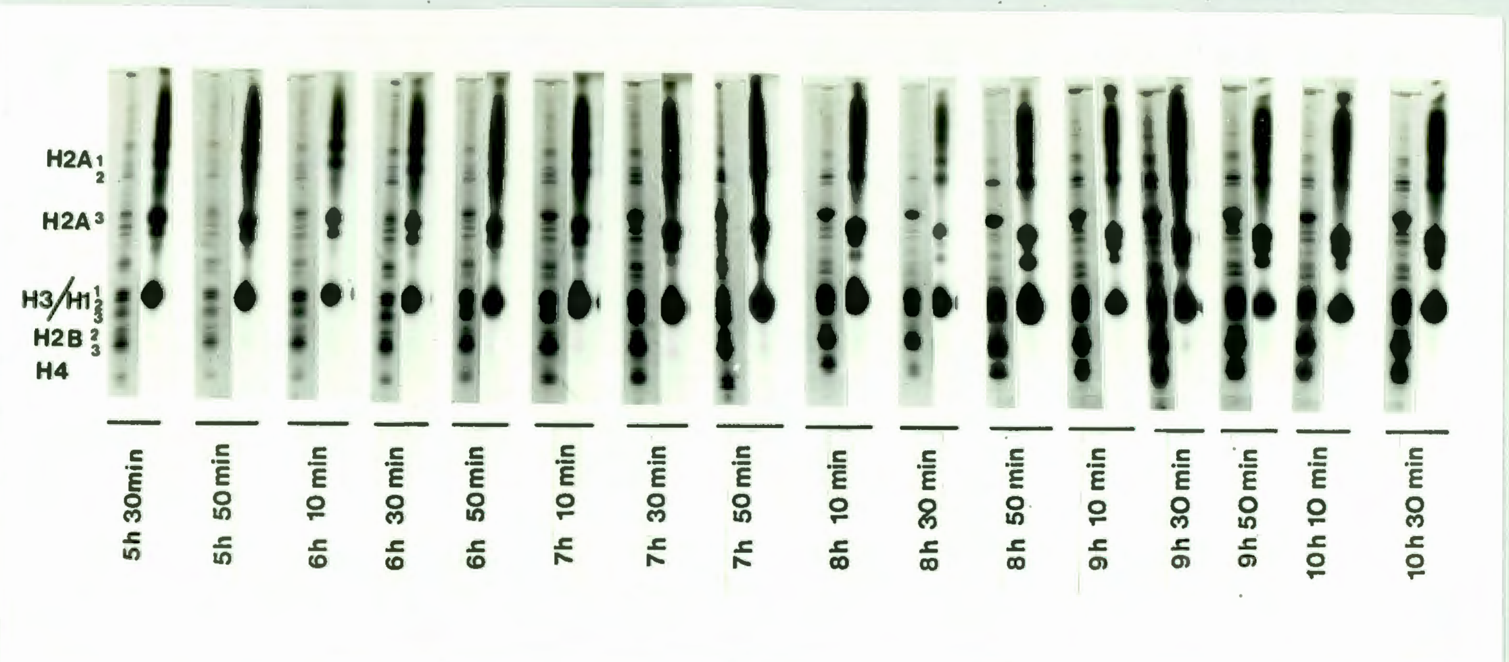


Figure 3.12: Comparison between the stained histone bands and the [^{32}P] labelled histone bands in the 8,5 M urea-Triton-polyacrylamide gel system.

Coomassie Brilliant Blue stained proteins = left;
Fluorogram = right;
Standard histone proteins = ^{3}H-lysine labelled

The times are when the [^{32}P] orthophosphate was added to the growing culture (section 5.6.4).

3.2.3 Acetylation of the Histones During the Ninth Cell Cycle

The acetylated forms of histones are generally found in transcriptionally active chromatin (Davie and Candido, 1978; Chahal et al., 1980; Perry and Chalkley, 1981). Acetylation and deacetylation of histones has been found to be rapid, thus providing the nucleosome with the possibility of re-establishing critical interactions shortly after they are broken (Jackson et al., 1975).

For HTC (hepatoma tissue culture) cells, the non-H1 histones showed, for some acetyl residues, an exceedingly rapid turnover of acetate (50% of acetate removed with a $t\frac{1}{2}$ = 3 minutes) as well as a slower turnover for other acetyl groups ($t\frac{1}{2}$ = 30-40 minutes) (Jackson et al., 1975). Experiments with HTC cells indicated that [^{3}H] acetate uptake and ..istone modification occured rapidly with an approximate $t\frac{1}{2}$ = 10-15 minutes (Jackson et al., 1975).

It has thus been difficult to define the level of initial acetylation with precision because of the rapid deacetylase reaction.

The use of sodium butyrate to induce hypermodification of histones by inhibiting the histone deacetylase (Sealy and Chalkley, 1978; Candido et al., 1978; Vivaldi et al., 1978) has provided a way of analysing the degree of acetylation more accurately over short intervals.

In order to investigate histone acetylation during the 9th cell cycle it would be desirable to block the deacetylase(s) with butyrate, otherwise the rapid turnover of the acetyl residues would require excessively high labelling of the embryos. If however butyrate were to be used, the concentration should be such as not to interfere too drastically with the growth of the embryo.

I examined and compared the incorporation of [^{14}C]-thymidine into the DNA of embryos grown with or without 5 mM sodium butyrate. This was done by growing two embryo cultures at the same time, one with 5 mM

sodium butyrate present in the growth medium and the control without sodium buytrate. At particular times 4 x 10^3 embryos were taken from the respective cultures, suspended in 5 ml sea water and labelled for 20 minutes with 0,04 Ci [^{14}C] thymidine (58 mCi/mMol). The embryos were collected by centrifugation after the 20 minute period of labelling, washed with cold calcium magnesium free sea water (CMFSW), precipitated with 5% TCA (w/v), collected on millipore filters and counted on the Packard Liquid Scintillation spectrometer.

Labelling was done on separate embryo cultures (4 x 10^3 embryos/5 ml) for 20 minutes consecutively between 5½ hour stage to 10½ hour stage.

The [^{14}C] thymidine incorporation into the DNA of the embryo culture grown with sodium butyrate, was 31% less than in the control (Fig. 3.13). The [^{14}C] thymidine incorporation into the DNA of the embryos grown with sodium butyrate followed the same pattern as the [^{14}C] thymidine incorporation into the DNA of the control (Fig. 3.13). This would indicate that the embryos developing in the presence of sodium butyrate, went through the same cell stages as the control at the same time. The acetylation of the histones in the sea urchin embryos _P. angulosus_ was examined during the ninth cell cycle. As described before, the ninth cell cycle is the period of switch over of synthesis from an early set of histones to a later set (section 3.1).

Acetylation of histones was examined with 5 mM sodium butyrate present in the growth medium and in all the extraction media. The presence of butyrate enabled me to sample all the histones that became acetylated in the respective periods of labelling during the cell cycle.

The aim of the work was similar to the phosphorylation study, namely, to determine the histones that become acetylated at that stage and whether the histone variants of the first set became preferentially acetylated to the second set of histones or _vice versa_. The pattern of acetylation of the histones was also examined during the ninth cell cycle which was compared to the cell stages.

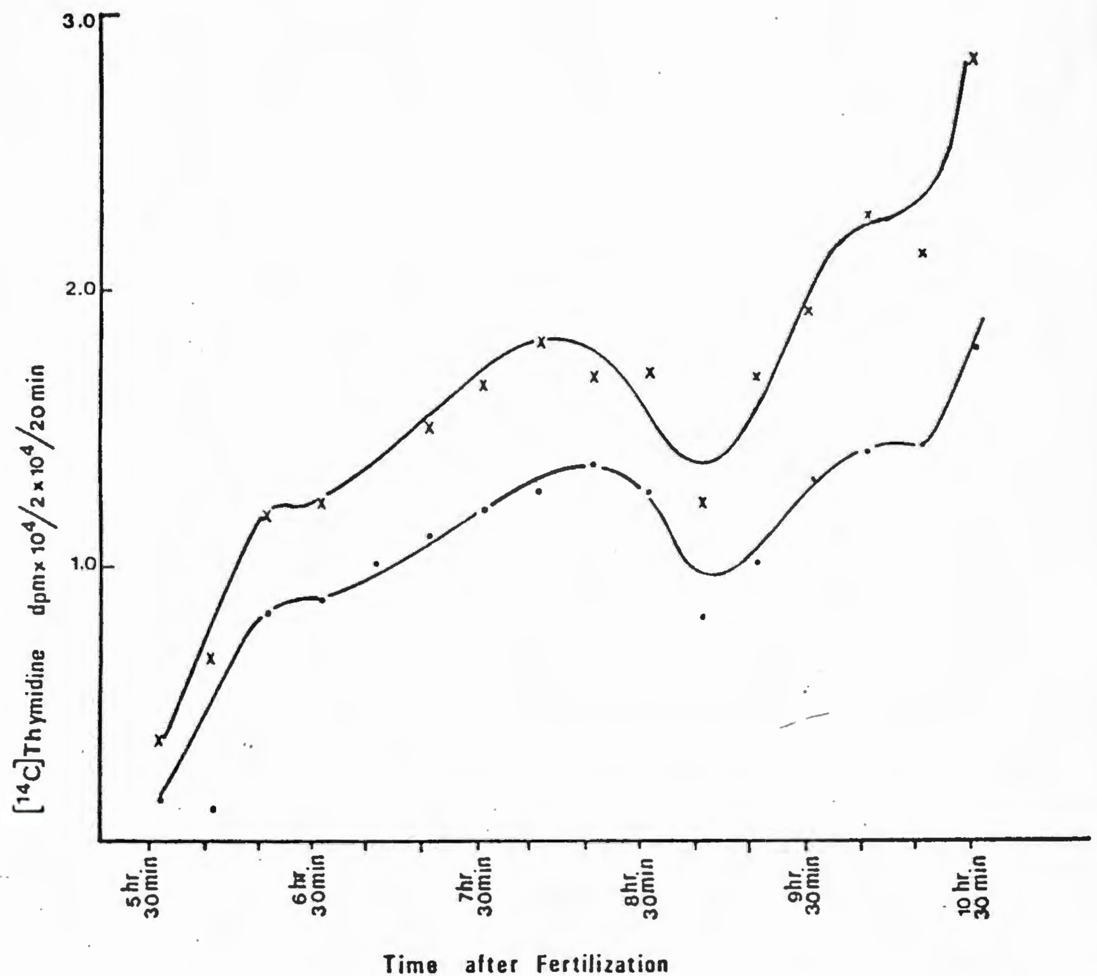


Figure 3.13 DNA Synthesis in the presence and absence of sodium butyrate.

Control (x) and 5 mM butyrate treated (o) sea urchin embryos were labelled with [^{14}C] thymidine (58 mCi/mmol). Sodium butyrate (5 mM) was added to a growing embryo culture 3 hours after fertilization. At particular times an aliquot of embryos (4 x 10^3 embryos suspended in 5 ml sea water) was taken and labelled for 20 minutes, with 0,04 μCi [^{14}C] thymidine. After the 20 minute labelling period the embryos were collected, washed with cold CMFSW, precipitated with 5% TCA (w/v), collected on millipore filters and counted on the Packard Liquid Scintillation counter.

Acetate labelling of the histones *in vivo* was done as described in section 5.6.3. Embryos were suspended in aerated sea water while being stirred, at a concentration of about 4 x 10^6 eggs per 100 ml.

5 mM sodium butyrate was present in the growth medium from 3 hours after fertilization. Sodium butyrate was prepared by adjusting n-butyric acid (Sigma Chemical co.) with 10 M sodium hydroxide to pH 7,0. The sodium butyrate was made up to a concentration of 5 M with distilled water.

The development of the sea urchin embryos grown with butyrate present in the growth medium, was monitored under the phase contrast microscopeand compared to a control. Development proceeded normally for the period 5½ to 10½ hours after the embryos were fertilized.

Separate 40 ml aliquots consisting of 16 x 10^5 embryos were taken at particular time intervals and labelled by the addition of 4 mCi [^{3}H] acetate (2,2 Ci/mMol). Nuclei were isolated from the labelled embryo culture immediately after the 20 minute period of labelling (section 5.2.1). The nuclei were frozen in liquid N_2 and kept until the following day, whereupon the histones from all the separate nuclei preparations were extracted simultaneously.

All nuclei and histone extraction solvents contained 5 mM sodium butyrate.

The whole histone complement was extracted with 0.25 M HCl. Examination of the acetylated histones was done similarly to the analysis of the phosphorylated histones (section 3.2.2).

The extracted histones were separated in the two gel systems, 3,8 M urea-Triton-polyacrylamide and 8,5 M urea-Triton-polyacrylamide (Fig. 3.14 and Fig. 3.15). The histones that became acetylated during the ninth cell cycle were $H2A_3$, H3 and H4 and to a lesser extent H1-1 and $H2B_1$ and $H2B_2$.

Figure 3.15 displays the separation of the [^{3}H] acetylated histones in the 8.5 M urea Triton polyacrylamide gel system and the respective fluorogram of the gel. Figure 3.15 (fluorogram) shows that the early H2A histone, $H2A_3$, became acetylated during the ninth cell cycle while only trace amounts of acetate associated with $H2A_1$ and $H2A_2$.

In the 8.5 M urea gel system, a large amount of label was located in the H3/H1 region of the gel (Fig. 3.15). Initially the label in the H3/H1 region appeared as a double band and from 8 hour 10 minutes as a single band. This was about the time that the H1-1 histone acetylation disappeared (Fig. 3.14). A band in the H4 region became heavily acetylated (Fig. 3.15). This dark band started to decrease in intensity after 7 hours 50 minutes.

A faint band was present in the $H2B_2$/$H2B_3$ region of the 8,5 M urea gel fluorogram (Fig. 3.15). In the 3,8 M urea gel the acetylated $H2B_2$ could be seen to migrate slightly faster than the unmodified $H2B_2$ (Fig. 3.14). Compared to the dark stained band in the region of the unmodified $H2B_2$, very little $H2B_2$ became acetylated (faint band migrating just ahead of $H2B_2$) (Fig. 3.14).

Histone $H2B_1$ became acetylated during the period 5½ to 10½ hours after fertilization (Fig. 3.14) while no acetylation of $H2B_3$ could be detected.

The relative amount of [^{3}H] acetate incorporated into the histones during the 5 hours of labelling was determined by scanning the fluorograms of the respective gels on the Vitatron Manual TLD 100 densitometer.

Figures 3.16 and 3.17 are a representation of the relative densities of the bands in the 3,8 M urea and 8,5 M urea gel systems. For each lane (time period), the density of the respective bands was calculated as a percentage of the total amount of absorption of the histone bands in that lane.

The relative amount of acetate incorporated into H1-1 dropped during the progression of the 9th cell cycle. Of all histones acetylated H1-1 acetylation was the least (Fig. 3.16). No acetylation of the other H1-histone variants could be detected.

The acetylation of the early H2A variant, $H2A_3$, was quite pronounced until 7½ hours, whereafter it dropped steadily (Fig. 3.16). $H2A_3$ became maximally acetylated at 8 hours 10 minutes (Fig. 3.16). Very little incorporation could be detected in the region of the later set of H2A histones, namely $H2A_1$ and $H2A_2$ (Fig. 3.15).

Histone $H2B_2$ became acetylated during the ninth cell cycle but as the embryo went into the 10th cell cycle the amount of acetate in the $H2B_2$ region became very faint. The $H2B_1$ histone, a histone belonging to the second set of histones, became acetylated during the ninth cell cycle, and stayed uniformly labelled throughout the labelled period (Fig. 3.16).

No [^{3}H] acetate became associated with the $H2B_3$ variant. Histone H4 acetylation followed the same pattern as that of $H2A_1$ and $H2B_3$, dropping after 7 hours 50 minutes (Fig. 3.16).

The H3 histone followed a completely different pattern. As the ninth cell cycle progressed towards mitosis, the H3 histone became more acetylated. The acetyl content associated with H3 increased even more as the embryo entered the 10th cell cycle (Fig. 3.16).

All the histones, except H3, became less acetylated after 8 hours (Fig. 3.16), i.e. the period when the 9th cell cycle entered mitosis (Fig. 3.7).

Histone H3 accepted more and more acetate groups as the embryo proceeded through the 9th cell cycle and entered into the 10th cell cycle.

The lane to lane comparison of the fluorogram and the protein fractions in the 3.8 M urea-triton gels (Fig. 3.18) and the 8.5 M urea-Triton polyacrylamide gels (Fig. 3.19) also clearly shows the programmed acetylation of the histone fractions with a preferential modification of the early set of histones.

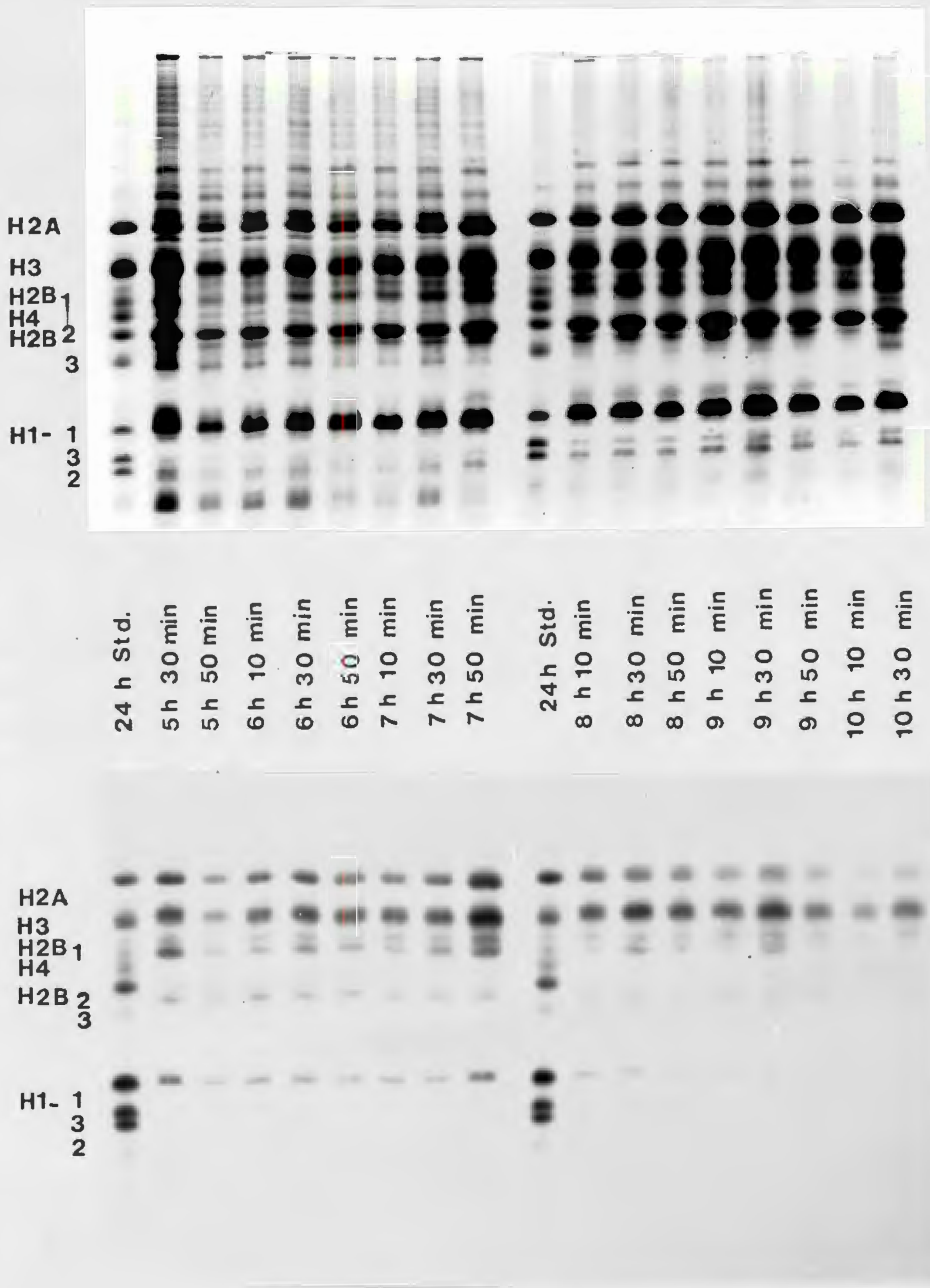


Figure 3.14 Separation of [^{3}H] acetate labelled histones in a 3,8 M urea, 6 mM Triton polyacrylamide gel.

4 mCi [^{3}H] sodium acetate (2,2 Ci/mmole) was added to 40 ml embryo cultures (4% cultures) at the times indicated. The embryo cultures were grown for 20 minutes after the addition of the label, whereupon the embryos were collected by centrifugation, nuclei isolated (5.2.1) and histones extracted (5.2.2). The embryos were grown in the presence of 5 mM butyrate from the 3 hour old stage. All extraction solvents contained 5 mM sodium butyrate.
Coomassie Brilliant Blue stained gel = Top;
Fluorogram = Bottom;
Standard histone = [^{3}H] lysine labelled histones

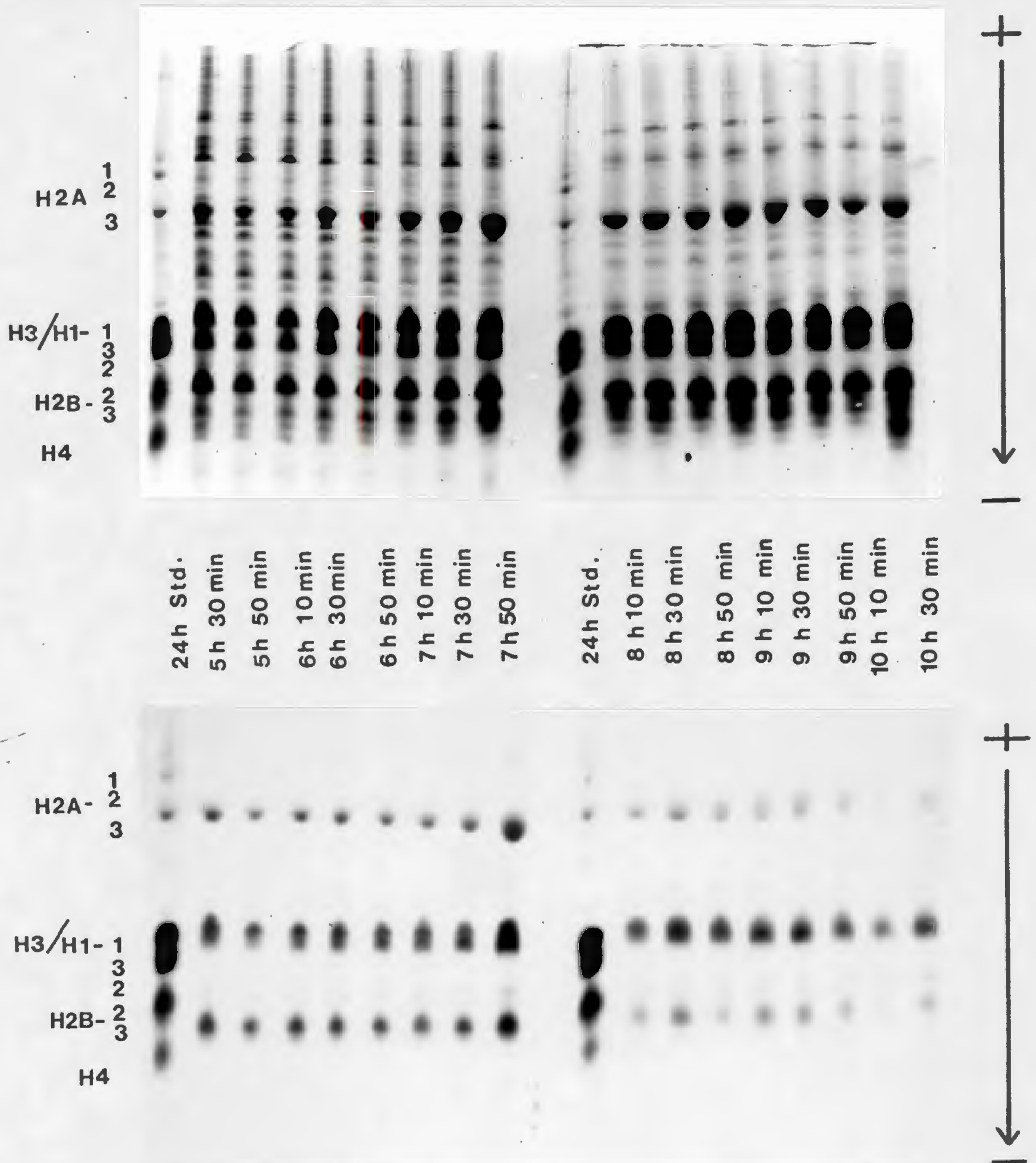


Figure 3.15 Separation of [^{3}H] acetate labelled histones in a 8,5 M urea, 6 mM Triton polyacrylamide gel.

4 mCi [^{3}H] sodium acetate (2,2 Ci|mmol) was added to 40 ml embryo cultures (4% culture) at the times indicated. The embryo cultures were grown for 20 minutes after the addition of the label, whereupon the embryos were collected by centrifugation, nuclei isolated (5.2.1) and histones extracted (5.2.2). The embryos were grown in the presence of 5 mM butyrate from 3 hour old stage. All extraction solvents contained 5 mM sodium butyrate.
Gels were prepared as described in section 5.3.1.
Coomassie Brilliant Blue stained gel = Top
Fluorogram = Bottom
Standard histones = [^{3}H] lysine labelled histones.

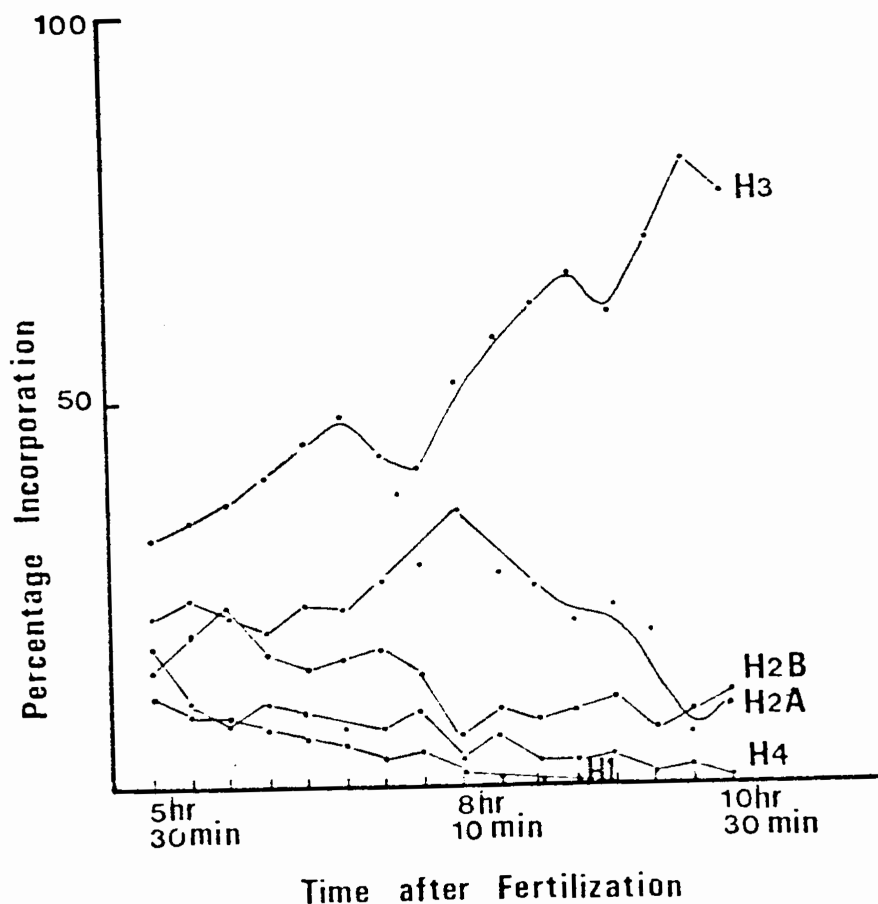


Figure 3.16 Relative amount of [^{3}H] acetate associated with the histone bands in the fluorogram of the 3,8 M urea gel.

The fluorogram was scanned on a Vitatron Manual TLD 100 Densitometer. The intensity of each band was calculated as a percentage of the total amount of absorption for each lane. H2B was plotted as the sum of the absorption for $H2B_3$ and $H2B_1$.

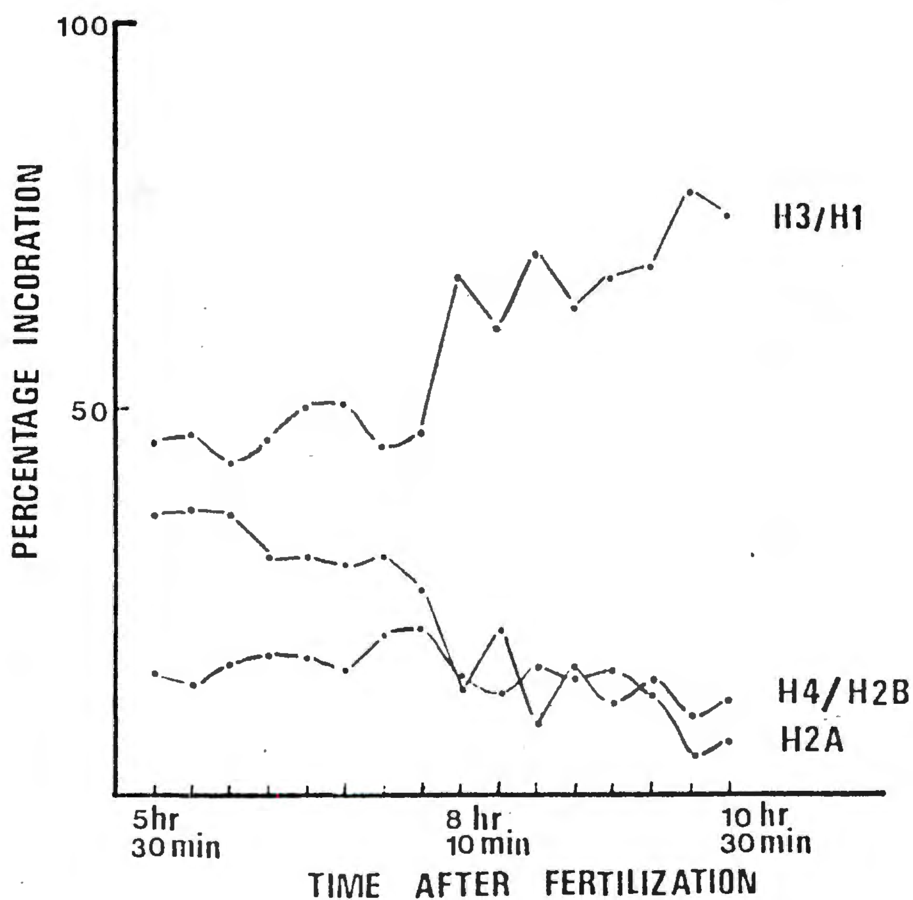


Figure 3.17 Relative amount of [^{3}H] acetate associated with the histone bands in the fluorogram of the 8,5 M urea gel.

The fluorogram was scanned on a Vitatron Manual TLD 100 Densitometer. The intensity of each band was calculated as a percentage of the total amount of absorption for each lane. H4 and H2B was plotted as one band.

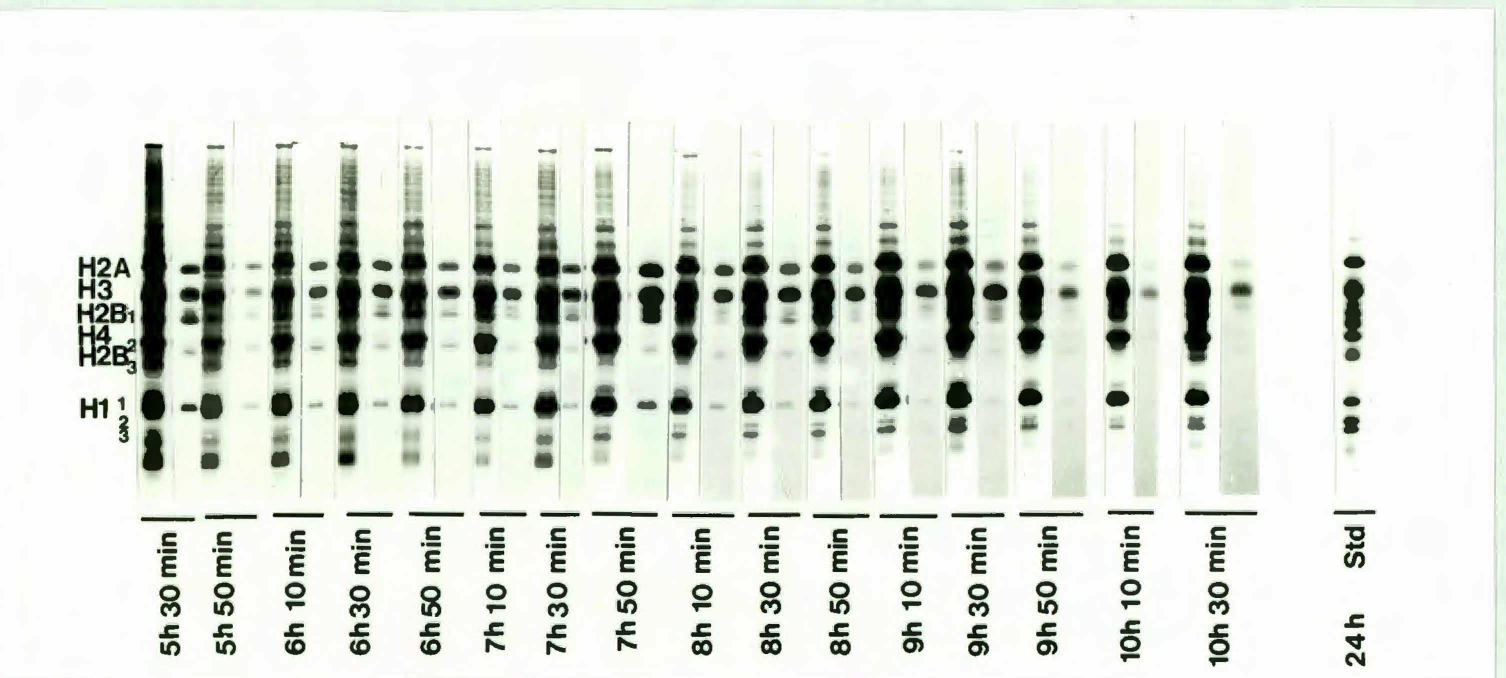


Figure 3.18: Comparison between the stained histone bands and the [^{3}H] acetate labelled histone bands in the 3,8 M urea-Triton-polyacrylamide gel system.

Times are when the [^{3}H] acetate was added to the growing culture.

Coomassie Brilliant Blue stained proteins = left lane;
Fluorogram = right lane;
Standard histone proteins = [^{3}H] lysine labelled histones.

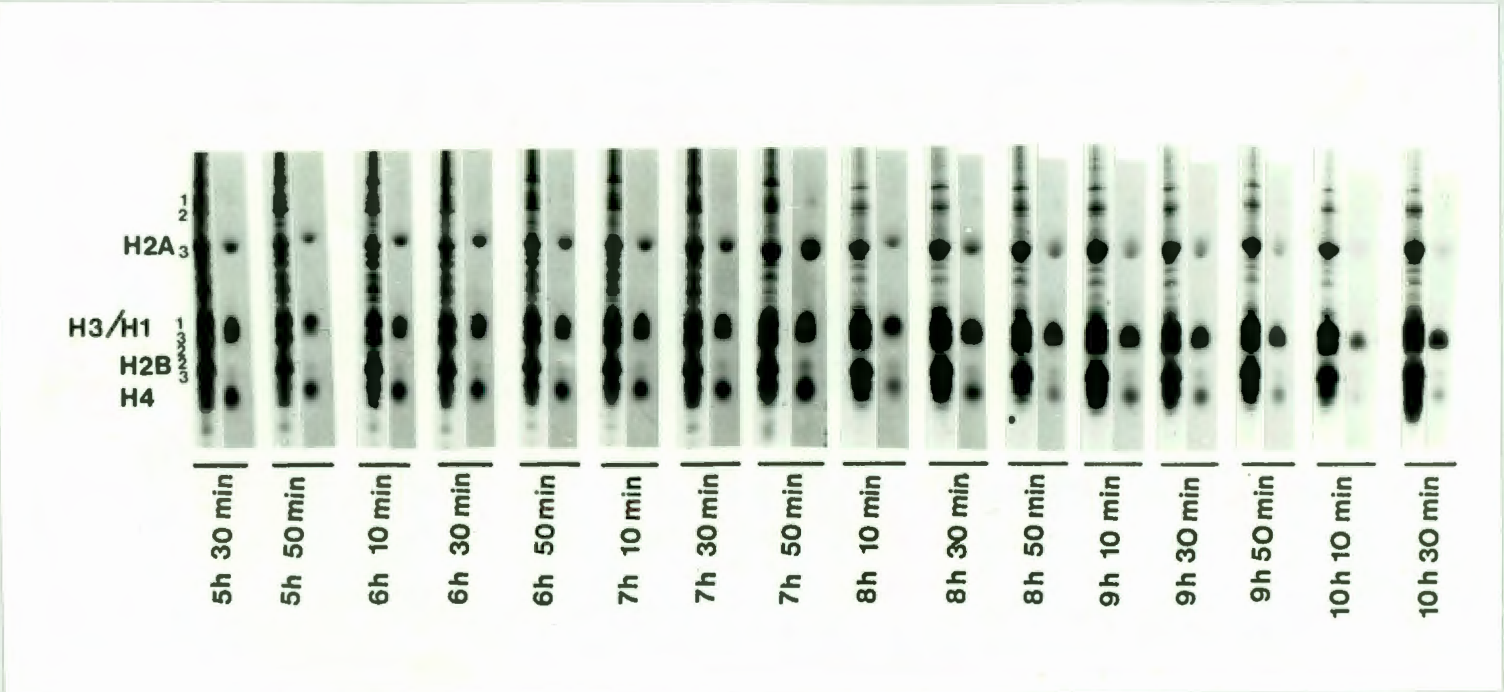


Figure 3.19: Comaprison between the stained histone bands and the [^{3}H] acetate labelled histone bands in the 8,5 urea-Triton-polyacrylamide gel system.

Times indicate the time after fertilization when the [^{3}H] acetate was added to the growing culture.

Coomassie Brilliant Blue stained proteins = left lane;
Fluorogram = right lane;
Standard histone proteins = [^{3}H] lysine labelled histones.

PART 4

4.1 Evolutionary and Developmental Aspects of the Primary Structure of the H1-histone Fraction

By now it is well established that the histone H1 structures exhibit a considerable degree of variation from species to species and within one species from tissue to tissue (Elgin and Weintraub, 1975).

Schaffner et al. (1978) reported that the H1-histones represent a challenge for identification since these histones evolve so rapidly.

The amino acid composition of *P. angulosus* embryo H1 histones shows these proteins to have a distinctly different composition to the sperm and gut histones of the same species (Table 4.1).

Embryo H1 histones have a less basic nature than the sperm histones in that they contain more glutamic and aspartic acid and considerably less arginine residues. The gut H1-histones have an amino acid composition intermediate between the sperm and embryo variants with respect to acidity and basicity.

The amino acid composition of *P. angulosus* embryo H1 histones fractions is more closely related to that of the H1-fractions of the other sea urchin embryo species, *Strongylocentrotus purpuratus* and *Arbacia lixula*, than to the sperm and gut histones from the same species (Johnson et al., 1973; Ruiz-Carrillo and Palau, 1973) (see Table 4.1).

The closer correlation between the H1-fractions from embryos of different species, suggests a particular role and function for the histones in the embryos which require the H1 histone fraction to be less basic. The higher acidity of embryo H1 histones and the lower content of proline and arginine may thus influence its structure and binding to the DNA (Bradbury et al., 1975) and therefore result in a looser packing of the chromatin.

Amino Acid	P. angulosus Gastrula H1 1	2	3	P. angulosus Sperm H1	Gut H1	S. purpuratus pluteus H1	H1	Arbacia lixula Swimming blastula H1
Aspartic Acid	2.7	2.4	2.2	1.8	4.1	2.0	3.4	4.8
Threonine	7.9	3.4	4.7	2.0	4.2	3.2	5.4	6.4
Serine	3.1	4.1	3.3	7.1	4.7	7.9	4.8	4.7
Glutamic Acid	11.0	5.9	3.9	2.3	6.5	8.9	8.3	6.4
Proline	6.6	8.1	7.7	8.6	7.3	6.1	6.7	5.7
Glycine	2.9	4.6	4.1	4.4	5.5	3.3	6.1	4.7
Alanine	20.7	25.6	30.1	29.4	22.0	27.3	20.7	21.9
Cystine	-	-	-	-	-	-	-	-
Valine	4.1	4.1	2.7	4.0	4.0	2.6	4.8	2.8
Methionine	0.8	0.7	0.5	1.7	0.9	0.7	tr	0.4
Isoleucine	2.6	2.0	2.1	0.8	2.6	1.3	2.7	2.9
Leucine	3.7	2.9	2.8	2.1	4.2	2.1	4.7	4.2
Tyrosine	0.4	1.1	2.2	0.8	1.3	0.5	1.2	1.0
Phenylalanine	1.3	0.7	0.9	0.4	1.1	0.7	1.4	0.8
Lysine	32.2	34.1	33.0	25.1	27.2	30.8	24.9	29.1
Histidine	0.4	0.6	0.4	0.9	1.0	tr.	0.8	1.5
Arginine	1.5	1.9	1.8	9.1	3.2	2.1	4.7	2.4
N-Terminal	Bl	Bl	Bl	Pro	Bl	Bl	Bl	
Lys/Arg	21.5	17.9	18.3	2.7	8.5	14.7	5.3	12.1
Lys + His & Arg / Asp + Glu	2.54	4.4	5.8	8.6	2.9	3.0	2.6	2.9

Table 4.1 The amino acid composition in molar ratios of the three H1 histones from *Parechinus angulosus* gastrula stage embryos, compared to *P. angulosus* sperm and gut H1-histones and the H1-histones from other sea urchin embryo species, *Strongylocentrotus purpuratus* pluteus and *Arbacia lixula* swimming blastula.
Sperm H1-data from Strickland et al., 1980
Gut H1-data from Brandt et al., 1979
S. purpuratus embryo H1-data from Johnson et al., 1973.
A. lixula embryo H1-data from Ruiz-Carrillo and Palau, 1973

This lower arginine content and higher acidic amino acid content of H1 histones might be a reflection of the genetic activity of embryo chromatin.

Puigdomenech et al. (1976) reported that the contracted state of sperm chromatin was directly related to the increased arginine content of the Ol (marine invertebrate sperm H1) histone.

Three chromatographically (CM-cellulose) separable H1-variants were present in the gastrula stage embryo of P. angulosus. These three fractions represented the three major H1-bands in Triton-acid-urea gels. H1-1 was the early H1-histone variant while H1-2 and H1-3 represented the H1-variants that were synthesized and associated with the DNA at the late-blastula-early gastrula stage. The synthesis of late H1 histones followed disappearance from the polyribosomes of mRNA for the early H1-variant and its substitution by the mRNA for the late H1-histone variants (Ruderman, 1973; Ruderman et al., 1974). This clearly points toward a transcriptional and not translational control mechanism for the switch in synthesis. H1-1 was the smallest of the H1 variants according to SDS gel electrophoresis (Fig. 2.9) and the least basic of the H1-variants (Table 3.1). H1-1 would appear to represent the opposite of sperm H1 in that it is associated with the DNA at a period when the DNA is in a loose configuration (Ozaki, 1971) and genetically most active.

All three H1-variants had an acetyl blocked amino terminal end in contrast to sperm H1 that has a proline amino end. The embryo H1-variants had two methionine residues while sperm H1 contained a total of four.

The embryo H1-variants were a complex mixture of at least 5 H1-like histones with closely related amino acid compositions, molecular weights and primary structures.

The early H1-variant, H1-1, separated into multiple bands in the low urea section of the Triton-acid-urea gradient gel (Fig. 2.5). Those bands might be the result of methionine oxidation and modification of H1-1 since H1-1 displayed a homogeneous character upon chemical and enzymatic fractionation and sequencing. 70% of the primary structure of H1-1 was sequentially determined.

Both H1-2 and H1-3 had a heterogenous character. This became obvious upon chemical fragmentation, gel electrophoesis of the large fragmens and sequence analysis of the fragmented H1-variants. Of the primary structure of H1-2, 29% was sequentially determined while 27 sequential Edman degradations of H1-3 histone were lined up and compared to that of other H1-histones.

The sequentially liberated amino acids of the sea urchin embryo *P. angulosus* H1-variants were compared to the H1 sequences of some reference histones. These were the H1-sequence of sperm from *P. angulosus*, *Echinolampas crassa*, *Sphaerechinus angulosus*, the DNA sequence of H1 from two clones (h19 and h22) from *Psammechinus miliarus*, trout testis H1 and rabbit (RTL-3) (Fig. 3.1).

The H1-gene sequence deduced from the h22-clone was found to code for the most divergent amino acid sequence of H1 histones when compared with reference hisones (Schaffner et al., 1978). The DNA sequence of the h22 clone for H1 differed from that of trout testis H1 and rabbit H1 with the exception of a region of 22 amino acids (codon positions 65-86) (Schaffner et al., 1978) which were nearly identical to the region 77-98 of the trout testis H1 (Macleod et al., 1977) and to region 87-108 of rabbit H1 (Jones et al., 1974). By comparing the amino acid sequences of H1 of the h22 clone of *Psammechinus miliaris*, it was noted that the h22 clone had many similarities to the sperm H1 but was clearly not the translation of a sperm histone gene (Strickland et al., 1980).

The comparison between the amino acid sequences deduced from P. miliaris clone h19 and h22 showed an average divergence of 12,4% (Busslinger et al., 1980). The H1-protein sequences deduced from DNA sequences of both clones (h19 and h22) suggested that these two proteins differ by 30%, (Busslinger et al,, 1980). Busslinger et al. (1980) found that the histone DNA clone h19 from P. miliaris and clones pSp2 and pSp17 from S. purpuratus (Sures et al., 1978; Grunstein and Grunstein, 1977) showed an average divergence of only 1,74% in the DNA sequences coding for H4, H3, H2A and H2B proteins. It was thus apparent that the DNA sequence divergence between h19 and h22 (12,4%), two clones from the species P. miliaris, was far greater than between h19 and S. purpuratus pSp2 and pSp17 (1,74%).

On the basis of hybrid stability studies, the late and early histone genes of Strongylocentrotus purpuratus were found to be considerably more diverged from one another than the early histone gene classes of two distantly related sea urchins, S. purpuratus and Lytechinus pictus (Kunkel and Weinberg, 1978).

Clone h19 constitutes a mini-family of histone genes since it was repeated only a few times (ca. 5 times) in the haploid genome of the sea urchin Psammechinus. The h22 unit, on the other hand, had a reiteration frequency two orders of magnitude greater than h19. Clone h22 was part of a maxi-family, representing the most common histone DNA unit in Psammechinus (Busslinger et al., 1980).

Busslinger et al. (1980) suggested that h19 might code for embryonic histone variants since a correlation between H2A of h19 (residue 50-75) was identical to a partial amino-acid sequence of an H2A variant of P. angulosus 18h embryo H2A.

Comparing the P. angulosus embryo H1-variants primary structure to that of the h19 and h22 clones, I would regard h19 plus h22 as genes coding for embryonic histone variants.

H1-1 has been sequentially degraded for 169 steps. A comparison between the sequence of H1-1 and the amino acid sequences deduced from the DNA sequences for H1 of the h19 and h22 clones revealed an 80% identity between embryo H1-1 and the H1-gene sequence from h19 and a 77% identity between embryo H1-1 and the h22 clone sequence for H1.

In the majority of positions where the embryo H1-1 sequence differed from the H1-gene of one or the other clone, it nevertheless corresponded to that of the other clone. Those positions were positions 10, 24, 28, 32, 34, 38, 42, 43, 47, 48, 53, 55, 65, 69, 72, 75, 79, 89, 96, 97, 109, 110, 118, 119, 122 and 127.

Positions where H1-1 differed from both the H1 sequence of the h19 and h22 clone were positions 39, 46, 58, 59, 62, 131, 136, 144, 145, 147, 149, 152 and from position 183 onwards. These changes in many cases represented conservative changes.

The carboxyl terminal sequence of embryo H1-1 displayed the characteristic lysine-alanine rich feature. In addition the embryo H1-1 contained threonine and proline in this region in contrast to sperm which contained serine, proline and arginine. Compositionally the carboxyl terminal tail of embryo H1-1 compared well with that of the H1 from the h19 and h22 clones but the sequence in that region differed from the H1 clone sequences except for positions 171-182. The carboxyl termini of both the H1's from h19 and h22 clones lacked the arginine-rich character of sperm H1-tail.

The sperm histones sequence was approximately 26 residues longer at the amino terminus than the embryo H1s and the histone H1 sequence deduced from the h19 and h22 clones. Furthermore, the carboxyl terminal tail of sperm H1 was arginine rich in contrast to embryo H1 and the H1 sequences deduced from the genes. These two structural characteristics of sperm histone H1 are probably responsible for the close condensation of chromatin required in the sperm cell.

The partial sequence analysis of H1-2 and H1-3 confirmed their identity as H1-histones.

Embryo H1-2 has been sequentially degraded for 66 steps while 27 sequentially degraded amino acids of the H1-3 fraction were lined up with H1-1 and the gene sequences from P. miliaris.

From position 22-41 the H1-2 and H1-3 proteins were found to be more closely related to each other than to embryo H1-1 or the H1 sequences deduced from the h19 and h22 clones. In positions 24, 25, 29 and 34 the H1-2 and H1-3 histones were found to be similar while they differed from the reference H1's. Position 25 was alanine in the two H1 histones, H1-2 and H1-3, while it was threonine in H1-1 and the two gene sequences from P. miliaris. Position 51 was alanine in the sea urchin sperm H1's. This indicates a tendency for the later H1's, H1-2 and H1-3, to have a primary structure intermediate between embryo H1-1 and sea urchin sperm H1.

The sequence of H1-2 has been determined from positions 53-93. This region of the H1-2 histone fraction showed a strong correlation between H1-2 and the reference histones up to position 88. From position 89 the H1-2 histone displayed a variable character. This area represents the head-tail connection and it is well known for its variable character (Hartman et al., 1977).

Hartman et al. (1977) proposed that calf thymus H1 has a random coil "nose" rich in lysine, alanine and proline at the amino terminal region. This region is followed by a hydrophobic globular region, the "head". The transition area between these two domains is in the vicinity of the His-Pro-Pro-sequence. On the amino side of this triplet (position 16-18 of the embryo H1-1, Fig. 3.1) there was very little homology between embryo sequence and the sperm histone H1 sequence from P. angulosus (Strickland et al., 1980). This domain on the amino side of the triplet (His-Pro-Pro-) of embryo H1-1 was identical to the H1 sequence deduced from the h19 clone and differed only in one position (position 10) from that of the H1 sequence deduced

from the h22 clone. The "nose" of the mebryos H1-1 plus that of the H1 sequences from the two genes were shoter by about 26 amino acids when compared to the amino terminal "nose" of sperm H1.

Strickland et al (1980) described a homology between P. angulosus sperm H1 and the gene sequence deduced from the h22 clone on the carboxyl side of the triplet. They found that between alignment position 42-113 of sperm H1 (corresponding to positions 16-87 of the gene and embryo H1-1), there were 34 identical amino acids and 11 conservative amino acid changes. The homology between Parechinus embryo H1-1 and the H1-sequences deduced from the two gene sequences was much higher, with 60 identical amino acids between the H1-sequence deduced from the h19 clone and 55 identical amino acids deduced fdrom the H1-sequence of the h22 clone. This area has been described as the "head" of the H1 and has been found to have a hydrophobic character, giving a globular structure in an aqueous environment. The basic residues would be positioned on the surface (Hartman et al., 1977). This part has been shown to contain all the secondary and tertiary structure of intact H1 and, moreover, to retain this structure after the removal of the N-terminal and C-terminal regions (Puigdomenech et al., 1980). The "head" constitutes the globular hydrophobic region and the region where H1 sequences have been conservative, reflecting the importance of protein-protein interactions in chromatin structure.

The rest of the H1-histone protein has been described as a random coil "tail" (Hartman et al., 1977). It has been domonstrated that this part of H1 acted as efficiently as the whole H1-molecule in DNA binding (Bradbury et al., 1975) and this section also showed cross-linking properties (Hartman et al., 1977).

Figure 4.1 Comparison of the amino acid sequence of the *P. angulosus* embryo H1-variants with the corresponding amino acid sequences of *Psammechinus miliaris* histone H1's deduced from the nucleotide sequences of the h19 and h22 clones, sperm H1 sequences from *P. angulosus*, *Echinolampas crassa*, *Sphaerechinus granulosus*, trout testis H1 and rabbit (RTL-3).

Identical residues are enclosed within solid lines; solid lines between sequences = gaps for alignment; dashed line = sections not sequenced.

H1 sequences deduced from the h19 and h22 clones - M.L. Birnstiel personal communications

P. angulosus sperm H1 sequence - Strickland et al., 1980

Echinolampas crassa sperm H1-sequence - W.N. Strickland personal communications

Sphaerechinus granulosus sperm H1-sequence - W.N. Strickland personal communications

Trout testis H1 - Macleod et al., 1977

Rabbit thymus (RTL-3)- Cole, 1977

One-letter symbols for amino acids:

Alanine = A; Arginine = R; Asparagine = N; Aspartic Acid = D; Cysteine = C; Glutamine = Q; Glutamic Acid = E; Glycine = G; Histidine = H; Isoleucine = I; Leucine = L; Lysine = K; Methionine = M; Phenylalanine = F; Proline = P; Serine = S; Threonine = T; Tryptophan = W; Tyrosine = Y; Valine = V.

```
                                                                                 6       10
PARECHINUS ANGULOSUS EMBRYO H1-No 1                              Blocked NH2 - - - - - K K V T T
                            H1-No 2                              Blocked NH2 - - - - - - - - - -
                            H1-No 3                              Blocked NH2 - - - - - - - - - -
PSAMMECHINUS MILIARIS       H1-GENE-h19 CLONE                              A E K E S S K K V T T
                            H1-GENE-h22 CLONE                              T D T - - A K K V T Q
PARECHINUS ANGULOSUS SPERM  H1         P G S P Q K R A A S P R K S P R K - S P R K S P R K A S A S P R R K A K R
ECHINOLAMPAS CRASSA  SPERM  H1         A A S P Q K R A A S P R K S P P K - S P R K S P K K K - - S P R K R - K -
SPHAERECHINUS GRANULOSUS SPERM H1      P G S P Q K R A A S P R K S P P K G S P K K S P - - - - - - - - - - - - - -
TROUT TESTIS H1                                                Ac A E V A - P A P A A A A P A K A P K K K A A A K
RABBIT (RTL-3)                      Ac S G A P A E T A A - P A —— P A E K S P - - A K K K K A A K K P G A G A A K

                                           15        20        25        30        35         40        45
PARECHINUS ANGULOSUS EMBRYO H1-No 1     K K P - A A H P P A A E M V T T A I T E L K E R N G - S S L N A I K K Y I
                            H1-No 2     - - - - - - - - - - - - M V V A A I G A L K A R G G - E E E Q A I - - - -
                            H1-No 3     - - - - - - - - - - - - M V V A A I T A L K E R G G - S S N Q A L K K Y K
PSAMMECHINUS MILIARIS       H1-GENE h19 CLONE  K K P - A A H P P A A E M V A T A I T E L K T R N G - S S L Q A I K K Y I
                            H1-GENE h22 CLONE  K K P - A A H P P A A E M V T T A I K E L K E R K G - S S R Q A I A N Y I
PARECHINUS ANGULOSUS SPERM  H1          A R A - S T H P P V L E M V Q A A I T A M K E R K G - S S A A K I K S Y M
ECHINOLAMPAS CRASSA SPERM   H1          A R S - A A H P P V I D M I T A A I A A Q K E R R G - S S V A K I Q S Y I
SPHAERECHINUS GRANULOSUS SPERM H1       - - - - - - - - - - - - M I R A A I T A M K E R K G - S S V A K I K S Y I
TROUT TESTIS H1                         P K K - S G - P A V G E L A G K A V A A S K E R S G V S L A A L K K S L A
RABBIT (RTL-3)                          R K —— A A G P P V S E M I T K A V A A S K E R N G - L S L A A L K K A L
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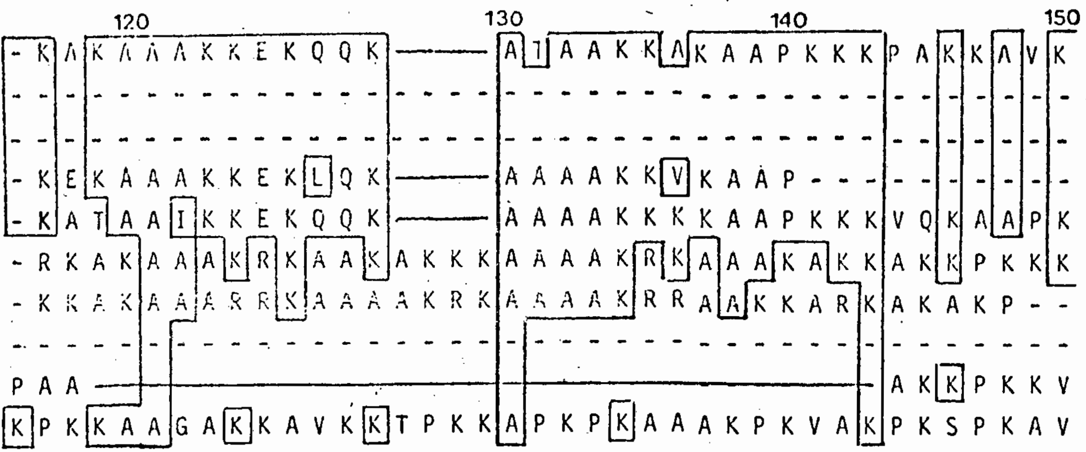
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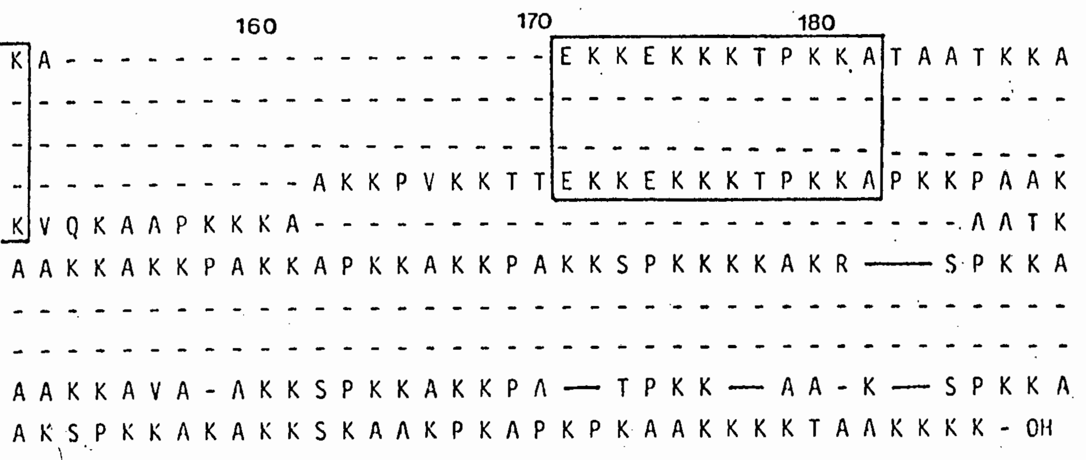
H1-No 2
H1-No 3
PSAMMECHINUS MILIARIS H1-GENE h19 CLONE - K E K A A A K K E K L Q K — A A A A K K V K A A P
H1-GENE h22 CLONE - K A T A A I K K E K Q Q K — A A A A K K K K A A P K K K V Q K A A P K
PARECHINUS ANGULOSUS SPERM H1 - R K A K A A A K R K A A K A K K K A A A A K R K A A A K A K K A K K P K K K
ECHINOLAMPAS CRASSA SPERM H1 - K K A K A A A R R K A A A A K R K A A A A K R R A A K K A R K A K A K P
SPHAERECHINUS GRANULOSUS SPERM H1
TROUT TESTIS H1 P A A — A K K P K K V
RABBIT (RTL-3) K P K K A A G A K K A V K K T P K K A P K P K A A A K P K V A K P K S P K A V


160 170 180
PARECHINUS ANGULOSUS EMBRYO H1-No 1 K A - E K K E K K K T P K K A T A A T K K A
H1-No 2
H1-No 3
PSAMMECHINUS MILIARIS H1-GENE h19 CLONE - A K K P V K K T T E K K E K K K T P K K A P K K P A A K
H1-GENE h22 CLONE K V Q K A A P K K K A - A A T K
PARECHINUS ANGULOSUS SPERM H1 A A K K A K K P A K K A P K K A K K P A K K S P K K K K A K R — S P K K A
ECHINOLAMPAS CRASSA SPERM H1
SPHAERECHINUS GRANULOSUS SPERM H1
TROUT TESTIS H1 A A K K A V A - A K K S P K K A K K P A — T P K K — A A - K — S P K K A
RABBIT (RTL-3) A K S P K K A K A K K S K A A K P K A P K P K A A K K K K T A A K K K K - OH

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                                                    50                  60                  70              80
PARECHINUS ANGULOSUS EMBRYO H1-No 1                E T N - F D V E M - D R Q L I K I K K A L K S G V E K G K L V Q T K G T G
                            H1-No 2                - - - - - - - - M - K K Q S V F K K K A L K S G V E K G Y L V Q V K G K G
                            H1-No 3                A A N - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
PSAMMECHINUS MILIARIS       H1-GENE-h19 CLONE      A T N - F D V E M - D R Q L L F I K R A L K S G V E K G K L V H T K G K G
                            H1-GENE-h22 CLONE      K A H - F D V E I - D Q Q L V F I K R A L R S G V A K G T L V Q T K G T G
PARECHINUS ANGULOSUS SPERM  H1                     A A N - Y R V D M - N V L A P H V R R A L R N G V A S G A L K Q V T G T G
ECHINOLAMPAS CRASSA SPERM   H1                     A A K - Y R C D I - N A L N P H I R R A L R N G V K S G A L K Q V S G V G
SPHAERECHINUS GRANULOSUS SPERM H1                  A A N - Y R V N M T N L Q - P H I R R A L R S G V A S G A L K Q V T G T G
TROUT TESTIS H1                                    A G G - T — D V - E K N N S R V K I A V S S L V T K G T L V Q T K G T G
RABBIT (RTL-3)                                     A A G G Y D V E K - N N S R — I K L G L L S L V S K G T L V E T K G T G

                                                                  90            100                 110
PARECHINUS ANGULOSUS EMBRYO H1-No 1                A S G S F K L N V Q A A K A Q A A E K A K K E - K E K A K L Q A Q R E K A
                            H1-No 2                A S G S F K L G K K P A A G R P D A - - - - - - - - - - - - - - - - - - -
                            H1-No 3                - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
PSAMMECHINUS MILIARIS       H1-GENE-h19 CLONE      A S G S F K L N V Q A A K A Q A S E K A K K E - K E K A K L L A Q R E K A
                            H1-GENE-h22 CLONE      A S G S F K L N M Q A A K A Q E A E K A K K E - K E K A K V Q A Q R E K A
PARECHINUS ANGULOSUS SPERM  H1                     A S G R F R V G A V A K P R R A K K T S A A A - K A K K A K A A A A K K A
ECHINOLAMPAS CRASSA SPERM   H1                     A T G R F R V G A V - K R S - A A S - A N K L - K A T R E K A R A R A K A
SPHAERECHINUS GRANULOSUS SPERM H1                  A T G R F R V G - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
TROUT TESTIS H1                                    A S G S F K L N K K A V E A K K P A K K A A A P K A K K - - - V A A K K -
RABBIT (RTL-3)                                     A S G S F K L D K K A A S G E A K P K P K K A G A A K P K K P A G A T P K
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                                                 190           200                       210               220
PARECHINUS ANGULOSUS EMBRYO H1-No 1              T P K T V T K K P A - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -OH
                            H1-No 2              - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -OH
                            H1-No 3              - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -OH
PSAMMECHINUS MILIARIS       H1-GENE-h19 CLONE    K S T P K K T P K K A ———— A A K K P I T A K P - - - - - - - K K P A A K A A K S K
                            H1-GENE-h22 CLONE    K A T P K K A T K K P ———— A A K K P K A S K P A A K K P A A K K P A A K V A K S K
PARECHINUS ANGULOSUS SPERM  H1                   K K A A G K R K P —————— A A K K A R R S P R K A G K R R S P K K A R K-OH
ECHINOLAMPAS CRASSA SPERM   H1                   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -OH
SPHAERECHINUS GRANULOSUS SPERM H1                - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -OH
TROUT TESTIS H1                                  T K A A - - - K P K A A K P K K A A K - - - - S P K K - - - V K K P A A A K K-OH
RABBIT (RTL-3)
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4.2 Schedule of Synthesis and Insertion of Chromatin Proteins

Whereas in S. purpuratus 3 different sets of histones appear to be synthesized in the development from fertilised egg to gastrula (Neurock, 1977) we found only evidence for two sets of histones imperative in the histone programme of the P. angulosus embryo.

No evidence of cleavage stage histones could be detected during the period immediately following fertilization through the cleavage stages of the embryo. Instead, the synthesis of the first set of histones was established 15 minutes after fertilization and continued to be synthesized during the early embryonic stages until early blastula. Thus, during the first stage in P. angulosus the chromatin became populated with histones $H2A_3$, $H2B_2$, H1-1 and minor amounts of H3 and H4 (Fig. 3.2). These forms of histones were the predominant histones present during the early embryonic stages until early blastula.

Stage 2 started with the onset of synthesis and association with DNA of the second set of hisones, comprising $H2A_1$, $H2A_2$, $H2B_1$, $H2B_3$, H1-2 and H1-3 (Fig. 3.1). The second set of histones first became associated with the DNA at about 8 hours after fertilization when the embryo was at early blastula stage. The onset of synthesis of the second set of histone variants coincided with the cessation of synthesis of the first set. The synthesis of the second set of histone variants occurred at a time in the embryonic development when cell division slowed down drastically (Fig. 3.6). In P. angulosus the switch in histone synthesis occurred towards the end of the ninth cell cycle. This cell cycle started at 5½ hours after fertilization and continued for 3 hours and 40 minutes to 9 hours and 10 minutes after fertilization when the embryo entered the 10th cell cycle (Fig. 3.7). All the variants made during the first stage were retained in the chromatin long after their synthesis ceased (as indicated by the dashed line in Fig. 3.1). Newrock et al. (1977) reported the retention in chromatin of cleavage stage (CS) subtypes at the prism

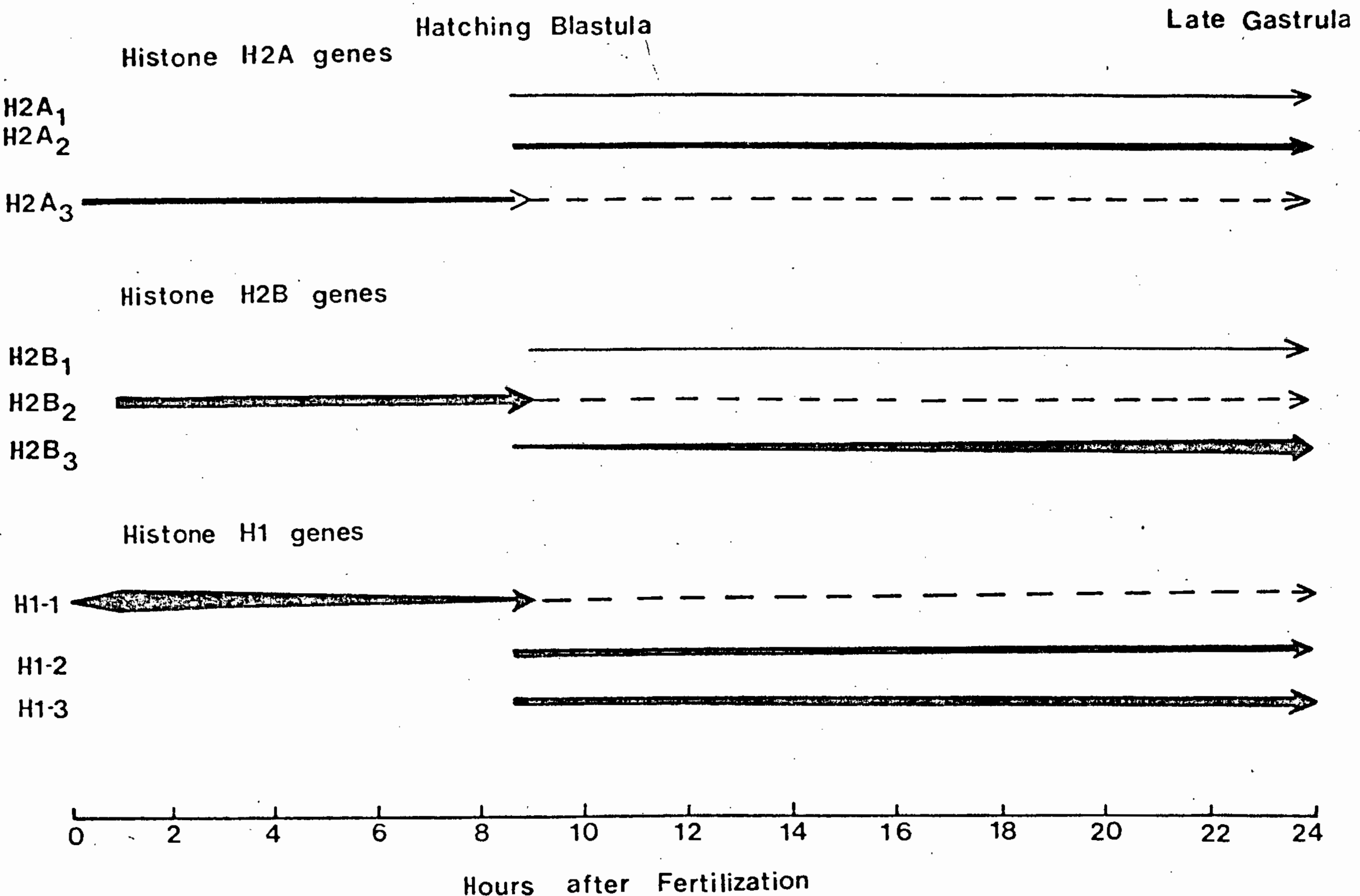

Figure 4.2: Schedule of synthesis and incorporation into chromatin of subtypes of H1, H2A-and H2B during the early development of P. angulosus, as detected by incorporation of [^{3}H] lysine. Heavy line indicates an abundant component; solid line shows the period during which a component was synthesized; dashed line shows the period during which a protein was no longer synthesized, but still maintained in the chromatin.

stage, 40 hours later. Poccia and Hinegardner (1975) concluded that the eventual lack of detection of the early histone variants is not because of degradation but because they gradually become overshadowed in quantity by the later histone variants. This long retention of early subtypes would indicate the high affinity of histones for the genome. The results of this investigation lead to the same conclusion proposed previously by Newrock (1977) namely:
any special functional properties that subtypes may have will continue to have an influence on the regions of the genome with which they are associated.

The increase in histone diversity during development can be correlated with changes in chromatin structure. The chromatin of the blastula, which contains the early set of histones, has been reported to be exceptionally susceptible to attack by nucleases (Spadafora and Geraci, 1976). This sensitivity has been reported to have declined by the pluteus stage (Keichline and Wassarman, 1977). The chromatin of sea urchin sperm, which contains its own unique set of histone variants, H1, H2A and H2B, displayed an unusually large nucleosomal repeat size (Keichline and Wassarman, 1977). Savic et al., (1981) reported that three general classes of DNA repeat lengths could be correlated with the three general classes of S. purpuratus sea urchin histone variants: (1) sperm histones and long spacers; (2) cleavage stage histones and short spacers; and (3) α and β possibly γ and σ histones and intermediate spacers.

4.3 Histone Phosphorylation and Acetylation during the Ninth Cell Cycle

The different histone variants probably have different functions in the dynamic as well as structural aspects of chromatin conformation.

Histone H1 may have an influence on the chromatin structure by way of H1 modification. There is evidence that gene activation, DNA replication and mitotic condensation of chromatin all require

phosphorylation of H1, and probably at different intramolecular sites (Langan, 1969; Lake et al., 1972; Bradbury et al., 1974; Hohmann et al., 1976). In mammalian cells, H1 subtypes differ from one another in the number of phosphorylation sites (Langan et al., 1971; Ajiro et al., 1976), providing a possible means for regional control of these chromatin functions (Newrock et al., 1977) (See Part I for a detailed discussion of these aspects).

An investigation of the phosphorylation and acetylation of the histone variants during the ninth cell cycle, (i.e. the period of histone switch over in synthesis from an early to late set of subtypes) showed that the early histone variants were the main substrates for modification at that stage.

All histone modifications were examined by separating the labelled histone variants electrophoretically in Triton-acid-urea gels at the two urea concentrations, 3,8 M urea and 8,5 M urea, (Figs. 3.9 & 3.10 and 3.14 & 3.15) which allowed optimum separation of the histone variants.

The labelled histone variants were identified by comparing them with [^{3}H] lysine labelled histone standards run in the same gel.

The main substrates for phosphorylation were the early H1-1 histone, the early $H2A_3$ histone, and histone H3. Two phosphorylated bands appeared in the region of $H2A_1$ and $H2A_2$, representing the later set of H2A histones, and they stayed equally labelled throughout the ninth cell cycle. The degree of $H2A_3$ phosphorylation during the ninth cell cycle showed a variation in the amount of phosphate groups associated with it. This became evident since phosphorylated $H2A_3$ separated into multiple bands that varied in intensity during the ninth cell cycle. Histone H-1 became heavily phosphorylated throughout the ninth cell cycle while very little detectable amounts of phosphate associated with the other H1-variants. No detectable amount of phosphate label became associated with either the H4 or H2B histones.

Thus the main histone substrates for phosphorylation during the 9th cell cycle were early histone variants H1-1 and $H2A_3$, while phosphorylation occurred in the region of the later H2A variants $H2A_1$ and $H2A_3$. As would be expected, histone H3 became heavily phosphorylated during the 9th cell cycle.

Acetylation was done in the presence of sodium-butyrate, a de-acetylase inhibitor. The subtypes that became acetylated during each period of administration of [^{3}H] acetate in the presence of sodium butyrate, would thus be a good representation of the histones acetylated in that space of time.

Histones that became acetylated represented the early set of histone variants plus histone H3 and H4.

Histone H3 was the preferential acceptor of [^{3}H] acetate and became increasingly more acetylated during the ninth cell cycle and even more as the developing embryo entered the 10th cell cycle.

Of the early histone variants $H2A_3$, $H2B_2$ and H1-1 became acetylated. The only histone of the later set that corresponded to an acetylated band was $H2B_1$. This histone was minimally labelled throughout the 9th cell cycle.

Histone H1-1 was acetylated the least during the ninth cell cycle and as the embryonic development proceeded through the cycle, H1-1 acetylation disappeared. H1-1 acetylation might be a reflection on the acetate incorporated into the amino-end of the acetyl-blocked H1-protein during synthesis. Therefore, as the synthesis of H1-1 ceased during the 9th cell cycle, the acetate incorporated into the H1-1 region disappeared.

Histones $H2A_3$, $H2B_2$ and H4 reached a maximum acetylated state at about 7 hours 50 minutes after fertilization, whereafter the acetylation dropped again. The decrease in acetate associated with $H2A_3$, $H2B_2$ and H4 corresponded to the time the 9th cell cycle entered mitosis.

The increasing acetylated state of histone H3 did not seem to correlate with any stage of the 9th cell cycle but might be a reflection on the genomic activity of the embryo at that stage.

These acetylation and phosphorylation studies done only reflect an initial examination of the histone variant modifications during a single cell division of the developing embryo. From these results it would appear that the first set of histones synthesized during the early stages of embryogenesis associated rapidly and stabily with chromatin. These histones remained associated with the chromatin even after they ceased to be synthesized. Such a situation should give rise to the synthesis of nucleosomes with their exact histone composition dependent upon the particular stage of sea urchin development. During the 9th cell cycle synthesis of the first set of histones ceased while the second set appeared in the chromatin. During this period of switch over in histone synthesis the main histone substrates for enzymatic modifications were the early histone variants. It thus appears that the nucleosomes containing the early histone variants differ from those containing the later histone variants not only in their histone content but to the extent that the histones become modified. This would indicate that the different sets of nucleosomes would thus differ functionally.

PART 5

MATERIAL AND METHODS

5.1 MAINTENANCE AND PREPARATION OF SEA URCHIN EMBRYOS

5.1.1 Source of Supplies

Sea urchins of the species *Parechinus angulosus* were collected at low tide amongst the intertidal rock pools along the Cape Peninsula coast. Places of collection were Dale Brook, Glencairn and Kalk Bay in False Bay and Bloubergstrand and Melkbosstrand in Table Bay.

5.1.2 Maintenance of Sea Urchins

The maintenance and handling of sea urchin adults and embryos was carried out essentially as described by Hinegardner (1967; 1975).

The sea urchins were collected in buckets and brought back to the laboratory for preparation. Sea urchins could be kept overnight, for "next day" preparations, by placing them upside down in trays with aerated lids in a 15°C constant temperature room. Unless they were already very near to spawning naturally, they did not spawn spontaneously overnight.

5.1.3 Collection of Gametes

Spawning was induced by injecting 3-5 ml 0,5 M KCl through the peristomal membrane into the coelomic cavity. The sea urchin was left upside down until it was possible to tell whether it was producing eggs or sperm.

The eggs were collected in sea water by inverting the injected females in a tray so that their genital pores were below the water.

The sperm were collected by placing the male in a petri dish, allowing the sperm to collect undiluted. The sperm was left undiluted up to the moment it was used for fertilization (Hinegardner, 1967).

Females were allowed to shed their eggs for ½ - 3/4 hour. The eggs were filtered through cheesecloth and allowed to settle by gravity sedimentation. The sea water supernatant was removed by suction and the eggs were resuspended in filtered sea water (Whatman 41). The washing procedure was done twice in order to get rid of spines, sand etc.

After washing and settling the eggs, they were resuspended in a known amount of sea water and 10 ml of this egg suspension was spun in a graduated centrifuge tube in a bench centrifuge. The final egg suspension was calculated accordingly and made up in sea water to be 4% - that is 4 ml of cenrifuged eggs per 100 ml of sea water. A 4% egg suspension was calculated to contain an average of 4×10^6 eggs per 100 ml sea water. A rough estimate of the number of eggs was made by counting on a Coulter counter. (M. Strickland and F. Davids - personal communication).

All sea water was filtered through Whatman 41 filter paper.

5.1.4 Fertilization and Development of Embryos

The spermcells were diluted 1000 times in filtered sea water and added to the egg suspension (4×10^6 eggs/100 ml) at about 1 ml sperm/100 ml egg suspension.

Fertilization was monitored under the microscope by the presence of the fertilization membrane. Only batches of eggs showing more than 95% fertilization were used for experiments.

The fertilized eggs were incubated at 20°C in filtered sea water (Whatman 41) at 4 x 10^6 eggs/100ml containing 100 mg/ml Penicillin G and 50 g/ml Streptomycin sulphate.

When large quantities of culture were grown (1-7ℓ), the embryos were allowed to develop while being gently stirred with rotating propellers. Simultaneously air was bubbled through the suspension. These measures were necessary to provide adequate oxygenation.

Small cultures were grown in 150 ml or 250 ml conical flasks fitted with cottonwool stoppers. The conical flasks were fitted on a Gyratory shaker and the egg suspension was swirled at a speed of about 220 rpm.

All cultures were grown in the 20°C constant temperature room.

5.2 ISOLATION OF NUCLEI AND HISTONES

Large volumes of embryo cultures were immobilized and settled by the addition of 3 ml 5% SDS / 5ℓ embryo suspension. Small cultures were centrifuged in SS34 centrifuge tubes at 750 rpm for 10 minutes.

5.2.1 Isolation of sea urchin embryo nuclei was done according to Kleichline and Wassarman (1979).

Embryos at the desired stages were centrifuged and washed four times in Ca^{2+}, Mg^{2+} - free sea water (CMFSW) containing 0.01% EDTA. (CMFSW = 28.0g NaCl; 0,8 g KCl; 0,2 g NaHCO : dissolved in 1ℓ deionized water).

These steps and all succeeding steps were done at 0-4°C. The pelleted, washed embryos were resuspended in 0.5 M sucrose, 10 mM Tris, pH 7,5, and the cells were dissociated by several passages of the loose plunger of a Dounce homogenizer. The suspension of cells

in 0.5 M sucrose was adjusted to a concentration of 1:20 relative to packed cell colume. Cells were lysed by the addition of an equal volume of buffer containing 20 mM NaCl, 10 mM $MgCl_2$, 20 mM Tris, pH 7,5, 1% Triton X-100, with gentle mixing. After a few minutes, when lysis was complete, the suspension of nuclei was centrifuged at 600 g for 10 minutes. The pellet was gently resuspended in a few drops of buffer containing 0,25 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$, pH 7,5. The volume was increased to several millilitres, and the suspension was centrifuged at 600 g for 10 minutes.

5.2.2 Selective Extraction of Histones

The histones were selectively extracted from the nuclei by a modification of Johns (1964; 1977) procedure. All extraction steps and washing involved the Dounce homogenizer with the extraction medium.

The nuclear pellet was washed three times with saline sodium citrate solution (SSC) (0.14 M NaCl; 10 mM Na_3-citrate; 5 mM citric acid; 0.15% Thiodiglycol; 1 mM phenylmethylsulfonyl-fluoride and 50 mM sodium disulfide pH 5,0 - the last two as protease inhibitors). This was followed by centrifugation at 10000 x g for 10 minutes in a refrigerated centrifuge. The pelleted material was then washed once in 0,35 M NaCl, pH 7,0 and twice more in SSC solution.

The H1 histone fraction was selectively extracted by homogenizing the pellet in 3-5 ml 5% perchloric acid (w/v)/ml packed eggs. The supernatant was dialysed against three changes of deionized water in the cold room, and freeze dried to yield the H1 histone fraction.

Following centrifugation, the precipitate was extracted with 0,25 N HCl in 80% ethanol (2,3 ml ethanolic - HCl per 1 ml of packed eggs). This step removed the H4, H3 and H2A histones plus some H2B.

Finally, the crude H2B fraction was extracted with 0,25 N HCl (2-3 ml 0,25 N HCl per 1 ml packed eggs).

These extractions were all done at +4°C. The total histone complement could be isolated by extraction with 0,25 M HCl.

5.2.3 Column Chromatography

The effluent from the columns was monitored at 206 nm on an LKB UVICORD III absorptiometer or by measuring individual fractions at 230 nm. Selected fractions were pooled, dialysed or desalted on a Sephadex column and freeze dried.

5.2.3.1 Ion Exchange Chromatography

The H1 fraction, selectively isolated with 5% perchloric acid (w/v), was fractionated on a carboxymethyl cellulose (Whatman CM-52) column (9 mm x 60 mm) with a linear NaCl gradient. The gradient buffer contained 50 mM Na-Acetate/HCl, pH 5,5. The NaCl gradient was 100 ml 0,3 M NaCl to 100 ml 0,55 M NaCl. The sample was dissolved in a small volume of 50 mM sodium acetate, 8 M Urea, HCl pH 5,5. Approximately 30 mg of crude H1 was placed on a CMC column per run. A typical elution pattern is shown in Fig. 2.10.

The flow rate was maintained using a peristaltic pump. The column was flushed with 1 M NaCl in 50 mM Na-Acetate buffer/HCl pH 5,5 after every run.

5.2.3.2 Gel Exclusion Chromatography

Sephadex G10 and G15 (16 mm x 900 mm), were used for desalting the H1 subfractions from the CMC column. The eluant was 0,01 N HCl.

5.3 CHARACTERIZATION OF THE H1-HISTONES

5.3.1 Polyacrylamide Gel Electrophoresis

Slab gels instead of the conventional tube gels were used. Slab gels were made by use of two glass plates (11,9 cm x 11,9 cm), held apart by teflon spacers (0,7 cm). The glass plates and spacers were held in place with wide fold back clips. The glass plates and spacers were sealed with a 1% agarose solution.

The power supply used was a Shandon Model Power supply, with a variable voltage (0-400 V) and current (0-80 mA) output.

Triton X-100-acetic acid urea gels were made according to Panyim and Chalkley (1969) and modified by Zweidler (1972) by the addition of Triton X-100.

Gels contained 15% acrylamide (w/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).

The urea concentration was varied to optimize the separation of the embryo histones and thiodiglycol was added to a final concentration of 0,1% in order to prevent oxidation of methionine (Zweidler, 1978). Riboflavin (1 mg/ml gel) was added to initiate the polymerization of the gel. Daylight or fluorescent light was used to activate the riboflavin. Polymerization was found to be complete after ½ hour. Better results were obtained by pre-electrophoresing the gels. This was done at 15 mA (constant current) until the voltage stabilized. Pre-electrophoresis was done with a sample buffer layered across the gel, to prevent diffusion of urea. Before the stacking gel was poured, the top of the running gel was rinsed thoroughly with running buffer. Electrophoresis was done at 15 mA (constant current) for 4½ hours or at 5 mA (constant current) overnight.

5.3.1.1 Stock Solutions

A: 60% acrylamide (w/v)
0.4% N,N'-methylene bisacrylamide (w/v)

B : 43,2% glacial acetic acid (v/v)
4% N,N,N',N',-tetramethylethylenediamine (TEMED)

C : Urea, molarity according to the histones to be separated.
Optimum urea concentrations routinely used for sea urchin embryo histones were 8,5 M urea and 3,8 M urea final concentration.

D : 0,9 M glacial acetic acid = Tray buffer

E : 10% Triton X-100 (w/v)

F : 0,1% Riboflavin (w/v)

All solutions were stored in the dark except solution C which was prepared immediately before use.

The 8,5 M urea and 3,8 M urea-polyacrylamide solutions were made up routinely the following way:

3,8 M Urea; 0,37% Triton; 15% Polyacrylamide

	Running gel 16 ml	20 ml	stacking gel
Urea (g)	3,7	4,6	0.9
H_2O (ml)	6,4	8,0	2,0
A (ml)	4,0	5,0	0,5
B (ml)	2,0	2,5	0,5
Triton X-100 (10%) (ml)	0,6	0,75	0,15
TDG (1μ)	20	25	5
Riboflavin (1μ) (1 mg/ml)	40	50	10

8,5 M Urea; 0,375% Triton; 15% Acrylamide

	Running gel 16 ml	20 ml	stacking gel
Urea (g)	8,16	10,2	2,0
H_2O (ml)	3,0	3,75	1,25
A (ml)	4,0	5,0	0,5
B (ml)	2,0	2,5	0,5
Triton X-100 (10%) (ml)	0,6	0,75	0,15
TDG (1μ)	20	25	5
Riboflavin (1μ) (1 mg/ml)	40	50	10

5.3.1.2 Staining and Destaining

Gels were stained, in a mixture of water/acetic acid/methanol (4:1:5) containing 0,25% Coomassie Brilliant Blue, for 1-3 hours.

Destaining was achieved in a solution containing water/acetic acid/methanol (8:1:1). Destaining was done by diffusion, changing the buffer regularly, until the gel backgraound became clear. This could be achieved in a day.

After photography, gels were dried under heat and a vacuum between Whatman 3 M chromatography paper and clear cronar film, for storage or fluorography.

5.3.2 SDS Polyacrylamide Gel Electrophoresis

SDS (sodium dodecylsulfate) was used for two dimensional gel electrophoresis.

SDS polyacrylamide gel electrophoresis was done according to the method of Laemmli (1970). This electrophoresis was used to determine the relative molecular weights of the H1 variants and it was used in the second dimension. Triton-acid-urea polyacrylamide gels were used in the first dimension.

5.3.2.1 Stock Solutions

a) Running gel

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

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 (w/v)

C : 10% ammonium persulfate (w/v) in H_2O (made up freshly daily)

D : TEMED

c) Sample Application Buffer
0,0625 M Tris HCl pH 6,8
2% SDS (w/v)
10% Glycerol (w/v)
0,001% Bromophenol blue (w/v)

d) Tray Buffer
0,025 M Tris HCl pH 8,3
0,192 M glycine
0,1% SDS

5.3.2.2 Gel Preparation

The separating gel consisted 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) ammonium persulfate and 0,6% (v/v) TEMED. The gel was poured leaving enough space for a stacking gel or strip of gel to be run in the second dimension.

Distilled water was carefully layered onto the SDS gel solution to obtain a straight interface.

The stacking gel consisted of 3% (w/v) acrylamide and 0,125 M Tris-HCl pH 8,8. The gel strip was layered across the SDS running gel and secured with stacking gel or 1% agarose.

Electrophoresis was carried out at 200 volts (constant) for 3-4 hours.

The gels were stained and destained as described under section 5.3.1.3.

The sample was dissolved at 2 mg/ml in the application buffer plus 5% (v/v) mercaptoethanol. The sample mixture was incubated in a boiling bath for 2 minutes before being applied to the gel.

When a strip from a Triton-acid-urea gel was used to be run into an SDS gel second dimension separation, the strip was equilibrated in sample buffer for 1-2 hours.

5.3.3 Acetic Acid/cetyltrimethylammonium Bromide Gels

CTAB (cetyltrimethylammonium bromide) gel electrophoresis was used as a cationic detergent second dimensional system for further separation and molecular weight difference determination of the H1-fraction. Acetic-acid-CTAB polyacrylamide gels were prepared as described by Panyim et al. (1977).

5.3.3.1 Stock Solutions

Solution A
30.0% acrylamide (w/v)
0.45% N,N'-methylene bisacrylamide (w/v)

Solution B
245 ml of 0,2 M acetic acid
255 ml of 0,2 M Na-acetate
2 g of CTAB
made up to 1ℓ with distilled H_2O

Running Buffer Solution B diluted 1:1 with distilled H_2O. The gel strips from the first dimensional acetic acid-urea-Triton gels were equilibrated in running buffer before being loaded onto the CTAB slab gel.

The gel strip was fixed on the CTAB gel with 1% agarose.

5.3.3.2 Gel Preparation

A 7,5% acrylamide gel was prepared by mixing 4,5 ml solution A, 8,1 ml solution B, 0,06 ml TEMED, 50µℓ Riboflavin (0,1%, w/v) and 5,4 ml distilled water. The gel was run at a constant voltage of 100 for 3½ hours.

The gels were stained and destained as described in section 5.3.1.3. Staining and destaining was done by heating the buffers to 50°C-80°C to prevent precipitation of the CTAB complex and to accelerate the procedures.

5.3.4 Analytical Polyacrylamide Gel Electrophoresis of Sea Urchin Embryo H1-Histone Fraction

5.3.4.1 0-6 M Transverse Urea Gradient Polyacrylamide Slab Gel Electrophoresis

The gel apparatus and the procedure for preparing gradient polyacrylamide gels, were as described by S. Schwager (1981).

A double chamber gradient maker, with a magnetic stirring bar in the mixing chamber, was used. The mixing chamber was connected to a peristaltic pump which in turn led, via tubing, to the space between two glass plates held apart by teflon spacers.

The gradient was layered between the glass plates by starting with the heavier solution. The inlet tubing was continuously raised in the slab gel space as the level of the polymerization mixture rose.

A linear 0-6 M urea gradient was made from the stock solutions as listed under 5.3.1.1.

Solutions I (no urea) and II (6 M urea) were placed in the respective chambers of the gradient maker.

Solution I : 15% (w/v) acrylamide, 0,1% (w/v) N,N'-methylene bisacrylamide, 5,4% (v/v) acetic acid, 0,5% (v/v) TEMED and 0,1% (v/v) thiodiglycol.

Solution II: Same as under I, except that the solution contained 6 M urea.

This urea gradient gel was repeated except that solutions I and II contained 6 mM Triton X-100.

8 ml of each of the solutions I and II were prepared and poured in the respective chambers of the gradient maker. 10 µl riboflavin (1 mg/ml) was added to each chamber. The stop cock, stirrer and pump were activated simultaneously and the gel mixture was pumped into the slab gel space at about 0,5ml/minute.

While pouring the gradient gel, the polymerization mixture was shielded from bright light in order to prevent polymerization. After the gradient gel was left for ½ hour in the light to set, the open side of the gel was sealed with a teflon spacer and the top spacer was removed.

A stacking gel, similar to solution I, was poured in at the top and a multi-toothed comb was used to make wells for the sample.

Electrophoresis was carried out at 100 V (constant) for 6 hours.

5.3.4.2 0-20 mM Transverse Triton X-100 Gradient Polyacrylamide Slab Gel Electrophoresis

The stock solutions were the same as listed under 5.3.1.1 and a transverse 0-20 mM Triton X-100 gradient slab gel was poured from the following solutions:

Solution I : 15% (w/v) acrylamide, 0,1% (w/v) N,N'-methylene bisacrylamide, 5,4% (v/v) acetic acid, 0,5% (v/v) TEMED and 0,1% (v/v) thiodiglycol.

Solution II : Same as under I, but the solution also contained 20 mM Triton X-100 and 0,5 M sucrose.

8 ml of each solution was prepared and poured in the respective chambers of the gradient maker. The gradient and stacking gel was prepared and run as described for the urea gradient gel (5.3.3.1).

This Triton X-100 gradient was repeated in the presence of 2,5 M urea.

Electrophoresis was carried out at 100 Volt (constant) for 6 hours and the gel was stained and destained as described (5.3.1.3).

5.3.4.3 12-18% Transverse Polyacrylamide Gradient Slab Gel

The stock solutions were the same as listed under section 5.3.1.1 and a transverse 12-18% acrylamide gel was prepared from the following solutions:

Solution I : 18% (w/v) acrylamide, 0,1% (w/v) N,N' -methylene bisacrylamide,5,4% (v/v) acetic acid, 0,5% (v/v) TEMED 0,1% (v/v) thiodiglycol and 3,8 M urea.

Solution II : 12% (w/v) acrylamide, 0,1% (w/v) N,N'-methylene bisacrylamide, 5,4% (v/v) acetic acid, 0,5% (v/v) TEMED and 0,1% (v/v) thiodiglycol plus 3,8 M urea.

Two different stock A solutions were prepared to mix in the ratio of A:B:C, 4:2:10. Solution A for the 18% acrylamide gel solution (I) contained 72% acrylamide (w/v) and 0,48% N,N'-methylene bisacrylamide (w/v).

Solution A for the 12% acrylamide gel solution (II) contained 48% acrylamide (w/v) and 0,32% N,N'-methylene bisacrylamide (w/v).

8 ml of each solution (I and II) was prepared for the preparation of the gradient.

Electrophoresis was carried out at 100 volts (constant) for 6 hours.

5.3.4.4 Transverse Crosslinker Gradient Slab Gel

A transverse crosslinker gradient slab gel was prepared with the acrylamide to N,N'-methylene bisacrylamide ratio ranging from 1:150 to 1:25.

A linear crosslinker gradient was made from the stock solutions as listed under 5.3.1.1.

Solutions I and II were prepared and placed in the respective chambers of the gradient maker for mixing of the gradient.

Solution I : 15% acrylamide (w/v), 0,1% (w/v), N,N'-methylene bisacrylamide, 5,4% (v/v) acetic acid, 0,5% (v/v) TEMED and 0,1% (v/v) thiodiglycol and 3,8 M urea .

Solution II : 15% acrylamide (w/v), 0,6% (v/v) N,N'-methylene bisacrylamide, 5,4% (v/v) acetic acid, 0,5% (v/v) TEMED, 0,1% (v/v) thiodiglycol and 0,5 M sucrose.

Two stock solution A solutions were prepared to mix in the ratio of A:B:C, 4:2:10.

Solution A for the 0,1% N,N'-methylene bisacrylamide gel solution (I) contained 60% acrylamide (w/v) and 0,4% N,N'-methylene bisacrylamide (w/v). Solution A for the 0,6% N,N'-methylene bisacrylamide (w/v) gel solution (II) contained 60% acrylamide (w/v) and 2,4% N,N'-methylene bisacrylamide (w/v).

8 ml of each solution (I and II) was prepared for the preparation of the gradient.

Electrophoresis was carried out at 100 volts (constant) for 6 hours.

5.3.5 Amino Acid Analysis

Hydrolysis of peptides and proteins was done by dissolving 100-500 µg of sample in 200 µl twice distilled constant boiling HCl containing 0,025% phenol, at 110°C for 24 hours. The glass tubes used (8 x 80 mm Pyrex) were specially cleaned by being heated in the glass blowers oven to about 600°C.

Before sealing the glass tubes, the sample was flushed with nitrogen and evacuated to about 0,02 mm Hg.

Free amino acids were identified on a Beckman amino acid analyzer using the sensitive scale (0,2). An internal standard of norleucine for the acidic amino acids was used and α-amino-β-guanidino-propionic acid for the basic amino acids was used. Quantitation was done by way of A/D converters between the 440 and 570 nm channels of the analyzer and a Hewlett Packard computer (Laboratory Data System 3352B).

No corrections were made for hydrolytic losses or incomplete cleavage.

For tryptophan determination, amino acid hydrolysis was done with twice distilled constant boiling HCl containing 4% thioglycolic acid. Lysozyme was used as a standard protein containing tryptophan.

5.3.6 End Group Analysis

5.3.6.1 Dansylation

Dansylation of proteins and peptides was done according to the labelling methods as described by Gray (1972).

5.3.6.1.1 Protein Labelling

1. Dissolve 50-250 μg protein in a 6 x 50 mm test tube in 50 μl of 1% SDS (w/v) by heating in a boiling water bath for 2-5 minutes. Cool.

2. Add 50 μl N-ethylmorpholine - mix (N-ethylmorpholine should be colourless).

3. Add 50 µl DNS-chloride, dissolved (50 mg/ml) immediately before use in water free acetone-mix.

4. Cover and incubate for 30 minutes at 45°C.

5. Fill tube with acetone to precipitate protein. Centrifuge.

6. Discard supernatant and dry pellet under vacuum.

7. Add 20 µl 5,7 N HCl, seal tubes in a gas flame. Incubate at 105°C for 6-15 hours. The shorter time gives a better recovery of proline but less complete protein hydrolysis. A 15 hour hydrolysis was routine.

5.3.6.1.2 Peptide Labelling

1. 0,5-5 nmoles peptide in a 4 x 50 mm test tube was dissolved in 5 µl 0,2 M NaHCO (pH must be 8,5-9).

2. Add 5µl DNS-Chloride (2,5 mg/ml in acetone).

3. Cover and incubate 30 minutes at 45°C.

4. Dry entire incubate under vacuum.

5. Hydrolyze in 10 µl 5,7 N HCl as for proteins.

5.3.6.1.3 Identification of DNS-derivatives

Stock solutions of standard amino acids were made 1 mg/ml in 95% ethanol (Pro, Ser, Thr, di-DNS-Lys and Arg) or acetone (Ala, Glu, Gly, Ile). For use, 20 µl of the stock solutions desired were mixed and the total volume made up to 400 µl. One spot of this mixture was spotted on the reverse side of a 5 x 5 cm F-1700-micro-polyamide sheet

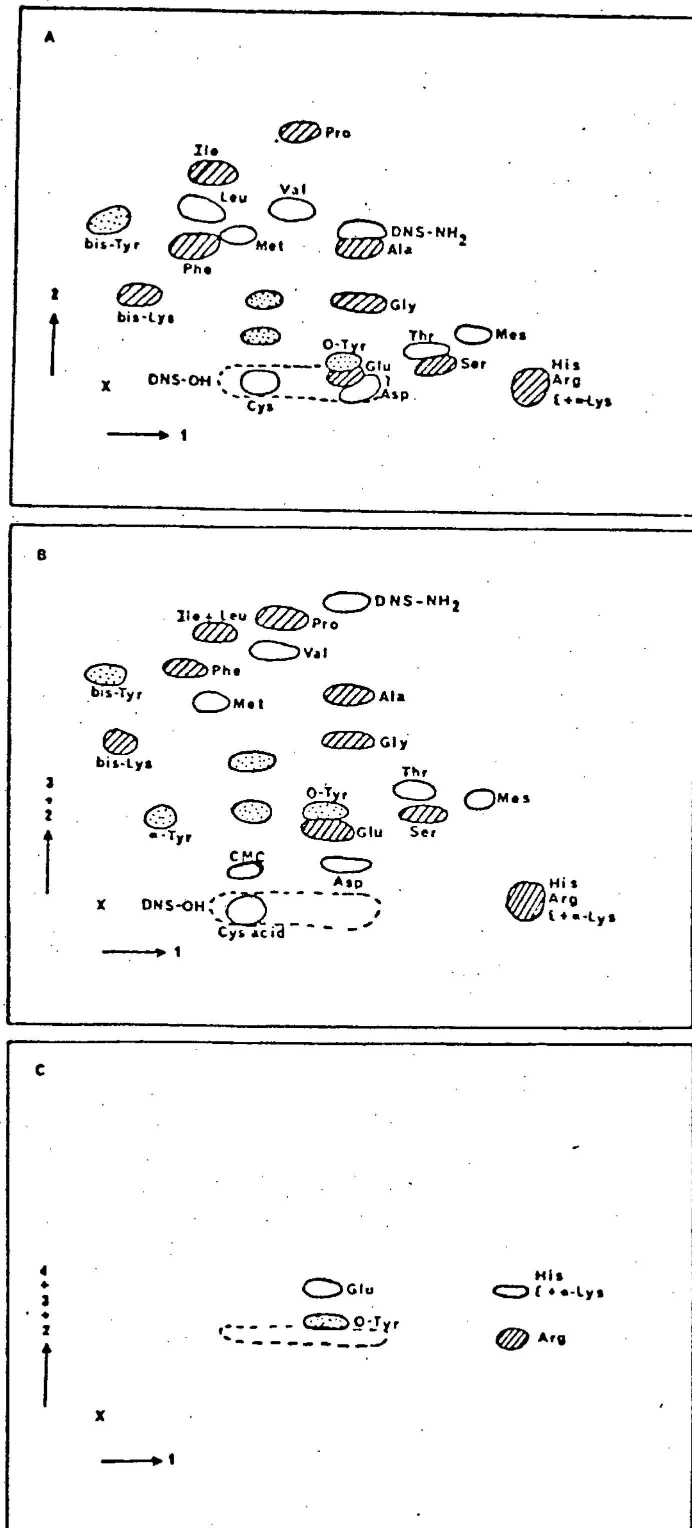

Figure 5.1: Thin layer chromatography of standard DNS-amino acids. For details see section 5.3.1.3. Cross hatched amino acids are those usually included in a standard mixture. Derivatives of tyrosine are stippled.

(Schleicher and Schull) and compared to the unknown spots after development using the four solvent systems described by Narita et al. (1975) (Fig. 4.1).

Solvent 1. 1,5% formic acid in water (v/v)
Solvent 2. Benzene-acetic acid (9:1 v/v)
Solvent 3. Ethyl acetate-acetic acid-methanol (20:1:1 v/v)
Solvent 4. 50 mM Na_3PO_4 in 25% aqueous ethanol

After hydrolysis of the peptide or protein sample, the dried residue was dissolved in 5 μl of 95% ethanol and spotted onto the polyamide sheet. The sheet was examined under an ultraviolet lamp after development in solvents 1 and 2. Resolution of DNS-glutamic acid and DNS-aspartic acid; of DNS-serine and DNS-threonine; of DNS-alanine and DNS-NH_4 was achieved after development in solvent 3. Development in solvent 4 separates DNS-arginine from ε-DNS-lysine. ε-DNS-lysine and and DNS histidine do not separate. Development in the 4th solvent discriminates between DNS-glutamic and O-DNS tyrosine which can be a problem if several tyrosines are present in the sample.

Figure 4.1 shows chromatograms of standard DNS-amino acids after development as described.

A blue DNS-OH spot can be seen on all chromatograms. DNS-derivatives of tyrosine show an intense yellow-brown colour. DNS derivatives of other amino acid show a yellowish white colour.

5.3.6.2 Hydrazinolysis

Hydrazinolysis was done according to Doolittle (1977). This was done for identification of an acetyl group at the amino-terminus of a protein or a peptide.

50-100 nmoles of peptide or protein were incubated with 200 μl hydrazine in a sealed and evacuated tube (8 x 80 mm - Pyrex) and incubated for 17 hours at 110°C.

The sample was dried and 100 μl 0,2 M Na_3 -citrate (pH 3,0) and 0,25 mg DNS Cl (10 mg/ml in acetone) was added.

This mixture was incubated at 45°C overnight and subsequently dried. The sample was dissolved in 100 μl water (2 x distilled) and the dansylated acetyl or formyl hydrazides were extracted 3 times with 100 μl chloroform. This organic solvent was dried, dissolved in 10 μl 95% ethanol and spotted onto F1700 micro-polyamide (5 cm x 5 cm) plates (Schleicher and Schull) and run in the solvents as described under section 5.3.6.1.3.

Figure 5.2 shows chromatograms of dansylated acetyl and formyl hydrazides relative to some DNS-amino acids.

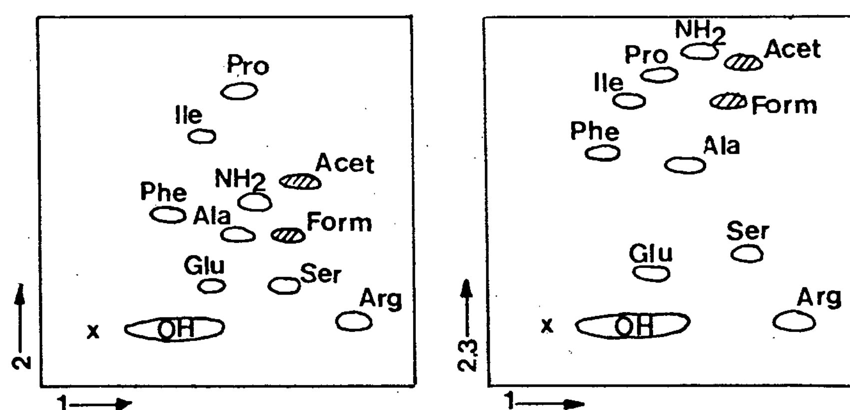

Figure 5.2: Positions of acetyl-DNS-hydrazide (Acet) and formyl-DNS-hydrazide (Form) on polyamide sheets using the solvent system as described under 5.3.6.1. The relative positions of some DNS-amino acids (not hydrazides) are shown. (From Takagi and Doolittle, 1974).

5.3.6.3 Digestion with Carboxypeptidase A and B

The protein and peptide samples were dissolved(5-10 mg/ml) in 100 mM NH_4HCO_3 , pH 8,0. The enzyme: substrate ratio for carboxypeptidase A was 1:200 (w/w) and for carboxypeptidase B 1:200 (w/w). The peptide/enzyme mixture was incubated at 37°C and 100 μl

aliquots were taken at appropriate times, acidified with 1 drop of glacial acetic acid and freeze dried. The free amino acids were quantitated directly on a Beckman amino acid analyzer. The results were corrected for background amino acid by analyzing control samples containing only carboxypeptidase or sample solution. The control samples were incubated in parallel with the enzyme-substrate mixture.

Sources of enzymes were: Carboxypeptidase A - Boehringer Mannheim GMbH from bovine pancreas; Carboxypeptidase B - Boehringer Mannheim GmbH from porcine pancreas.

5.4 FRAGMENTATION OF H1 HISTONES

5.4.1 Chemical Methods

Cyanogen bromide cleavage at methionine (Gross, 1967; Witkop, 1968). The protein was dissolved at a concentration of 10 mg/ml in 70% formic acid (w/v). Then a 100 fold molar excess (based on methionine) of CNBr (in 70% formic acid) was added and the mixture incubated while stirring, in the dark, at room temperature for 24 hours. Excess CNBr was evaporated under a stream of N_2 in a fume hood, the sample diluted 1:10 with distilled water, and freeze dried. Separation of the samples was achieved on CM-cellulose columns.

5.4.2 Enzymatic Methods

5.4.2.1 Trypsin - digestion of maleylated or citraconylated material was achieved at arginine residues. Maleylation of lysine residues: The peptide or protein was dissolved (5 mg/ml) in 100 mM $NaHCO_3$, pH 8,5. A 20 fold excess of maleic anhydride (recrystallized twice from chloroform) was added slowly as a solid while the pH was maintained at about pH 8,0 with 2 M NaOH. The protein or peptide was subsequently separated from excess reagent on a Sephadex G10 column with 100 mM NH_4 HCO_3 pH 8,5 as the eluant. The protein or peptide fraction was freeze dried.

Citraconylation (Atassi and Habeeb, 1972): protein (40 mg/ml) was dissolved in H_2O and the pH was adjusted to 8,2. Aliquots of citraconic anhydride were added to the solution, while magnetically stirred, at 30 minute intervals. A total of 10 µl citraconic anhydride /5mg peptide or protein was added. The pH was maintained at 8,2 with 5N NaOH. When the addition of citraconic anhydride was completed, the reaction was left for a further 2 hours at room temperature at pH 8,2.

The freeze dried peptide or protein with its lysine groups blocked was redissolved (10 mg/ml) in 100 mM NH_4 HCO_3 pH 8,5. Trypsin (Merck no. 24581 TPCK treated) was added in a 1:100 ratio (w/w). After 2 hours incubation at 37°C the mixture was fractionated on a Sephadex G50 column with 100 mM NH_4HCO_3 as the eluant.

Demaleylation of peptides (Kasper, 1975) was performed in 30% acetic acid (w/v) at 40°C for 48 hours. After freeze drying the peptides were desalted on a Sephadex G10 column.

Decitraconylation (Atassi and Habeeb, 1972); Removal of citraconic blocking groups was carried out by dissolving the protein on peptide (5 mg/ml) in 50 mM acetate pH 4,2 at 40°C for 4 hours or by dialysis against 10 mM HCl, pH 2,0, at room temperature for 6 hours. The peptides or protein were freeze dried afterwards.

5.4.2.2 *Staphylococcus aureus* digestion at glutamic acid residues. To a protein or peptide (10 mg/ml) dissolved in 100 mM ammonium acetate pH 4,0, *Staphylococcus aureus* protease (Miles No. 36-900) (Houmard and Drapeau, 1972; Drapeau, 1976) was added in an enzyme to substrate ratio of 1:30. After incubation for 18 hours at 37°C the mixture was freeze dried. Fractionation of the peptides was on a CMC column.

5.4.3 Purification of Peptides

5.4.3.1 Gel Exclusion on Sephadex and Biogel

Various types of Sephadex or Biogel were packed in columns and applied to the separation of peptides, according to their separation properties. The usual eluant was 0,01 M HCl pH 2,0 containing 0,001% toluene as a preservative. Maleylated or citraconylated material was eluted from columns with 100 mM NH_4HCO_3 pH 8,5 in order to prevent demaleylation before the peptides were separated from trypsin.

Protein or peptide samples were usually dissolved in a small volume of 6 M urea in 10 mM HCl and then layered carefully onto the column. The flow rate was maintained by a constant hydrostatic head (Mariotte flask). Fraction size was controlled by a drop counter or by taking timed fractions. The absorption of the eluates was registered at 206 nm and 280 nm on an LKB UVICORD III absorptiometer or by measuring individual fractions at 130 nm.

Pooled fractions were freeze dried.

Descriptions of particular column runs are given in the figures in the text.

5.4.3.2 Ion Excange on CMC (Whatman CM-52)

CMC columns were equilibrated in 50 mM Na acetate/HCl pH 5,5. The elution order depended on net positive charge.

Pooled fractions were freeze dried and then redissolved in a minimum volume of 10 mM HCl and desalted by gel exclusion on appropriate types of Sephadex.

CMC columns were run with the use of peristaltic pumps in order to keep the flow rate constant.

5.5. SEQUENCE ANALYSIS

5.5.1 Automatic (Spinning Cup) Edman Degradation Sequencing

A Beckman model 890 sequencer was used to sequence the peptides. Automatic sequential Edman degradation (Edman and Begg, 1976) was done as described by Brandt and von Holt (1974).

Improvement of the sequence vacuum system was achieved by the fitting of a cold (liquid N_2) trap between the pump and the sequencer.

5.5.1.1 Operation of the Sequencer

5.5.1.1.1 Purification of the Chemicals

N-N-dimethylallylamine (DMAA) was purified and prepared according to Edman and Henschen (1975). Purification for the other chemicals was also done as described by Edman and Henschen (1975) while purification of chlorobutane was done as previoously described by Strickland et al (1978a). Quadrol buffer (N,N,N',N'-tetrakis (2-hydroxypropyl-ethylene diamine) (Edman and Henschen, 1975) was used in the protein program instead of DMAA buffer.

Quadrol and DMAA buffer were treated with amino-ethylcellulose before use on the sequencer. The buffers were stirred overnight in the presence of aminoethylcellulose. The buffers were filtered through a sinterglass filter before being used on the sequencer. The aminoethylcellulose serves as a map for free aldehydes (Frank, 1979).

5.5.1.1.2 Programs

Brandt et al. (1976) demonstrated that proline residues cleave more slowly than other residues during sequencing. Therefore the number of cleavage steps at these residues was increased. Double cleavage at proline residues improved overlaps due to incomplete cleavage at proline residues.

Due to the tendency of very hydrophobic peptides to be washed out of the sequencer cup by the solvents used, these peptides were made less hydrophobic. This was done by modifying the ε-amino groups of the lysine with 4-sulfophenylisothiocyanate (S-PITC) (Braunitzer, 1970).

A carrier protein, the blocked H4 histone from Parechinus angulosus sperm was used to prevent small peptides being washed out of the sequence cup during the peptide program.

The programs used were basically the same as described by M. Strickland (1978) except for the following changes.
Quadrol buffer was used in the protein program instead of 3-dimethylamino-1-propyne (DMAP) buffer.
N-N-dimethylallylamine (DMAA) instead of 3-dimethylamino-1-propyn (DMAP) was used during the peptide program.

In the peptide program the reaction time of the cleavage step 56 was increased from 80 to 240 seconds.

In the protein program the ethylacetate wash at step 56 was increased from 40 to 100 seconds.

The programs as used during sequencing are described in Tables 5.1 and 5.2.

TABLE 5.1 PROTEIN PROGRAM

Step	Program Statement	Step Time (sec)	Cup Speed	Step	Program Statement	Step Time (sec)	Cup Speed
1	Stop slew	2	L	40	Delay	82	H
2	Delay	6	L	41	Vac. restricted	200	H
3	Blank	2	L	42	Vac. rough	40	H
4	R4 vent.	14	L	43	Vac. fine + Fraction collector step	300	H
5	R4 press.	14	L	44	Delay	6	L
6	R1 vent.	14	H	45	R3 vent. + Fraction collector vent.	20	L
7	R1 pressurize	14	H	46	R3 press.	14	L
8	R1 deliver	6	H	47	R3 deliver	26	H
9	Blank	2	H	48	Reaction	190	H
10	Vac. restricted	30	H	49	Vac. restricted	40	L
11	Delay	6	H	50	Vac. rough	50	L
12	N_2 dry	100	H	51	Vac. fine	20	L
13	R_2 deliver	18	H	52	Delay	4	L
14	Reaction	300	H	53	S3 vent.	30	L
15	R_2 deliver	4	H	54	S3 press.	30	L
16	Reaction	300	L	55	S3 deliver + collect	150	H
17	R_4 deliver	4	L	56	Delay	100	H
18	Reaction	300	L	57	Vac. restricted	60	H
19	R5 deliver	10	L	58	Vac. rough	60	H
20	Reaction	300	L	59	Vac. fine	60	H
21	R5 deliver	100	L	60	Delay	4	H
22	Blank	2	L	61	R3 vent	14	L
23	Vac. restricted	60	L	62	R3 press	14	L
24	Delay	4	L	63	R3 deliver	26	H
25	N_2 dry	300	L	64	Reaction	90	H
26	Vac. restricted	200	H	65	Vac. restricted	40	L
27	Vac. rough	200	H	66	Vac. rough	50	L
28	Vac. fine	100	H	67	Vac. fine	20	L
29	Blank	6	H	68	Delay	4	L
30	S1 vent.	30	H	69	S3 vent.	0	L
31	S1 press.	30	H	70	S3 press.	0	L
32	S1 deliver	200	H	71	S3 deliver + waste	200	H
33	N_2 dry	200	H	72	Delay	100	H
34	Vac. restricted	30	H	73	Vac. restricted	60	H
35	Vac. rough	140	H	74	Vac. rough	40	H
36	Delay	3	H	75	Vac. fine	200	H
37	S2 vent.	30	H	76	Vac. fine	2	L
38	S2 press.	30	H	77	Vac. fine	2	L
39	S2 deliver	440	H	78	Vac. fine	0	L

PROGRAMS for protein sequencing

Reagent 1 = phenylisothiocyanate 5% in heptane
Reagent 2 = Quadrol buffer, 0,2 M = N,N,N[1],N[1] - tetrakis - (2 hydroxylpropyl)-ethylenediamine. (Edman and Henschen, 1975). Quadrol buffer diluted with water was mixed with n-propanol in a ratio of 1:1
Reagent 3 = Heptafluorobutyric acid
Reagent 4 = DMAA buffer (Edman and Henschen, 1975). 0,3 M N, N-dimethylallylamine in water was first diluted with n-propanol in the ratio 4:5 and then adjusted to pH9 with trifluoroacetic acid.
Reagent 5 = N_2
Solvent 1 = benzene. Solvent 2 = ethyl acetate. Solvent 3 = butylchloride.

TABLE 5.2 PEPTIDE PROGRAM

Step	Program Statement	Step Time (sec.)	Cup Speed	Step	Program Statement	Step Time (sec.)	Cup Speed
1	Stop slew	2	L	40	S1 deliver + collect	200	H
2	Delay	6	L	41	Delay + collect	30	H
3	Blank	2	L	42	Blank	2	H
4	R4 vent.	14	L	43	Vac. restricted	30	H
5	Delay	14	L	44	Delay	6	H
6	R4 press.	14	L	45	N_2 dry	200	H
7	R1 vent.	14	H	46	Vac. restricted	100	H
8	R1 press.	14	H	47	Vac. rough	300	H
9	R1 deliver	6	H	48	Vac. fine	300	H
10	Blank	2	H	49	Blank	2	H
11	Vac. restricted	30	H	50	Delay	6	H
12	Delay	6	H	51	S3 vent.	30	H
13	N_2 dry	60	H	52	S3 press. + Fraction collector vent and step	30	H
14	R4 deliver	20	H	53	R3 vent.	14	H
15	Reaction	4	H	54	R3 press.	14	H
16	R4 deliver	0	L	55	R3 deliver	16	H
17	Reaction	300	L	56	Reaction	240	H
18	R5 deliver	4	L	57	N_2 dry	40	L
19	Reaction	300	L	58	Vac. restricted	20	L
20	R5 deliver	10	L	59	Vac. rough	70	L
21	Reaction	300	L	60	Delay	6	L
22	R5 deliver	120	L	61	S3 deliver	25	L
23	Blank	2	L	62	N_2 dry	130	L
24	Vac. restricted	60	L	63	Vac. restricted	62	L
25	Delay	4	L	64	Vac. rough	62	L
26	N_2 dry	400	L	65	Vac. fine	62	L
27	Vac. restricted	100	H	66	Blank	2	L
28	Vac. rough	200	H	67	Delay	6	L
29	Vac. fine	600	H	68	S3 deliver + collect	150	H
30	Blank	2	H	69	Delay	30	H
31	Delay + F/C step	6	H	70	Blank	2	H
32	S1 vent	30	H	71	Vac. restricted	60	H
33	S1 press. + F/C vent	30	H	72	Delay	6	H
34	S1 deliver	20	H	73	N_2 dry	200	H
35	Blank	30	H	74	Vac. restricted	60	H
36	Blank	2	H	75	Vac. rough	100	H
37	Blank	2	H	76	Vac. fine	100	H
38	Blank	2	H	77	Vac. fine	0	L
39	Delay	6	H	78	Vac. fine	200	L

PROGRAMS for peptide sequencing

Reagent 1 = phenylisothiocyanate 5% in heptane
Reagent 2 = Quadrol buffer, 0,2 M = N,N,N[1],N[1] - tetrakis - (2 hydroxylpropyl)-ethylenediamine. (Edman and Henschen, 1975). Quadrol buffer diluted with water was mixed with n-propanol in a ratio of 1:1
Reagent 3 = Heptafluorobutyric acid
Reagent 4 = DMAA buffer (Edman and Henschen, 1975). 0,3M N,N-dimethylallylamine in water was first diluted with n-propanol in the ratio 4:5 and then adjusted to pH9 with tri-fluoroacetic acid.
Reagent 5 = N_2
Solvent 1 = benzene. Solvent 2 = ethyl acetate. Solvent 3 = bytylchloride.

5.5.2 Solid Phase Sequencing

The solid phase sequencer used was made and operated as described by Alk et al. (1981).

Cyanogen bromide peptides with a methionyl residue at the carboxyl end were sequenced on the solid phase sequencer.

The method of attachment of carboxyl-terminal homoserine peptides to a solid state resin was as described by Horn and Laursen (1973).

The method involved lactonization of the homoserine residue with trifluoroacetic acid and subsequent aminolysis of the lactone with amino glass beads.

The excess glass bead amino groups and peptide amino groups plus the unprotected peptide N-terminal amino-groups were blocked with methyl isothiocyanate.

The sample was degraded using an automatic sequencer and procedures as described by Alk et al. (1981).
The solid phase sequencer was adapted to automatically convert thiazolinones to phenylthiohydantoin (PTH) amino acids.

The phenylthiohydantoins were identified and quantitated using High Pressure Liquid Chromatography as described under section 5.5.6.

5.5.3 Identification and Quantitation of PTH-amino acids

The thiazolinones from the liquid phase sequencer first had to be converted to PTH-amino acids,. while the solid phase sequencer was programmed to convert the thiazolinones. To each tube in the sequencer fraction collecter 0,1 mg of dithioerythritol was added to improve the recovery of PTH-serine and threonine. PTH-norleucine (100 nanomoles) was also added as an internal standard.

5.5.3.1 Conversion of Amino Acid Thiazolinones

The amino acid thiazolinones were converted to phenylthiohydantoin derivatives essentially as described by Wittmann-Liebold et al. (1975).

Fractions from the sequencer fraction collecter were dried and 0,2 ml of 20% trifluoroacetic acid (TFA) was added. After flushing with nitrogen, the mixture was incubated at 80°C for 10 minutes. The TFA was evaporated under a stream of nitrogen in a 50°C waterbath and the dried residue was taken up in methanol.

5.5.3.2 High Pressure Liquid Chromatograph

Quantitation of PTH-amino acids was done by the internal standard method of the computer dedicated to the Hewlett Packard Model 1084 High Pressure Liquid Chromatograph.

In a single run 18 PTH amino acid derivatives and the internal standard PTH-norleucine can be resolved (Fig. 5.3). The PTH-derivatives of the unresolved fraction (PTH-lysine, PTH-phenylalanine, PTH-isoleucine and PTH-leucine) were identified on an isocratic run on the HPLC (Fig. 5.4).

In the HPLC system used, the elution time of the acidic amino acids can be shortened by increasing the pH whereas the elution time of the basic amino acids can be delayed by increasing the molarity of the buffer at a given pH.

The columns used for the separation of the PTH-amino acids (4.6 x 100 mm) which were much shorter than the ones previously used (4.6 x 250 mm), had the advantage of speedy analysis of the PTH-amino acids, but had a much shorter life-span than the longer columns.

The columns were developed with a 14 minute gradient of 95%A + 6%B to 51%A + 49%B at flow rate of 1,7 ml/minute (Fig. 5.3).

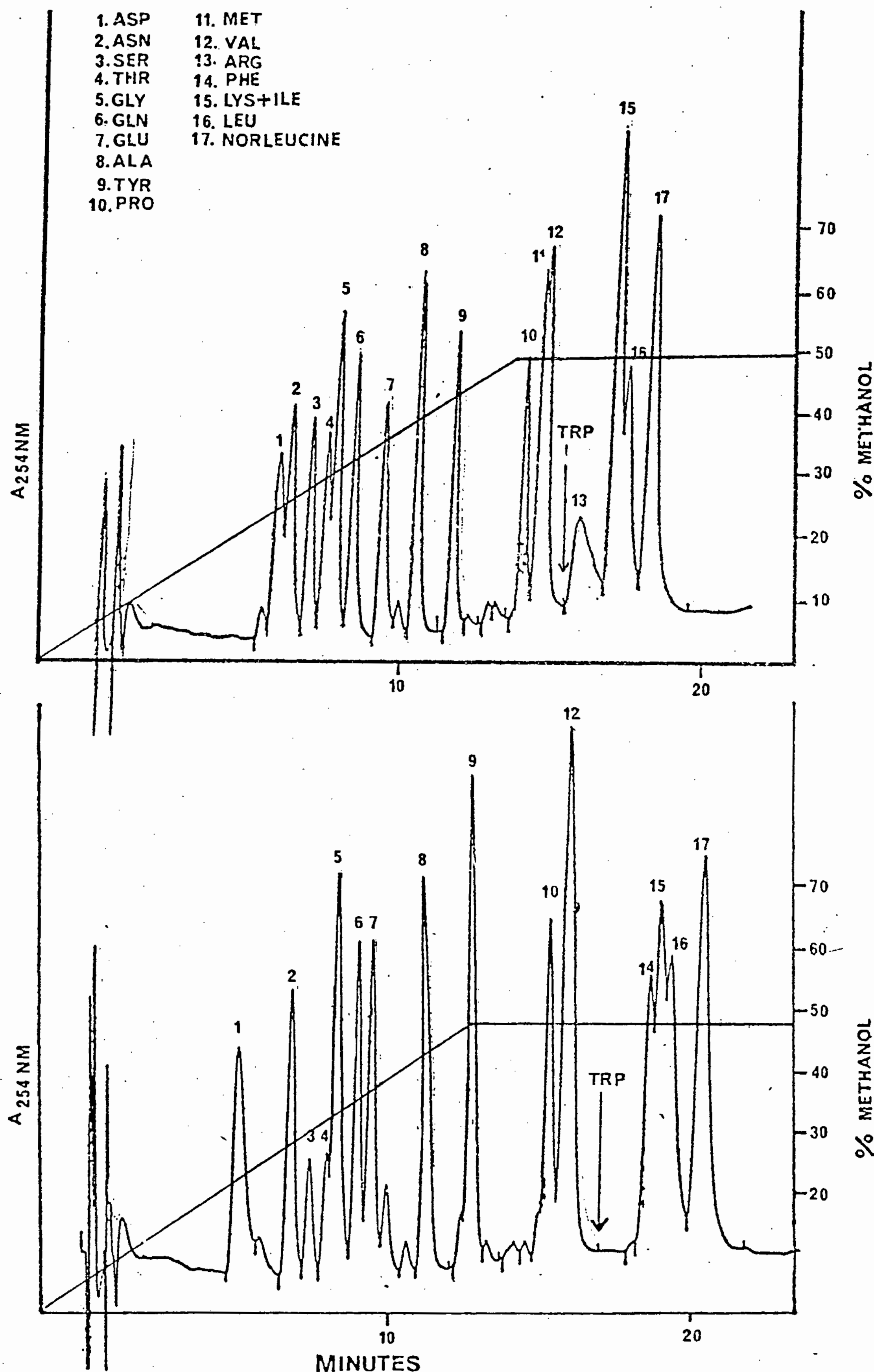

Figure 5.3 High pressure liquid chromatograph of a mixture of PTH-amino acids containing 50 nmoles of each amino acid. A Hewlett Packard Model 1084A Liquid chromatograph with a Merck Lichrosorbe RP 18 column no. 79913B (4,6 x 100 mm) with a particle size of 7 µm was used. Eluant A: 5 mM acetate buffer adjusted to pH 4,02 with 1 N NaOH, eluant B: AR methanol (Merck): gradient 6-49%B, temperature 30°C, flow rate: 1,7 ml/minute, attenuation: $2^6 = 64$ AU x 10^{-4} /cm.

Two column were used in order to identify arginine, and phenylaline; valine and methionine. Isoleucine was identified on an isocratic run.

The isocratic run was done with 49% A and 51% B at a flow rate of 1,9 ml/minute (Fig. 5.4). In order to identify arginine and phenylalanine, and valine and methionine I had to use two separate columns (Fig. 5.3).

1. Lys
2. Phe
3. Ile
4. Leu
5. Norleucine
6. Arg

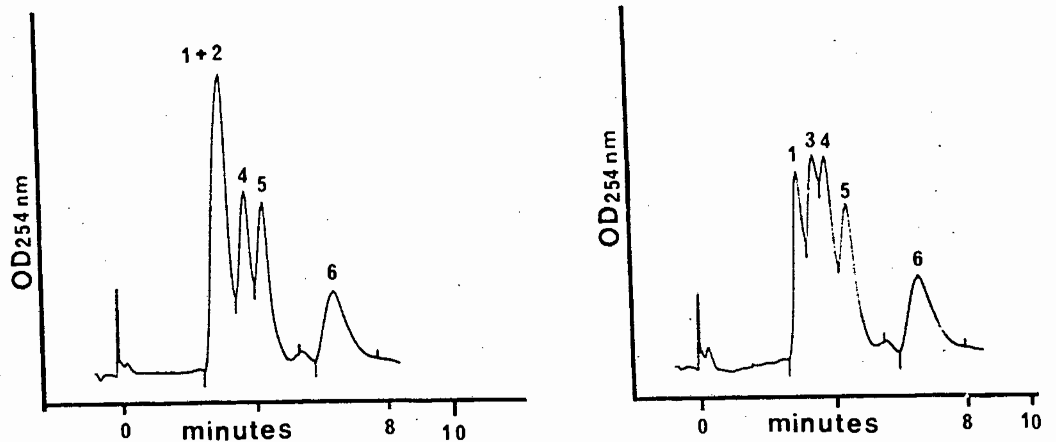

Figure 5.4: Isocratic run with a Hewlett Packard Model 1084A Liquid Chromatograph with the same columns used for the gradient run.

The flow rate was 1,9 ml/minute and the run was done with 49% acetate buffer (A) and 51% buffer B (methanol).

Phenylalanine and lysine did not separate on the isocratic run, but they did separate on the gradient run.

In the two runs shown, A was done without PTH-isoleucine standard and run B with PTH-isoleucine standard but without PTH-phenylalanine.

5.6 THE HISTONE PROGRAM AND INVESTIGATION OF CELL CYCLE HISTONE MODIFICATIONS

5.6.1 Histone Labelling with [^{3}H] Lysine

Embryos of Parechinus angulosus were grown as described in section 5.1.4. Labelling of the histones was done by adding 200 µCi [^{3}H] lysine (80 Ci/mmol; Amersham U.K.) to a 100 ml growing culture containing approximately 4 x 10^6 embryos (4% solution).

Separate embryo cultures (100 ml cultures) were incubated with 200 µCi [^{3}H] lysine (80 Ci/mmol), added at the following times after fertilization: 15 minutes, 1, 2, 4, 6, 7, 8, 9, 12, 18, 21 and 24 hours. The embryos were grown to 26 hours, collected and the nuclei and histones were extracted as described (section 5.2.1 and 5.2.2).

The [^{3}H] lysine label was taken up by the embryos within one hour from the time of addition (M. Strickland- personal communication).

The histones were displayed on Triton-acid urea gels (described under section 5.3.1) and radioactive histones were demonstrated by fluorography (section 5.6.5).

5.6.2 Time Course of Thymidine Incorporation

5.6.2.1 The Accumulative Incorporation of (^{14}C) thymidine in growing embryos was done from 3 hour old stage to 10 hours after fertlization.

(^{14}C) thymidine (specific activity 58 mCi/mmol; Amersham U.K.) was pumped into the growing culture with the use of a peristaltic pump at a rate of 0.2 µCi/25 ml culture/20 minutes. The culture was a 4% culture, containing approximately 4 x 10^6 embryos/100 ml. A 200 µl (8 x 10^3 embryos) sample was taken from the growing culture every 10

minutes, starting from 3 hours after fertilization to 5 hours, and thereafter a sample (200 μl) was taken every 20 minutes up to 10 hour old embryo stage.

The samples taken were mixed immediately with 3 ml cold 5% TCA (w/v), washed, centrifuged and washed twice more with 5% TCA (w/v) containing 1 mM cold thymidine.

The precipitate was collected and suspended in scintillation fluid 0.5% PPO (2,5 diphenyloxazole), 0,5% SDS in 7% (v/v) H_2O, 30% Triton-X-100 (w/v) made up with technical toluene). The samples were counted on a Packard Tri Carb model 3385 Liquid Scintillation Spectrometer.

5.6.2.2 Pulse Labelling of DNA with ^{14}C-Thymidine

DNA Labelling with [^{14}C] thymidine was done on embryos for short preiods between the 5½ hour old stage and 10½ hour old stage.

This was done by adding 0.04 μCi [^{14}C] thymidine (58 mCi/mMol; Amersham U.K.) to a culture containing 4 x 10 embryos/5 ml. The embryos were incubated for 20 minutes in the presence of [^{14}C] thymidine, collected, washed with CMFSW, centrifuged and precipitated with 5% TCA (w/v), collected on millipore filters, suspended in scintillation fluid and counted on the Packard Liquid Scintillation Spectrometer.

This labelling was done on separate embryo cultures (4 x 10^3 embryos/5 ml) at consecutive 20 minute intervals between the 5½ hour old stage to the 10½ hours old stage.

This labelling experiment was paralleled by a similar experiment except for the presence of 5 mm sodium butyrate in the developing culture medium.

5.6.3 [^{3}H] Acetate Labelling of Sea Urchin Embryo Histones During an Early Cell Cycle

Cultures labelled with [^{3}H] sodium acetate were grown in the presence of 5 mM butyric acid and the histones were extracted with 5 mM butyric acid present in all the extraction solutions. Butyric acid acts as an deacetylase inhibitor.

Separate cultures of 40 ml (4% cultures) each, fertilized at the same time, were labelled with 4 mCi [^{3}H] sodium acetate (specific activity: 2.2 Ci/mmol, Amersham U.K.) for 20 minutes consecutively between 5½ hours to 10½ hours after fertilization. After the 20 minute labelling period of a 40 ml culture, the embryos were collected, nuclei and histones extracted as described (section 5.2.1 and 5.2.2) and label was added to the next 40 ml culture for a 20 minute period.

The isolated histones of the various 20 minute consecutively labelled cultures were compared by running them on Triton-acid-urea gels at a urea concentration of 3.8 M and 8.5 M. Photographs of the Coomassie stained gels were taken and thereafter the gels were prepared for fluorography as described (section 5.6.5). A photograph of the X-ray plate was taken to compare with the photograph of the Coomassie stained gel.

5.6.4 [^{32}P] Labelling of Histones During the 5½ hour to 10½ hour Cell Cycle

Growing embryo cultures were labelled with [^{32}P] orthophosphate for 20 minute periods in a similar way to the [^{3}H] acetate labelling.

150 µl [^{32}P] (1 mCi/ml) was added to 15 ml cultures containing approximately 4 x 10^5 embryos/15ml (=4% culture). The embryos were grown in the presence of [^{32}P] orthophosphate for 20 minute periods, whereafter the embryos were collected and nuclei and histones

extracted in the presence of 50 mM sodium bisulfite (a phosphatase inhibitor). Labelling was done for 20 minutes at a time, on separate cultures between 5½ hours to 10½ hours after fertilization.

The histones of the various 20 minute periods were run on Triton-acid-urea-gels and the radioactivity was demonstrated by fluorography of the gels.

5.6.5 Fluorography

Fluorography was done as described by Chamberlain (1979). The Triton-X-100 urea gels containing the labelled amino acids were soaked in several changes of deionized water in order to get rid of the methanol and acetic acid.

Method

1. Soaked gel in 10 volumes of 1 M sodium salicylate (Riedel-De Haen AG Seelze Hanover), pH 5-7, room temperature, for 30 minutes. The gel was laid on H_2O-wetted Whatman 3M paper with Photra C-41 clear cronar film (0,1 mm thick) covering it.

2. Dried at about 80°C on a hot plate under a vacuum for 2 hours.

3. The dried gel was exposed to Kodak X-Omat RP-film at about -130°C (in liquid N_2 freezer).

Development of film

The X-ray plate was developed in D-19 developer (Kodak) for 5 minutes, put in a stop bath (2½% acetic acid, v/v) for 30 seconds and fixed in Amfix for 8 minutes.

5.6.6 Photography of Gels

Gels and the X-ray plates were placed on an opaque light box and photographed with a Pentax camera fitted with a 50 mm macro lens and a Vivitar red filter (25A) for gels and Vivitar blue filter (25A) for X-ray plates. Technical Pan film (black and white) was used at 1/15 second at F8 for gels and F11 for X-ray plates.

Film was developed with Kodak HC 110 diluted 1 + 19 for 8 minutes at 20°C.

Negatives were printed on high contrast 3.1 M Ilfospeed paper.

Abbreviations

A_{230}	=	absorbance at 230 nm
CTAB	=	cetyltrimethylammonium bromide
CMC	=	carboxylmethyl cellulose
CNBr	=	cyanogen bromide
DMAA	=	N,N-dimethylallylamine
DMAP	=	3-dimethylamino-1-propyn
DNS) DANSYL)	=	1-dimethylaminonaphtalene-5-sulfonyl
DTE	=	dithioerythritol
HFBA	=	heptafluorobutyric acid
PPO	=	2,5 diphenyloxazole
PTH	=	phenylthiohydantoin
PITC	=	phenylisothiocyanate
Quadrol	=	N,N,N',N'-tetrakis-(2 hydroxylpropyl)-ethylenediamine
SDS	=	sodium dodecyl sulfate
S-PITC	=	4-sulfophenylisothiocyanate
TCA	=	trichloro acetic acid
TEMED	=	N,N,N',N'-Tetramethylenediamine
Tris	=	tris-(hydrozymethyl)-amino methane

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