STUDIES ON PROTAMINE BIOSYNTHESIS IN HERRING TESTES

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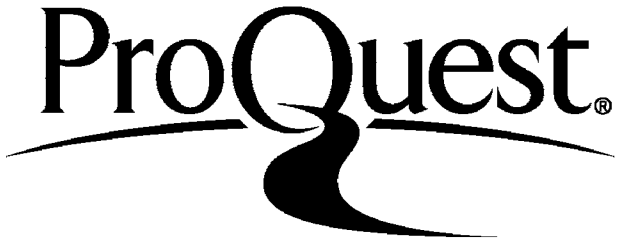

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ABSTRACT

Protamines are small basic proteins found in association with nuclear DNA in the sperm of certain species. The biosynthesis of protamine has been studied in testes material from the European herring (Clupea harengus).

The herring testes were classified by the Hjort maturity scale into arbitrary maturity stages as immature (stage I), intermediate (stages II-IV) and mature (stages V-VI). Bulk protamine was purified from acid-soluble nuclear proteins of mature sperm by successive chromatography on ion-exchange and gel exclusion columns. Characterisation of this protamine by further ion-exchange chromatography showed the presence of three components. However, difficulties were encountered in extension of these methods to analysis of the relative rates of protamine component synthesis as a function of development. Enzymatic dephosphorylation of newly-synthesised protamine was found necessary prior to radioactive component analysis, since the presence of phosphate groups made interpretation of the results difficult. Treatment with alkaline phosphatase changed the elution profiles previously obtained with mature protamine and so a method for analysis of radioactive protamine components could not be developed.

Using analytical electrophoresis on 20% polyacrylamide gels, protamine was first detected at stage II, increased during subsequent stages and reached a maximum at stages V and VI. Greatest protamine synthesis occurred at stage III. Testes from stages II-IV were therefore used as a source for mRNA isolation and characterisation. Microsomal RNA was separated into high and low molecular weight RNA by differential salt precipitation and these fractions tested for mRNA activity in a cell-free system. Both fractions exhibited activity, over 95% of the total being in the high molecular weight RNA. The latter was separated into poly A (+) and poly A (-) fractions by chromatography on oligo (dT) cellulose. All fractions exhibited messenger activity, the poly A (-) RNA comprising over 80% of the total. Sucrose gradient analysis of protamine mRNA under denaturing conditions showed that its apparent large size was artifactual. However, the presence of the messenger in poly A (+) and poly A (-) forms was shown to be real by elimination of possible RNA degradation and overloading of the oligo (dT) cellulose column.

TABLE OF CONTENTS

TABLE OF FIGURES

TABLE OF TABLES

TABLE OF APPENDICES

GLOSSARY OF ABBREVIATIONS

Hn RNA	Heterogeneous nuclear RNA
mRNA	Messenger RNA
cDNA	Complementary DNA
tRNA	Transfer RNA
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulphate
EDTA	Ethylenediaminetetra acetic acid
PCA	Perchloric acid
TCA	Trichloroacetic acid
PPO	2,5-Diphenyloxazole
POPOP	1,4-bis[2(5-Phenyloxazolyl)]benzene

INTRODUCTION

Different cell types are characterised by the possession of unique populations of proteins. Since all the cells of a given organism contain identical DNA sequences, the establishment of these unique arrays implies differential gene activity i.e. different structural genes are expressed in phenotypically different cells or in the same cell at different stages of development. This further implies that the way in which these genes are expressed is under strict cellular control and the mechanism and control of this process is a major problem currently commanding the attention of many molecular biologists.

According to the central dogma, information flow from genome to protein can be expressed as follows:

$$DNA \rightleftarrows RNA \rightarrow PROTEIN$$

In eukaryotes, this process is very complex, involving a large number of component molecules and steps, many of which are both physically and temporally separated from one another. This involves transcription of nuclear DNA, followed by processing of the transcripts, transport of the resultant molecules across the nuclear membrane and finally translation. The process is discussed in three sections, transcription of RNA, processing of Hn RNA, and translation of messenger RNA.

1. Transcription of RNA in eukaryotic systems

Transcription involves the polymerisation of nucleoside triphosphates into RNA, a process catalysed by RNA polymerase under the direction of DNA. It is usually assumed that once the enzyme is attached to the DNA template eukaryotic RNA synthesis proceeds along similar lines to those observed in prokaryotic systems. However, since the structural and functional organisation of the chromosome is still not well defined, we have no idea of how or where the enzyme becomes attached to the template in the first place. It is therefore difficult to envisage specific models for the mechanism and control of transcription. Nonetheless, it is obvious that some sort of control process must occur, otherwise the amount and types of RNA produced would place impossible demands on the degradative machinery of the cell. For this reason, numerous attempts at defining possible

models have been attempted.

Studies on transcription require that methods of analysing the transcription products are available. A brief survey of the methodology currently used is therefore appropriate.

(a) Autoradiography:

This technique is used when it is necessary to visualise nascent RNA molecules i.e. to localise regions of the genome which are actively undergoing transcription.

(b) Base composition analysis:

Analysis of base composition of the transcription products enables very crude statements to be made about the nature of the RNA molecules synthesised, since the major classes of RNA have characteristic base compositions.

(c) DNA/RNA hybridisation:

This technique is used to detect the presence of specific RNA sequences in a mixture of RNAs. Either simple DNA/RNA hydridisation or competitive hybridisation techniques are used. The former is based on the principle that two nucleic acid strands of complementary nucleotide sequence (e.g. DNA and its transcription product) will hybridise to one another, given the correct experimental conditions. The latter relies on the fact that if two RNA molecules contain similar sequences, then hybridisation of one of these to its complementary DNA(cDNA, rDNA or cellular DNA) will be inhibited by the presence of the other (the two types of RNA used in experiments on transcription would be a well defined cellular RNA and the <u>in vitro</u> transcription products).

Hybridisation is the most widely used of the available techniques for RNA analysis, due to its flexibility.

$\frac{R_0}{D_0 C_0} t_{\frac{1}{2}}$ All hybridisation reactions are characterised by the expression (concentration of nucleic acid x time for the reaction to be half complete). For a given concentration of nucleic acid, the speed of reaction is dependent on the concentration of the sequences under study in the nucleic acid sample. Thus, long hybridisation times are required for sequences that are only present as a small percentage of the total e.g. DNA and RNA sequences coding for proteins. The concommitant increase in the possibility of nucleolytic degradation can be alleviated by the presence of high nucleic acid concentrations to drive the reaction forward, but this is not always feasible. For

this reason, the available hybridisation techniques will only allow efficient study of sequences which form a significant fraction of the sample investigated e.g. genes and transcription products for 5S, rRNA and histone mRNAs. Even so, the practical necessity of using intermediate reaction times raises the possibility that closely related, but non-identical sequences may react, which reduces the specificity of the technique. It is therefore necessary to use highly stringent experimental conditions to avoid unnecessary difficulties in interpretation.

Methods for the synthesis of cDNA from pure mRNA have provided powerful tools for the study of unique gene sequences and are becoming widely used. The use of the cDNA probe reduces the necessity for long hybridisation times mentioned above, since cDNA contains a much higher percentage of the gene sequence in question than would an equivalent amount of cellular DNA.

Despite the limitations of all available methods, much work has recently been reported on the control of transcription. Since selective transcription implies that the polymerase (and/or any additional regulatory molecules) recognises specific DNA sequences for initiation, it is possible to formulate two generalised models for possible control mechanisms.

(1) Specificity resides in the enzyme (or any associated regulatory molecules). The polymerase could have specific initiation factors associated with it, comparable to the bacterial σ factor. Alternatively, the enzyme itself could have several distinct, specific forms. (The presence of multiple enzyme forms would not be flexible enough to control large numbers of genes, but could possibly regulate groups of genes; this effect could be enhanced further by the initiation factors).

(2) The structure of the chromosome could affect transcription by modifying the efficiency with which the RNA polymerase binds to different initiation sites. Differential binding efficiencies will already be imposed by the structure of the DNA itself. This effect could be enhanced in either a positive or negative way by interaction of regulator molecules with the template e.g. by alteration of the conformation of the template.

These models are not mutually exclusive, however, and it is quite probable that the regulation of eukaryotic transcription is sufficiently complicated to require a combination of both.

1.1. RNA polymerase as a suitable point of control

Studies on RNA polymerase have primarily utilised three systems, intact cells (or animals), isolated nuclei and purified enzyme preparations.

1.1.1. Studies with isolated nuclei

During the course of these studies, it was noted that the types of RNA molecules produced depended on the ionic strength of the medium, the nature of the cations present or the presence of amanitin. By manipulation of these parameters, either nucleolar synthesis of rRNA or nucleoplasmic synthesis of HnRNA could be favoured (1 - 4). These results suggested the presence of at least two polymerase activities, which may or may not be associated with one molecule. Purification of nuclear extracts has demonstrated the physical independence of the activities (5,6). The enzymes catalysing synthesis of nucleolar rRNA and nucleoplasmic HnRNA have been called enzymes I, (or A) and II, (or B) respectively. Measurement of the relative proportions of the activities in different nuclear fractions has confirmed that enzyme I (or A) is located in the nucleolus and enzyme II (or B) is in the nucleoplasm (7). Enzyme purification also yielded another activity, in addition to those expected. This has been called enzyme III (or C), which is sensitive only to high levels of amanitin. Monitoring of 4 and 5S RNA synthesis as a function of amanitin concentration shows that this synthesis is inhibited only at high levels of amanitin, implying that enzyme III (or C) is responsible for the synthesis of 4 and 5S RNAs (8).

Thus, three classes of RNA polymerase can be demonstrated, each with its own characteristic location and set of properties (Figure 1.1).
N.B. Since the nomenclature on RNA polymerases is somewhat confusing, only the amanitin dependent nomenclature will be used.

1.1.2. Studies with intact cells (or animals)

In general, these studies support the conclusions reached in section 1.1.1. It can be shown that α amanitin does not affect rRNA synthesis either in X. laevis stage four oocytes (9,10) or in cultures of chick embryo fibroblasts (11), RFL-T fibroblasts (12) or Chinese hamster ovary cells (13), although it does seem to affect HnRNA synthesis (11,12,13). However, there is at least one exception to this trend. Mouse and rat liver rRNA synthesis is drastically repressed by treatment of the

Figure 1.1 Nomenclature and localisation of eukaryotic DNA-dependent RNA polymerases

Class of enzyme			
Nomenclature based on sensitivity to α amanitin	Nomenclature based on chromatographic properties on DEAE-Sephadex	Localisation	RNA type synthesised
A insensitive to amanitin	I	Nucleolar	rRNA
B sensitive to low concentrations of amanitin (10^{-9} - 10^{-8}M)	II	Nucleoplasmic	Hn RNA
C sensitive to high concentrations of amanitin (10^{-5} - 10^{-4}M)	III	Nucleoplasmic and cytoplasmic	4S,5S RNA

animal with amanitin (14 - 19). This effect is not observed when liver nuclei are isolated from such animals and assayed *in vitro* for RNA polymerase activity (17,20), although the amount of class A activity associated with the nucleoli decreases (21). It may be that liver cells are aberrant in this context, since the amanitin accumulates in these cells to an unusually high degree after *in vivo* administration.

The discovery of three enzyme classes having different localisations and performing different synthetic functions has obvious regulatory significance. Each enzyme may recognise and transcribe a group of genes through interaction with characteristic initiation sequences common to each gene within a particular group. This type of control would allow simultaneous transcription of related genes which may be located on different portions of the genome. If specificity could be demonstrated conclusively with respect to each enzyme class, it should be possible to postulate that gene expression is controlled, at least in part, by manipulation of the relative concentrations and/or activities of these enzymes. Manipulation of enzyme levels has been demonstrated in several systems. It is known that polymerase B is present in larger amounts in rapidly growing *D. discoidium* cells than in non-growing cells (22). The quantity of enzyme also appears to depend on the nutritional or hormonal state of the cells. For example, the yield of polymerase B in calf thymus has varied over a hundred-fold range (23,24). The levels of polymerase B thus appear to be governed by cellular requirements. Recent work with normal and tumour infected BALB/C cells has shown that the levels of enzymes A and C are also dependent on the state (i.e. tumourous or not) and the age of the cells (25).

None of the experiments described in sections 1.1.1 and 1.1.2. are without difficulties, however. The nuclear system has low activity and studies with isolated nuclei and whole cells cannot exclude the possibility that the observed specificity is in fact due to compartmentalisation. To circumvent this problem, totally reconstituted systems for *in vitro* transcription have been devised, although it should be remembered that these systems suffer from the inherent disadvantage of being unphysiological.

1.1.3. In vitro studies with purified enzymes

Demonstration of the specificity of an in vitro reaction requires that a given enzyme can be shown to select, from the large number of sequences available, only those sequences which it is meant to transcribe. Several conditions must therefore be fulfilled. Firstly, the distribution of different RNA types should alter when different enzyme classes are used to transcribe the same template. (The methods used and their disadvantages have been described in section 1). Secondly, the enzyme should bind and terminate at the correct positions on the DNA i.e. transcripts produced in vitro should be identical structurally to their in vivo counterparts. This could be shown, in part, by sequence analysis of the 5' and 3' ends of the in vitro and in vivo transcripts. Thirdly, only sequences produced in vivo should be observed in vitro. One approach to this would be to show that only the sense strand of DNA is transcribed (asymmetric transcription).

Two approaches to in vitro transcription studies have been utilised, using either DNA or chromatin as the template.

1.1.3.1. DNA as template

Whenever DNA is isolated degradation is always a problem due to the large size of the eukaryotic genome. This is very important, since it has been shown that RNA polymerases from several sources can initiate transcription at breaks or gaps in DNA (26 - 28). A degraded template could support spurious initiation, thus nullifying attempts to demonstrate specificity. All experiments of this type must therefore use a DNA template of the highest integrity and an RNA polymerase preparation free of all DNAse activity (29).

When undegraded DNA is used, it is found to possess poor template activity on incubation with preparations of E. coli polymerase or eukaryotic enzymes A and B (27, 30 - 33). The low activity appears to be due to failure in initiation (34, 31, 32). Several possible explanations for this observation can be devised.

Purification of the enzymes may cause elimination of protein factors necessary for initiation. Proteins which stimulate transcription of native DNA by various classes of polymerase have been found (35 - 41) and purified (40, 42, 43) but these proteins appear to act at the elongation step (34, 42, 43, 44). Thus, a specific initiation factor (or factors) has yet to be definitively demonstrated.

Alternatively, DNA could genuinely have poor template activity. This makes experiments designed to demonstrate enzyme specificity much more difficult practically, and it is therefore not really surprising that the results of such experiments have not been really definitive. No obvious tendency to specific transcription was seen when X. laevis rDNA versus bulk DNA were incubated with polymerases A, B and C (45, 46). However, in other reports, polymerase A did appear to select rDNA sequences from high integrity X. laevis DNA enriched in rDNA, whilst polymerase B did not (47 - 49). Thus, an apparent contradiction arises, though the variability of the results may be explained by the fact that neither of these experiments is without experimental deficiencies. In the former, the integrity of the template may not have been high enough to exclude the possibility that spurious initiation was obscuring the transcriptional pattern obtained. In the latter, this criticism does not apply, but the degree of symmetric transcription was not investigated and the polymerase A preparation was impure. It is therefore possible that other factors e.g. contaminating proteins, could have been responsible for the observed specificity. Strand selectivity has been demonstrated with yeast class A enzyme, but not with class B. Enzyme A showed a sense:nonsense transcription ratio of 2:1 with yeast DNA and also with rDNA-enriched yeast DNA (50). However, the magnitude of the effect was not really definitive. A more impressive ratio of 5:1 in favour of one strand has been reported when polymerase B transcribes mouse satellite DNA. Polymerase A was equally efficient with both strands (51).

It should be noted that the experiments with mouse satellite DNA described in the last paragraph illustrate the in vitro synthesis of RNA sequences not normally transcribed in vivo. This implies that control mechanisms active in vivo are relaxed in vitro.

Thus, no firm evidence exists to prove that the enzyme/DNA interaction is template specific; whilst results like asymmetric transcription support the hypothesis, transcription of RNA not observed in vivo (mouse satellite RNA) would suggest that some other molecules are also involved in controlling specificity.

1.1.3.2. Chromatin as template

The variable specificity observed in vitro with naked DNA might be due to the fact that it is not the natural template. In the cell, DNA exists complexed with other molecules (RNA and protein) and it is possible

that these molecules alter the conformation of the DNA to promote more efficient binding of the RNA polymerase. It can be postulated that any potential specificity resident in the enzyme can only be realised if the binding sites are in the correct conformation. Therefore, attempts to prove specificity in the enzyme/template interaction might be more successful with chromatin as the template, and many studies of this type have been reported, despite the fact that chromatin generally appears to be less active as a template than the corresponding naked DNA.

The first *in vitro* experiments were carried out using RNA polymerase from *E. coli*. In several systems, the RNA transcripts were analysed by competitive hybridisation with RNA isolated from the tissue concerned. Effective competition was observed, implying that similar RNA sequences were produced *in vitro* and *in vivo* (52 - 55).

However, these experiments have been criticised on the grounds that a bacterial enzyme would not necessarily recognise initiation and termination signals present in eukaryotic chromatin. Kinetic studies on the transcription process catalysed by bacterial polymerase have suggested that the enzyme binds efficiently to the chromatin, but does not automatically commence transcription. On the other hand, eukaryotic enzymes bind less efficiently, but always proceed with transcription. These results imply that the interaction process is indeed different (56). If this is the case, then the prokaryotic and eukaryotic polymerases would be expected to produce different populations of RNA molecules from the same eukaryotic template. The demonstration that these enzymes had activities which were additive rather than competitive, supports this view (57), and more recent experimentation with eukaryotic RNA polymerases has also led to a similar conclusion (58 - 61). However, the fact that eukaryotic and bacterial enzymes may transcribe different DNA sequences is not sufficient to suggest that the former are any more selective than the latter. In this context, it should be noted that in the experiments described above (56 - 61) precise determination of the nature of the *in vitro* transcripts was not attempted.

Experiments to determine whether different eukaryotic RNA polymerases selectively transcribe homologous chromatins are somewhat contradictory. In some systems, class A and B enzymes do appear to be selective. Rat liver enzyme A appeared to transcribe homologous nucleolar chromatin more efficiently than enzyme B (62). However, enzyme

specificity was not observed when DNA was used as the template. This implies that some component bound to the chromatin alters the template to favour binding of enzyme A. On the other hand, enzyme A did not transcribe whole rat chromatin (57). The reason for this difference is not clear, although the presence of varying amounts of scission in the template preparations may be responsible, as in the former experiment no estimate of the accuracy of transcription was made and in neither experiment was any attempt made to determine whether initiation occurred at single-stranded breaks or double-stranded regions of DNA. The absence of differential activity between enzymes A and B in other systems also implies that the above results may be artifactual (63, 64). In this context, it is important to note the demonstration that *in vitro* transcription of *X. laevis* chromatin by homologous polymerases A and B was not asymmetrical with respect to 5S and rRNA genes. Both strands were transcribed (63). This is in contrast to the *in vivo* situation, where only one strand is utilised by enzymes C and A respectively. It would therefore appear that eukaryotic RNA polymerases are no more selective in transcribing chromatin than their bacterial counterparts despite the presence of chromatin-bound components.

The general conclusion from the work presented must be that *in vitro* interaction between RNA polymerase and either chromatin or DNA has not been proven to possess the specificity observed *in vivo*. Since there is no positive evidence that RNA polymerase is capable of selectively binding to particular sites on the template, we are forced to postulate that the components of the chromatin itself may regulate specificity of DNA transcription.

1.2 Chromatin structure as a suitable point of control

In order to demonstrate that specificity resides in the chromatin structure, it is necessary to show that changes in this structure result in the synthesis of different, specific populations of RNA. Two major approaches have been used to examine this postulation. Firstly, isolated chromatins from different cell types have been analysed for possible differences in chemical composition. Secondly, RNA populations synthesised *in vitro* using various chromatin preparations have been examined.

1.2.1. Analysis of chromatin components as a function of cell type

Excluding DNA, chromatin components can be divided into three classes, histones, non-histone proteins and chromosomal RNA.

The histones consist of a small group of basic proteins which are very similar in structure and exhibit little cell specificity. This similarity implies the presence of genetic pressure to counteract any divergence during evolution, a situation which could occur if this group of proteins had a specialised function dependent upon primary structure. Such a limited range of structures would be unlikely to have the necessary flexibility to control the transcription of a complex eukaryotic genome, and it seems likely that these proteins have a structural, rather than a regulatory role.

In contrast to the histones, the non-histone proteins (hereafter called NHP) are a very heterogeneous group. This heterogeneity makes them plausible candidates for regulatory molecules. However, their study is complicated by their tendency to aggregate and also by the lack of a suitable assay for distinguishing between true chromosomal proteins and cytoplasmic contaminants. Numerous preparative methods have been developed for the isolation and characterisation of NHP from different cell types (65). Preparations of total NHP have shown little tissue specificity (66 - 69). These results do not necessarily preclude a regulatory role for NHP, since currently available methods may not be sensitive enough to detect changes in regulatory molecules comprising a small proportion of the total NHP. However, preparations of selected fractions of NHP have often shown marked tissue specificity (70 - 73). The disadvantage of this approach is the assumption that a given sub-group of proteins is equally extractable from all chromatin types, which is not necessarily true (for discussion, see 65).

Recently, much interest has been focussed on chromosomal RNA (cRNA). (74,75).

This RNA is thought to be heterogeneous within a given tissue and to be organ-specific. (75,76).

The appearance of new sequences of cRNA has also been correlated with large increases in chromatin template activity. (77).

Thus, cRNA would appear to be a suitable candidate for a regulatory agent. Evidence has been presented that it is a discrete class of RNA characterised by distinct hybridisation properties and high levels of dihydropyrimidines. Other workers have failed to demonstrate this

hybridisation, or to find dihydropyrimidines in cRNA and it has been suggested that this RNA is an artifact arising from degradation of tRNA, rRNA or HnRNA (78 - 81).

It appears, therefore, that the question of the significance of cRNA is unresolved.

1.2.2. In vitro studies with isolated chromatin

A complementary approach to the study of dissociated chromatin components isolated from different cell types would be to investigate the RNA populations synthesised *in vitro* using a variety of chromatin preparations. These preparations can be of several types:-

(a) Intact chromatin

(b) Chromatin reconstituted from its various components under defined conditions

(c) Chromatin selectively depleted of certain macromolecules e.g. proteins

Studies with intact chromatin demonstrated that the species of RNA synthesised *in vitro* were dependent on the source of the chromatin (53, 55, 82, 83). However, these experiments were carried out using bacterial RNA polymerase and the RNA transcripts were analysed by low Cot hybridisation methods. The theoretical disadvantages of this approach, discussed in section 1, make interpretation of the results problematical. Recently, the synthesis of the cDNA probes (DNA complementary to given mRNAs), have enabled some study of unique gene sequences to be made, which support the above conclusions (84). These experiments still do not circumvent the objection to bacterial polymerase, but since the eukaryotic enzyme does not appear to transcribe any more selectively than its bacterial counterpart, this may not be too significant, though it should always be borne in mind.

The demonstration of organ specificity in chromatin forms the theoretical justification for studies of *in vitro* transcription of reconstituted chromatin. These are designed to determine which of the chromatin components are responsible for the observed effect on transcription. Purified chromatin components, prepared either from the same or different chromatins, were allowed to reconstitute under defined conditions. These experiments suggested that histones act in a non-specific way to repress chromatin activity, since chromatin reconstituted from DNA and histones alone exhibited virtually no template activity (85). These results are supported by the demonstration

that removal of histones from chromatin caused an increase in template activity (86). It has been suggested, however, that histone-induced repression may be due to the relative insolubility of nucleohistone, which results in decreased availability of the template for enzyme binding (87). It is therefore necessary to ensure that the experimental conditions employed in this type of experiment result in the maximum possible solution of the chromatin.

The repressed state of nucleohistone can partly be relieved by the presence of NHP. When chromatin was reconstituted by the addition of NHP to DNA, either before or simultaneously with the addition of histones, it exhibited transcriptional activity similar to that of intact chromatin. However, when NHP were added to nucleohistone preparations, the template activity obtained remained at the repressed level characteristic of the nucleohistone itself (88). This implies that NHP govern template activity by controlling the binding of histones to DNA rather than by removing previously bound histones. These findings are in contrast to those of Wang et al., who have shown that addition of NHP to heterochromatin (fully repressed chromatin) resulted in a stimulation of template activity proportional to the quantity of NHP added. Similar additions to euchromatin (derepressed chromatin) had far smaller effects. This implies that NHP can exert regulatory influence on repressed chromatin (89 - 92).

One possible explanation for this contradiction may be provided by the demonstration that phosphorylation appears to be important to NHP action, since dephosphorylation of calf uterine NHP prior to reconstitution has been shown to reduce their ability to reverse histone-induced repression. Other experiments have also shown that phosphorylated NHP can partially derepress the template activity of nucleohistones (93 - 95).

In addition to derepressing the chromatin, NHP also appear to confer some degree of specificity on the observed transcription. The studies of Wang et al. mentioned above have shown that heterologous NHP can cause new regions of the genome to become available for transcription. The selection of DNA sequences to be exposed therefore depends on the source of the NHP (90). These results are supported by others using chromatin reconstituted with components from different rat tissues (96). Chromatin reconstituted from DNA, liver NHP and thymus histones synthesised RNA typical of liver, whilst that reconstituted from DNA, thymus NHP and liver histones synthesised RNA typical of thymus.

The idea that specificity resides with the NHP has been challenged by Bonner's group, who maintain that it is the cRNA present in the NHP preparations which confers selectivity of transcription, since this selectivity was lost when the cRNA was degraded prior to reconstitution (97, 98). This proposal has yet to be rigorously proven.

In addition to studies with the reconstitution of chromatin, it is also possible to study the effect of selective depletion of certain macromolecules from the chromatin on in vitro transcription. Removal of histones resulted in a large increase in template activity, although removal of specific histone classes showed no consistent pattern (99 - 103). The latter effect may be due to methodological differences. The residual DNA/NHP complex was either more or less active than naked DNA, depending on the use of eukaryotic or bacterial RNA polymerases, respectively (54, 101, 86, 104, 105).

Thus, the evidence presented on transcriptional control is, almost without exception, somewhat contradictory in nature. This is due to the theoretical and practical limitations which have been emphasised throughout. These difficulties are not surprising when one considers the enormity of the problem.

The first major obstacle is the complexity of the system. Even the simplest eukaryotic cell is vastly more complicated than a prokaryotic cell, a fact reflected in several of its properties. The cell generation time is, in general, far longer than in prokaryotic cells. This results in mutant cells being produced with low frequency, the numbers being further reduced by the fact that such mutations are frequently lethal. The application of genetical analysis, so successful in studies on prokaryotic regulation, is therefore less feasible with eukaryotic systems and studies in this field have been almost exclusively biochemical in nature.

This presents its own problems, however, since such an approach relies heavily on isolation of the various components and presently available methodology has not been able to meet the challenge. It is extremely difficult to isolate components without introduction of artifacts. These include scission of high molecular weight DNA, rearrangement of protein distribution in chromatin, differential extractability of the components from chromatin.

Once isolated, the components have to be purified in order to investigate the connection between a putative regulatory molecule and the activation of a specific gene, or group of genes. This is frequently a major problem, since non-histone proteins and cRNA each behave as a heterogeneous collection of molecules. Hopefully, the advent of better preparative methods and probes for the study of single gene sequences will improve this situation.

Preparation of pure components enables reconstituted *in vitro* systems to be used. The effect on transcription of varying component concentrations should give valuable insight into the possible regulatory significance of these molecules. However, such studies are hampered by the difficulty of adequately reproducing *in vivo* conditions *in vitro*. Even if all the component molecules were to be available in a pure and native state, it is difficult to exclude the possibility that some important molecule is limiting or entirely absent. There is also always the problem associated with destruction of a compartmentalised structure, which may permit certain unphysiological interactions to occur. These difficulties are reflected in the fact that *in vitro* transcriptional activity is only linear for a short period of time and that premature termination appears to occur. This results in a low yield of RNA transcripts, which may also not adequately represent the DNA sequences available for transcription, a fact that hampers subsequent comparisons with HnRNA prepared from the tissue in question.

Thus, we are clearly a long way from establishing the interrelationship and regulatory significance of these components. For these reasons, firm conclusions would be premature. Nonetheless, certain tentative conclusions can be made from the evidence available. That histones act as non-specific repressors of gene activity is implied by all *in vitro* experiments; however, the question of whether specificity resides in the NHP or cRNA is controversial and must for the present remain open.

These results imply that interaction of DNA with histones normally maintains the DNA in a predominantly repressed state. In this case, expression of a given gene is only possible by specific derepression of that gene through the mediation of a specific activator molecule. Suitable candidates for the activator molecules are the NHP and cRNA.

Despite the lack of information on the structural and functional organisation of the chromosome, several models have been formulated to explain how the hypothetical activator molecules could bring about selective gene transcription.

1.3. Models for control of transcription

Any model for transcriptional control must be able to explain the following observations:

(1) that isolated functional genes are structurally dispersed throughout the genome, and so a mechanism to coordinately control these genes must be provided;

(2) that the transcription products are of larger size than necessary to code for the RNA (protein) in question and also contain large amounts of repeated sequences.

Thus, all models postulate the existence of two types of sequences in DNA, structural genes coding for RNA or protein and non-informational sequences which interact with suitable regulatory molecules. It is assumed that each regulatory sequence is located adjacent to the structural gene under its control and that this gene is transcribed only when the regulatory site is activated. The possession of a common regulatory site for functionally related genes would allow their coordinate control by a single regulatory (activator) molecule. The transcription of the regulator sequences would also explain the large size of the transcripts.

The most comprehensive model currently available is that of Britten and Davidson (106, 107). In this model, the regulatory sequences are called receptor sites and coordinated regulation of functionally related genes is effected by interaction of one regulatory molecule (activator) with common receptor sites. The activator could either be RNA or protein. In each case, genomic sequences coding for these molecules must exist and these are termed integrator sites. In the former case, the integrator site transcript is the activator, whilst in the latter, it is the mRNA from which the activator is produced. The model, as it stands, does not link the production of these activators to the physiological requirements of the cell. This is remedied by the postulation of sensor sites, which control the transcription of the integrator sites in a similar manner to the control of structural gene expression exerted by the receptor sites. These sensor sites are

activated by cellular effectors e.g. protein-hormone complexes. The whole process is shown diagrammatically in Figure 1.2.

However, the expression of a given gene, or group of genes, may be demanded in response to different cellular effectors. It is clearly wasteful to duplicate the whole mechanism with several different sensor sites and the necessary flexibility can be obtained by the presence of several different receptors per structural gene and/or by several different integrator sites per sensor site. The former would allow the expression of a given gene under the influence of a small number of different activators. The latter would allow the expression of groups of genes in response to one cellular effector. It is also possible to express a particular group of genes in response to different cellular effectors by repeating integrator sites under the control of more than one sensor site, an analogous situation to the presence of common receptor sites adjacent to different structural genes. This results in a hierarchical arrangement: small changes are controlled at the receptor site level and extensive changes at the integrator site level. (Figure 1.3).

Although the model does not differentiate between RNA or protein as the activator molecule, RNA does have several advantages in this capacity. Firstly, the entire control mechanism could be contained within the nucleus; this could not occur with proteins as activators, as it would be necessary to transport and translate the integrator site RNA. Secondly, coordinate activation of a group of genes requires coordinate transcription of integrator sites. Use of proteins as activators requires that this control is also extended to translation of those RNA transcripts. However, a choice between RNA and protein will have to await more definitive evidence on the role of non-histone proteins and cRNA in the control of transcription.

Irrespective of the nature of the activator molecule, any successful model must explain how a particular sequence of DNA is recognised. With RNA activators this recognition would probably occur via base pairing, although it is then necessary to explain how the DNA helix unwinds sufficiently to allow such pairing to occur. This problem is not alleviated by use of protein activators, since one must then explain how they interact with the DNA already in close association with proteins. It is possible that the organisation of the chromatin is such that receptor sites are always accessible to activators. This means

Figure 1.2

Schematic representation of the Britten-Davidson Model

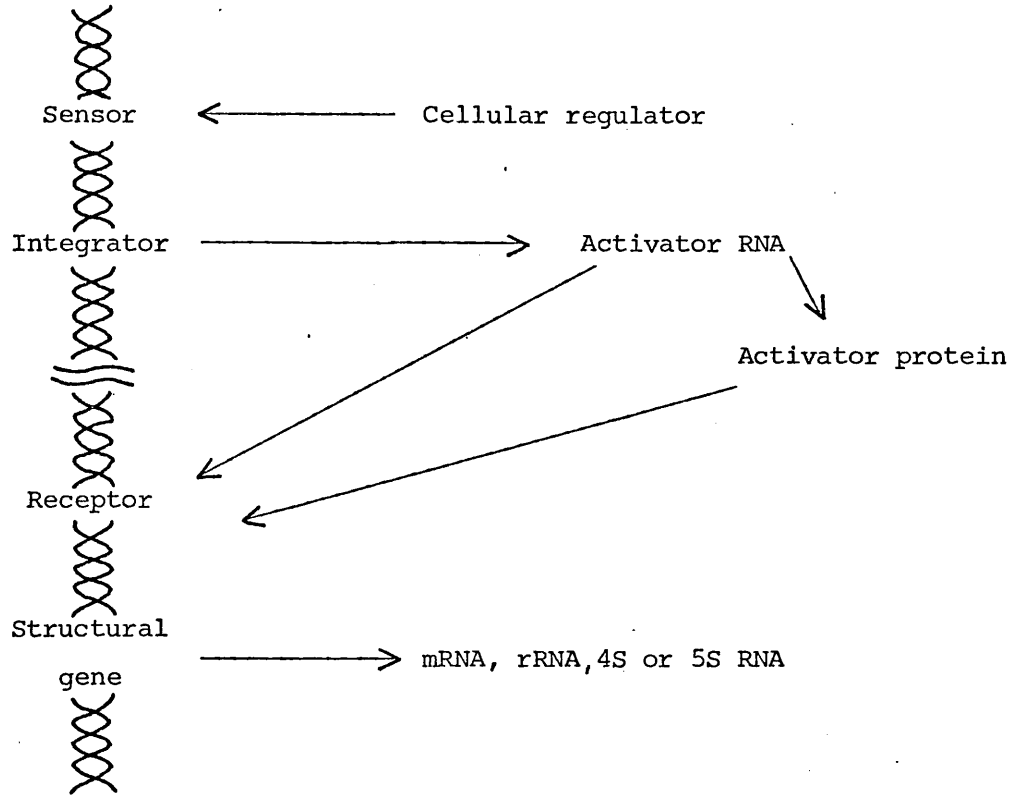
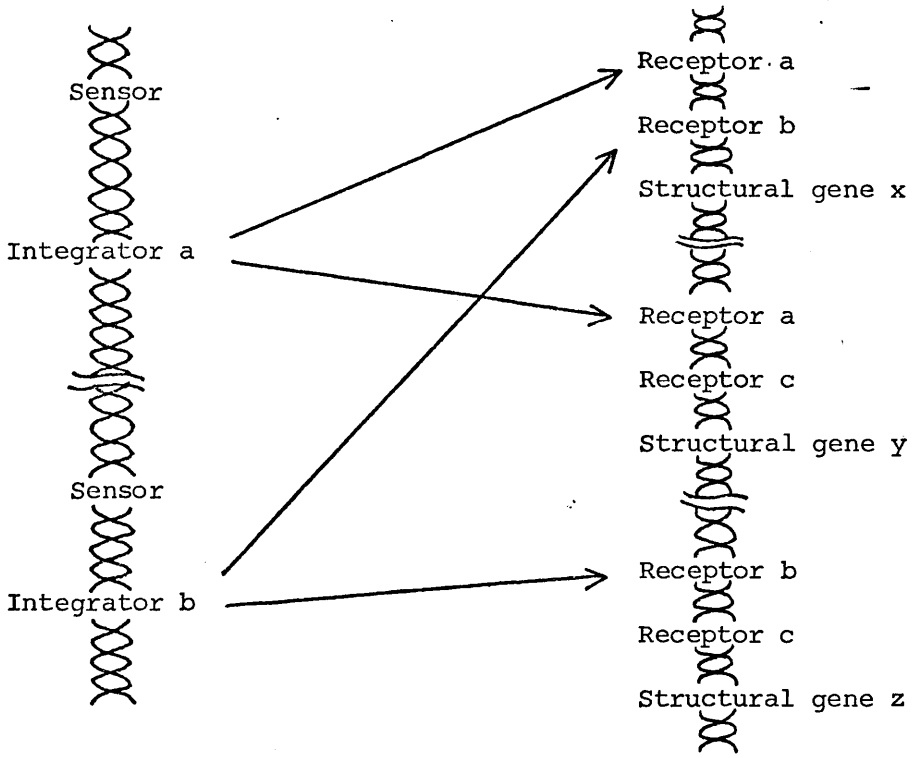
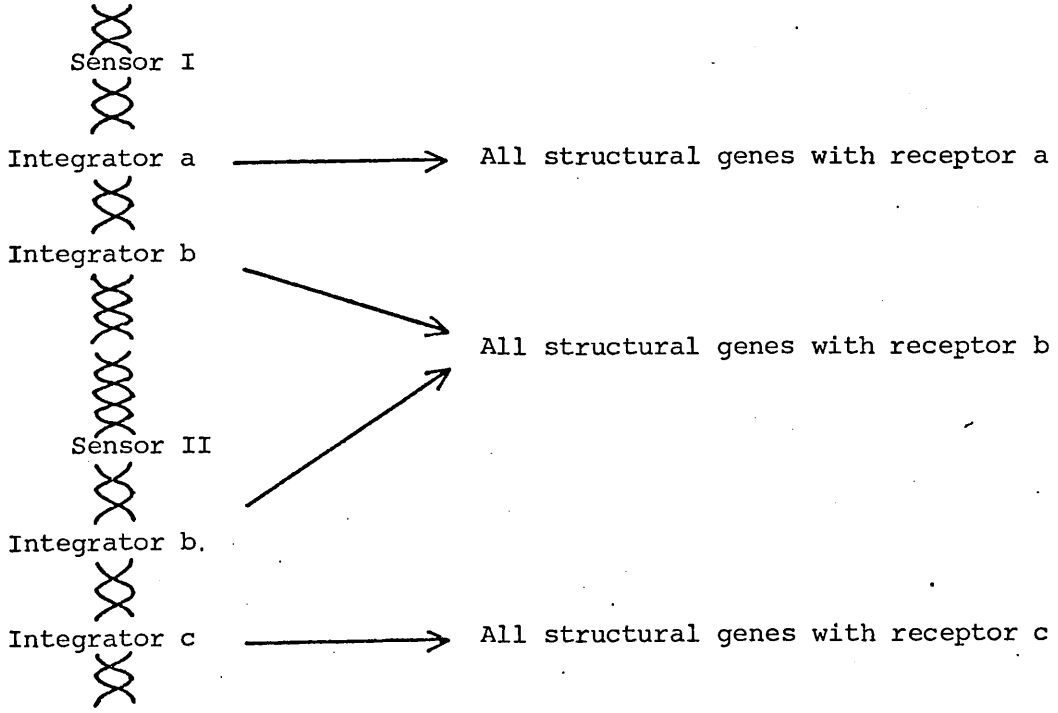


Figure 1.3

Control of gene activation at the receptor site level



Control of gene activation at the integrator site level



that the chromatin is structurally programmed to permit transcription and it is the presence of cellular signals which determine the sequences to be transcribed.

Thus, the structure of chromatin must also be taken into consideration when models are devised. It is known that in diptera, the interphase chromosomes consist of densely-coiled band regions and less dense interband regions (108). The latter actively synthesise RNA and are called "puffs", whilst the former do not support RNA synthesis (109, 110). Similar conclusions have been reached with rat liver. It is possible that transcription has either proceeded from the band to the interband regions or is in the process of proceeding from the interband to the band regions. Each of these models has generated a model for transcriptional control. The former has been proposed by Crick (111), who postulates that the regulatory DNA is in the compact band region. The arrangement of the chromatin in this region maintains the DNA in a single-stranded state suitable for activator binding (the pre-programming effect mentioned above). However, such structures have not been detected in isolated chromatin, a fact which argues against this model.

The possibility of interband-band transcription predicts that the interband region contains the recognition sites. These are called address sites and Paul has postulated that they contain non-histone proteins which are responsible for maintaining the chromatin in a less compact form suitable for initiation of transcription (112). Once initiation is accomplished, destabilisation of the band regions could occur to allow transcription to proceed. Thus, this model suggests that the difference between interband and band regions is due to the presence of activators bound to the address sites and that inter-conversion between the two is possible. This implies that address sites (and presumably RNA polymerase binding sites) can occur in both inter-band and band chromatin, unlike Crick's model, which assumes that inter-band regions would have few functional address or enzyme binding sites. The recent demonstration that euchromatin can support <u>in vitro</u> transcription by homologous RNA polymerase B and that the frequency of enzyme binding sites is similar in euchromatin and heterochromatin implies that Paul's model is the more plausible (113). However, it has some deficiencies. It would be unlikely that binding of one activator molecule could cause the extensive uncoiling often observed. Therefore, multiple address sites may be involved. Evolutionary divergence of some

of these sites and structural genes could result in a large amount of repeated DNA between an initiation site and the ancestral structural gene. Transcription of this DNA would explain the existence of HnRNA and the presence of repeated sequences at the 5' end of this RNA. The inclusion of a particular gene product in a given set of proteins could be explained by similar means to the Britten-Davidson model.

The generality of these models emphasises the lack of definitive information on the roles of the chromatin components in regulation of gene expression and further evidence will be required before any assessment of their validity can be made.

2. Processing of HnRNA to mRNA in eukaryotic systems

Studies on the processing of HnRNA to mRNA involve monitoring of the newly-synthesised RNA in its passage through the various intermediary stages. Two main approaches have been used, kinetic and structural analyses. The kinetic approach involves *in vivo* labelling of HnRNA with RNA precursors and following the appearance of radioactivity in the various precursor mRNA intermediates as a function of time. There are two possible ways to illustrate this: the first is continuous labelling, when radioactive precursor mRNAs appear in a sequence corresponding to that occurring in the processing mechanism; an alternative method involves pulse-chase experiments, where the radioactivity is followed after new RNA synthesis has been stopped by the action of actinomycin D or by dilution with excess cold nucleoside. Kinetic experiments on mRNA precursors have not been particularly successful due to practical limitations. Continuous labelling is of limited use since the HnRNA molecules have a complex size distribution and it is difficult to differentiate between nascent molecules and complete molecules which have been partly processed. The HnRNA is also rapidly obscured by a vast excess of labelled rRNA precursors; this problem can be alleviated in some systems by selective repression of rRNA synthesis using low doses of actinomycin D, but the effect of this treatment is only worked out for a small number of systems (114, 115). Problems with actinomycin also occur in pulse-chase experiments. Here the drug is used to stop all transcription prior to monitoring of label through the intermediates. Whilst transcription is effectively repressed, actinomycin D also appears to affect the actual processing

mechanism itself (116). The alternative method, dilution with excess cold nucleoside, produces results which are difficult to interpret since the presence of internal pools causes continued incorporation into RNA. Detection of the intermediates and final products is also difficult due to the short half-life of the HnRNA (117) and the fact that the final product (RNA of cytoplasmic size) represents only a very small fraction of the original transcripts (about 10%) (118 - 120, 117). A further limitation is the fact that use of labelled RNA precursors is an unspecific way to follow intermediates, since no differentiation between RNA sequences is possible. The structural approach is therefore more precise, since it involves monitoring of specific RNA sequences through the various precursor stages.

The presence of mRNA sequences in precursor molecules can be detected either by comparing alkaline hydrolysis 'fingerprints' of the precursors and products, by competitive hybridisation experiments, or by hybridisation with complementary DNA synthesised from a pure mRNA (cDNA probe). The technique of fingerprinting relies on the identification of common oligonucleotide fragments, a process which can be facilitated by the presence of suitable internal markers. The methyl groups of rRNA provide such markers (121).

Unfortunately, mRNA sequences contain no groups able to perform such a function. More serious drawbacks are the facts that mRNA sequences comprise only a small fraction of the precursor molecules (which means that the fingerprints of product and precursor would not necessarily correspond) and that it is difficult to obtain mRNA of high enough specific activity or in sufficient quantity for studies of this kind. The problems associated with competitive hybridisation methods and the use of cDNA probes have been discussed in section 1 but will be briefly summarised here. It is probable that all protein-coding genes (except histone genes) are present in the genome in small numbers (122 - 128). Thus, these experiments require either long hybridisation times (undesirable due to increased likelihood of nucleolytic degradation) or high nucleic acid input (quantities of which are not always available). The specificity of the technique is also reduced by the presence in both HnRNA and mRNA of repeated sequences, which represent only a small percentage of mRNA but a much larger percentage of the HnRNA. It is therefore possible that a given mRNA and HnRNA may appear to compete effectively but that this effect is in fact due to hybridisation of these

repeated, but not necessarily identical, sequences. cDNA probes hybridise more efficiently than total cellular DNA since they contain a higher percentage of the sequence under study, but nevertheless are still subject to the reservations above.

All methods used to study the processing of HnRNA assume the availability of a pure mRNA. Thus it is essential to isolate, purify and characterise specific mRNAs. Experience has shown, however, that this is not an easy task. Functional mRNA represents a minute fraction of the total cellular RNA (129) and, due to its tendency to be hydrolysed by RNAse (130), is very difficult to isolate intact. Purification from non-informational RNA is often a major problem, and has been made more difficult by the fact that until recently very little was known about the physical and chemical properties and base composition of any mRNA. Purification of mRNA also requires that a suitable assay method is available. Development of these methods has been a relatively recent occurrence.

These difficulties place a number of restrictions on the choice of a suitable system for the isolation and characterisation of a mRNA. These can be summarised as follows: the tissue source should be as homogeneous as possible with respect to cell type; both the properties and primary structure of the protein in question should be known, to facilitate the identification of a protein synthesised under the direction of a putative mRNA; the tissue should produce this protein in large amounts, relative to others, so that a large population of different mRNAs is not present to make purification of the desired one more difficult; the tissue should be available in large quantity.

The stringency of these restrictions has recently been reduced by the rapid increase in the sophistication of available methodology for isolating and characterising mRNA. Advances in isolation and purification have included the introduction of powerful RNAse inhibitors e.g. diethylpyrocarbonate and heparin, the use of zonal rotors, the immunoprecipitation of specific polysomes and the isolation of poly A containing mRNAs by affinity chromatography. Zonal rotors have been used for the large scale preparation of mRNA whose size enables a ready separation from contaminant rRNA and tRNA (e.g. 9S globin mRNA and 14S lens crystallin mRNA (131, 132)). Specific polysomes have been precipitated from a heterogeneous population by utilising the reaction between the antibody to the particular protein under study and the nascent chains forming on the polysomes synthesising that protein. This has enabled a considerable enrichment

of mRNA over that obtained from total polysomes (e.g. ovalbumin mRNA (133 - 135)). Probably the most significant advance has been the discovery of a stretch of poly A at the 3' end of many mammalian mRNAs (136 - 140). This has enabled their purification from rRNA and tRNA (which do not contain poly A) by hybridising this sequence to the complementary nucleotide sequences (poly U or poly dT) which have been rendered immobile by attachment to fibreglass (136, 141), cellulose nitrate filters, sepharose (138) or cellulose (142 - 144). This method is extremely useful, since it permits purification of mRNAs whose size makes it difficult to separate them from rRNA (e.g. immunoglobulin mRNAs).

The development of systems for the assay of a putative mRNA has had a somewhat chequered history. The first system tried was prepared from E. coli but this proved unsuccessful since bacterial tRNA cannot form an initiation complex with bacterial ribosomes and eukaryotic mRNA. It was therefore necessary to turn to eukaryotic systems. The development of these systems has now reached the point where a wide variety is available. These systems are divided into two groups. The first consists of cell-free systems such as those from rabbit reticulocytes (134, 145 - 149), Krebs ascites cells (131, 150 - 152, 144) and wheat germ cells (153, 154), all of which can translate exogenous mRNA. The second group contains in vivo systems such as X. laevis oocytes and eggs (153 - 160), where exogenous mRNA is introduced by microinjection. The latter systems are very useful, since they largely circumvent objections raised against cell-free systems on the grounds of their unphysiological nature (due to lack of definition of ionic conditions, elimination of compartmentalisation etc.). Since the protein synthesising apparatus of the oocyte remains intact, it is not surprising that translation of added mRNA is much more efficient than in the cell-free systems. It should be noted, however, that practical difficulties in handling oocytes must be taken into account.

The variety of available systems has several practical advantages. Since it is necessary that the translation apparatus does not normally synthesise the protein to be monitored (in order to eliminate the possibility of endogenous mRNA activation and enable the separation of the product from endogenous proteins), certain systems may sometimes be more suitable than others. It is also known that certain mRNAs compete badly with endogenous messengers for the ribosomes of the reticulocyte lysate,

resulting in a high background and low observed incorporation into the desired protein (161). This makes subsequent product analysis difficult. When this occurs e.g. with duck globin mRNA, the wheat germ or ascites systems, with their very low levels of endogenous protein synthesis, are often used. Another advantage of these low backgrounds is that they enable TCA-precipitable radioactivity to be used as a measure of mRNA activity, providing it is first shown that the observed stimulation of radioactive incorporation into proteins generally is paralleled by a quantitatively similar increase into the protein concerned.

In these circumstances, it is hardly surprising that mRNAs for globin (162, 163), ovalbumin (134), myosin (164, 165), crystallins (131, 166), immunoglobulins (167, 168), histones (148, 152), fibroin (169), α casein (170, 171) and many other proteins have now been reported in the literature. The availability of pure mRNAs has allowed rapid progress in the study of mRNA production.

2.1. Mechanism of HnRNA processing and transport

One of the characteristics proposed for mRNA is that it should be the most rapidly synthesised of all RNA classes. Examination of rapidly labelled nuclear RNA shows that it is often present as giant molecules of heterogeneous size (HnRNA). The size of these molecules is much larger than necessary to specify the common proteins studied. However, by analogy with the large precursor rRNA molecules, this is less unusual than was at first thought. For reasons stated in section 2, kinetic studies on these molecules have not been very successful, though they have been used to divide duck erythroblast HnRNA into three broad classes on the basis of size and stability (161). Since no compelling kinetic evidence exists for the processing of HnRNA to mRNA it becomes very important to structurally relate the two.

Despite the limitations of methods for structural comparisons discussed in section 2, much work has been reported in this area. The presence of globin mRNA sequences in duck erythrocyte HnRNA has been demonstrated by the fact that this HnRNA stimulates globin synthesis in a Krebs ascites cell-free system (172). The possibility that mRNA could have aggregated with the HnRNA during its isolation was excluded by gradient centrifugation of HnRNA under denaturing conditions prior to assay. This causes dissociation of contaminant mRNA and its removal

on the gradient. Similar conclusions have been reached using cDNA probes. Duck erythroblast HnRNA bound cDNA for globin, confirming the presence of globin mRNA sequences, subject to the denaturing conditions described above (173). Competitive hybridisation experiments have shown that polysomal and HnRNA compete for the same sites on cellular DNA (174). In addition to these results, inferential evidence for a precursor-product relationship can be derived from the presence of a long stretch of poly A at the 3' end of HnRNA and mRNA in several systems (not histone specifying RNA) (175 - 177).

Thus it appears that HnRNA may be the informational precursor to mRNA, although the point is at the moment controversial. The argument centres around the possibility that high molecular weight RNA is an aggregation artifact. When total RNA from hormone stimulated oviduct is denatured by heat treatment (65^{o}) in SDS buffer, no large messenger precursors were detected using a cDNA probe of sufficient sensitivity to detect one molecule per cell. All the ovalbumin mRNA sequences were present as molecules of sedimentation coefficient 18S (178). Similar experiments on duck erythroblasts localised globin mRNA sequences in 14S RNA molecules (179) in contrast to other experiments mentioned above (161). (It has been suggested that high molecular weight RNA from erythroblasts is unstable and is rapidly processed to smaller molecules, which might resolve the apparent difference). On the other hand, HeLa and rat ascites cells contained high molecular weight RNA which was not extensively disaggregated on dimethyl sulphoxide gradients (177, 180). This implies that the integrity of these molecules is preserved. One limitation with these experiments lies in differentiating between high molecular weight RNA which is the result of aggregation and that which dissociates on denaturation because it has been partially processed whilst remaining intact in a three-dimensional sense due to base pairing. Controls to eliminate these possibilities have shown that both bona fide high molecular weight RNA and partly processed RNA occurred in HeLa cells. It should be noted, however, that in the experiments on HeLa cells, poly A-containing HnRNA has been selected and no attempt to locate a specific sequence was made . . . Work with the ovalbumin system was done by selecting a specific mRNA sequence from total RNA. This problem has partly been resolved by analysing HeLa cell RNA under similar conditions to oviduct RNA, when a certain amount of decrease in size was observed, though not to less than 40S (178).

Unless the absence of high molecular weight RNA in the oviduct is due to very rapid processing to the size observed (c.f. erythroblast RNA), it must be concluded that the post-transcriptional shortening of high molecular weight RNA is not proven to be a general phenomenon. Different systems may well have different methods of producing mRNA.

However, in those systems where HnRNA appears to be real, a certain amount of progress has been made towards understanding the arrangement of mRNA sequence(s) and the nature of the non-coding regions. In order to carry out work of this kind, markers for both the mRNA and the non-coding regions must be found.

The messenger sequence acts as its own marker, since it can be defined by its hybridisation rate with DNA. This property has enabled the location of mRNA sequences at the 3' end of HnRNA. Starting at the 3' end, progressively longer stretches of HnRNA were hybridised quantitatively to DNA. Up to a length of 3,000 - 4,000 nucleotides, the 3' fragments hybridised at a rate equal to that of mRNA. After this, the rate increased until it was characteristic of HnRNA at a length of 8,000 nucleotides, whereafter the rate remained constant (181). Since mRNA has a length of approximately 3,000 nucleotides, it would appear to be localised near the 3' poly A stretch. These results also imply that there is only one mRNA sequence per HnRNA molecule, a conclusion supported by evidence from duck erythroblasts (173).

Alternatively, the messenger sequence can be defined by competitive hybridisation with mRNA isolated from the tissue in question. When oligonucleotide fragments from the 5' and 3' ends of HnRNA were tested in this way, it was found that the 3' fragments hybridised very slowly and could virtually be excluded from the DNA by mRNA. The 5' sequences, on the other hand, hybridised much more rapidly but mRNA did not compete. This implies that the mRNA sequence is located at the 3' end and the non-coding, more highly reiterated sequences at the 5' end (182).

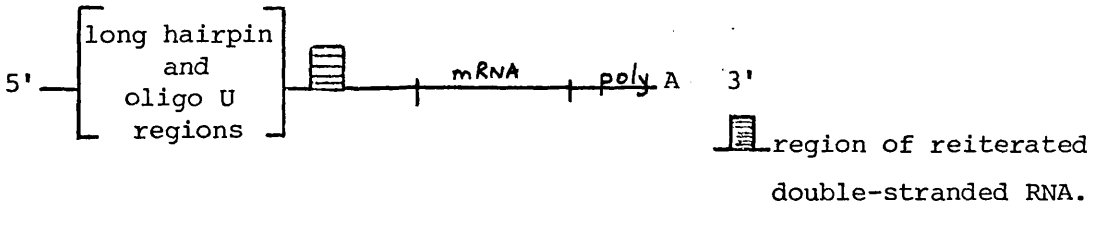
The non-coding regions are characterised by oligo A (183) and oligo U fragments (184, 185), double-stranded sequences (186 - 188) and highly-reiterated sequences (187). Study of these regions is still in its infancy, but certain facts have emerged. Oligo A regions have been shown to occur internally in poly A HnRNA. It was found that actinomycin D prevented the synthesis of oligo A but had no effect on the terminal polyadenylation of previously synthesised RNA, which implies

that these oligo A fragments are transcribed in the normal way (183). Oligo U regions have been shown to be present in high molecular weight poly A RNA molecules. As these molecules became shorter, some of these regions disappeared and were not found at all in mRNA. Thus, it appears that they are confined to the 5' end of the molecule (181). The double-stranded RNA regions can be divided into two classes according to size, namely 'long' and 'short' hairpins. The 'long' hairpins comprise about 1% of heavy (>30S) HnRNA and are absent from lighter (10-30S) HnRNA. The 'short' hairpins comprise about 2% of HnRNA and are present in all HnRNA. The function of these fragments is not well understood (189). If these double-stranded regions were melted, they hybridised with DNA at low Cot values, which implies that these sequences are transcribed from reiterated DNA. This conclusion is supported by studies on double-stranded DNA. These involve isolation of sheared DNA which has been melted and reannealed for very short times, such that only intra-strand renaturation takes place. If DNA hairpins prepared in this way were melted, immobilised and hybridised with melted 'long' RNA hairpins, then DNA/RNA hybrids were formed at the same rate as RNA/RNA renaturation. This implies that this DNA is the template for the double-stranded RNA (187). One interesting property of these RNA sequences is the capability of the denatured 'long' hairpins to hybridise with undenatured HnRNA or mRNA. Since the hairpins in both RNAs were still intact, the observed hybridisation must have occurred with single-stranded regions which were complementary to some hairpins. Since sequences complementary to 'long' hairpins appear to be present in HnRNA and mRNA to the same degree, it is tempting to suggest that they occur between the mRNA and the non-coding sequences and may play a role in processing (190). The experiments designed to map the non-coding region, (181), mentioned above, are relevant in this context. These imply that reiterated sequences occur in the middle of HnRNA. Since the rate of hybridisation did not increase when 3' fragments of length greater than 8,000 nucleotides were used, this suggests that reiterated sequences are interspersed with less rapidly hybridising sequences in regions of the HnRNA molecules further than 8,000 nucleotides from the 3' end.

At this point, it should be noted that the foregoing results have either been obtained with poly A-containing HnRNA or total HnRNA. Thus, it is a little difficult to make direct comparisons between experiments

since the starting material is different. In addition, many important aspects of the processing mechanism have not been examined because of technical difficulties. For example, no results presented here have proven that the mRNA(s) are produced by progressive degradation of HnRNA molecules i.e. HnRNA is a direct physical precursor to mRNA. Thus, that portion of the process between formation of the small HnRNA and appearance of the mRNA in the cytoplasm remains obscure. Further, the proportion of HnRNA molecules which contain mRNA sequences and the number of such sequences per HnRNA molecule is unknown, although some evidence has been presented that only one messenger sequence is present (173). Finally, the nature of the non-poly A HnRNA molecules has yet to be clarified. Further experimentation will be necessary to determine whether these molecules are not yet polyadenylated, are excess RNA left over after excission of the mRNA(s), are mRNA-containing species which are never polyadenylated, or are non-mRNA-containing species which are never polyadenylated.

Notwithstanding these deficiencies in our knowledge, several tentative structures have been proposed for HnRNA, the general features of which are summarised below:



(The oligo A sequence has not been included in this diagram, as its position in the molecule has yet to be determined)

Schemes for the processing mechanism have also been formulated. Due to the absence of concrete data, these schemes vary in detail, although they all have several points in common. All assume that the HnRNA associates with proteins on release from the chromatin and that these RNA/protein complexes are gradually reduced in size by progressive degradation at the 5' end of the HnRNA. At some point during this process, a poly A tract is added to the 3' ends of certain HnRNA molecules. Finally, RNA molecules of cytoplasmic size are transported across the nuclear membrane. Thus, having arrived at a

somewhat generalised view of the structure and processing of HnRNA, it is possible to examine putative control points. However, since extremely little is known about the mechanisms of HnRNA transport, any attempts to define control of this process must be purely hypothetical.

2.2. Control of HnRNA processing and transport

Although the processing and transport mechanisms are still far from clear, many attempts have been made to define possible control points i.e. post-transcriptional control. One possibility is the association of HnRNA and mRNA with proteins. It is known that these RNA molecules, like rRNA, are found in the cell as ribonucleoprotein (RNP) particles (191 - 193). A heterogeneous collection of nuclear RNP particles has been isolated according to size from rat liver or Ehrlich ascites cells. On treatment with RNAse, these RNP particles were converted to a single 30S species containing RNA fragments and a 30S protein moiety. This suggests that the protein moieties are bound to the RNA in an analagous manner to the binding of ribosomes in polysomes (194 - 196). That this RNA is genuine HnRNA was demonstrated by hybridisation to DNA in competition with HnRNA and mRNA from the tissue concerned (197, 198). The 30S protein moiety has been called informofer and is an aggregation product of one protein called informatin. RNP particles of similar size have been isolated from several other systems (199, 200). When nuclear RNP particles were radioactively labelled for different times, it was shown that their size decreased as a function of time, as would be expected if non-coding RNA were being removed (197). However, these results shed no light on the distribution of informofer proteins along the HnRNA molecules. Thus, some attempts have been made to implicate the mRNA sequences in HnRNA-informofer interactions. RNA isolated from 30S RNPs completely competed with HnRNA or mRNA, whilst mRNA only partially competed with RNA from 30S particles. These results suggest that RNP-derived RNA contains both coding and non-coding sequences (197, 198). However, in all these experiments, low Cot values were used, so that the hybridisation measured concerned reiterated sequences. It is therefore possible that only these sequences are associated with informofer. Experiments where individual mRNA sequences are monitored would be more easily interpreted and the use of cDNA probes may also prove useful.

From the results presented so far, Samarina et al. (201) have implied that HnRNA, on being synthesised, associates with informofers and remains in that state during nuclear processing. They regard the function of the informofers as two-fold, prevention of newly formed HnRNA from binding to histones in chromatin and induction of that conformation of HnRNA to allow the correct processing sites to become available for nucleolytic attack. It is also possible that the induced conformation simultaneously decreases the likelihood of unspecific degradation. Putative processing enzymes, such as a specific RNAse and a poly A synthetase, have been demonstrated in association with 30S particles, which appears to lend support to this theory (202, 203).

If these suggested functions are correct, it is unlikely that the informofers would be required in the cytoplasm. Thus, one would expect a different protein spectrum from cytoplasmic mRNP than from nucleoplasmic HnRNP. This postulation has been investigated with globin RNPs (161). SDS gel electrophoresis of proteins from HnRNP, free cytoplasmic RNP and polysome-bound RNP from duck erythroblasts showed that the proteins were indeed different. Thus, informofer protein would appear to be confined to the nucleus. However, any experiment of this type suffers from the disadvantage that mRNA sequences represent only a small percentage of the total HnRNA and thus messenger-associated proteins would be present in small quantities relative to informofer. The possibility cannot be excluded that electrophoresis is not sensitive enough to detect these minority molecules and that in fact mRNA sequences in HnRNA are associated with non-informofer proteins, which may or may not be changed on transfer to the cytoplasm.

At this point, it should be noted that whilst the evidence presented is consistent with informofer involvement in HnRNA processing, it does not differentiate between mechanistic or regulatory involvement. In order to qualify as putative regulatory agents, any class of molecules should be sufficiently heterogeneous to possess the flexibility required for regulation. There should also be specificity in its mode of action.

Reconstitution experiments have shown that informofers preferentially bind to HnRNA rather than to rRNA (204, 205). Thus a degree of specificity in the interaction of informofer with RNA can be demonstrated. The question of compositional heterogeneity is somewhat unclear. Georgiev's group have shown that informofer consists of only one protein, although some evidence has been presented that it is heterogeneous (206, 207). The

point is therefore undecided. If, however, only one species is confirmed, then the informofer-RNA interaction is more likely to be mechanistic and specificity must be a function of some other molecule. There are several possible ways in which this could occur.

The HnRNA could affect informofer binding if it was different for different HnRNA molecules, either by virtue of a totally different primary sequence or by different arrangement of those sequences which appear to be common to many HnRNA molecules. This preferential binding would confer additional resistance to degradation on certain HnRNAs. If this were true, then some molecules would be inherently more stable and would be processed automatically, while others would be degraded. This would provide a means for differentiating between one HnRNA and another i.e. the DNA carries the information necessary to determine the stability of the HnRNA/informofer interaction.

The other possibility is that the HnRNA sequence specifically interacts with some other molecule (promoter) which in turn promotes the correct conformation for informofer binding. In order to avoid the activation of the wrong mRNA, it is necessary that the HnRNA/promoter interaction should be specific, the most obvious place for the interaction being the mRNA sequence itself. Thus, the amount of HnRNA preserved would depend on the concentration of the promoter. This implies that the cell always transcribes more of one mRNA than is necessary and simply preserves a variable amount under different physiological conditions. This is one of the prerequisites for post-transcriptional control. It would therefore be of interest to find out whether an HnRNA molecule containing a specific mRNA sequence is detectable when that mRNA is not present in the cytoplasm. In this context, studies on HnRNA utilising cDNA probes are of great interest. These probes can be used to detect the presence in HnRNA of small quantities of the mRNA sequence from which they were prepared. Thus, information on the presence or absence of a specific HnRNA can be obtained by hybridisation of a specific cDNA with HnRNA isolated from a cell not containing the mRNA under study. Experiments of this type have been carried out with unstimulated chick oviduct cells in which the level of ovalbumin sequences was virtually undetectable in both nuclear and polysomal RNA fractions i.e. post-transcriptional control was not operating to a significant degree (208). A somewhat different situation occurs in Friend cells, which can be induced to synthesise globin by dimethyl sulphoxide. HnRNA isolated from these cells before and after induction with DMSO was shown to contain similar amounts of globin mRNA

sequences (209). Thus, precursor to mRNA was clearly synthesised at all times, implying that post-transcriptional control is operative. Further work with this system has shown that the level of globin-specific HnRNA after induction was higher than was at first thought (210). Thus, not only is more HnRNA preserved for processing but more specific HnRNA may also be transcribed i.e. both transcriptional and post-transcriptional control are operative.

However, it should be noted that measurement of pool sizes of specific HnRNAs under various conditions is not enough to make definitive conclusions about the existence of post-transcriptional control, since pool sizes are dependent on the balance between synthesis and degradation. Enhanced degradation in non-stimulated cells may make the pool size of mRNA-containing HnRNA too small for efficient detection. Increases in both transcription and processing could result in no net change in pool sizes between stimulated and non-stimulated HnRNA, but a large increase in cytoplasmic levels of mRNA. Thus, other criteria must be applied for studies of this sort. This has been attempted for 3T6 cells (211). These cells contain more poly A-containing mRNA in the growing than in the resting stage. Since the stimulation into growth phase causes no change either in the total content of nuclear poly A or in the proportion of HnRNA that is polyadenylated, it appears that neither increased transcription, or increased polyadenylation is responsible for the observed increase. Differential stability of mRNAs has also been eliminated. This implies that the extra mRNA is produced by an increase in the preservation of nuclear poly A-containing HnRNA. This conclusion is supported by comparison of the steady state ratio of cytoplasmic and nuclear poly A during the transition to growing phase, which shows that the value characteristic of resting cells increases gradually until that characteristic of growing cells is reached. However, it remains to be determined whether the observed difference in HnRNA preservation is merely quantitative, or whether it exhibits any specificity. One limitation of this experiment is its reliance on poly A to quantitate the amounts of the various fractions of RNA. Thus, no information can be obtained on non-poly A containing molecules.

The general conclusion on post-transcriptional control is that it has yet to be definitively proven or disproven. The different results obtained with different systems may be due to experimental limitations, or indeed may be relevant. There is no reason to suppose that all cells

regulate their mRNA production in exactly the same way, or that different mRNAs in the same cell are subject to the same control mechanisms. A wide spectrum of combinations of transcriptional and post-transcriptional control may well occur. Further work will be necessary before the universality of these control mechanisms and the balance between them can be evaluated.

Unfortunately, since very little is known about the transport of mRNA from the nucleus to the cytoplasm, possible control mechanisms are at the moment purely hypothetical. The poly A sequence has been implicated in control of processing and/or transport, since inhibition of poly A synthesis with cordycepin prevents the appearance of mRNA in cytoplasmic polysomes. The involvement of proteins in post-transcriptional control has already been mentioned and recent studies on poly A/protein complexes are important in this context. Poly A from rat liver HnRNA has been shown to occur in association with a protein moiety which causes the complex to sediment at 10-30S. The fact that no poly A could be detected in the 30S particles implies that this protein is not informofer (201). It has also been shown that poly A from several mRNA molecules exists in association with protein and that the resultant poly A/protein complex has similar sedimentation properties to the nuclear complex (212, 213). It has yet to be demonstrated that this protein is the same in both nuclear and cytoplasmic poly A/protein particles. If the same protein accompanies the poly A during its transport to the cytoplasm, then this protein may play some regulatory role. As before, it is necessary to distinguish between mechanistic and regulatory function. However, if the same protein is associated with poly A regions of different mRNAs then its significance as a putative regulatory molecule decreases and the specificity must reside in some other molecule. It may be that particular poly A-associated proteins determine differential transport rates for different mRNAs. As yet there is no evidence for specific poly A-associated proteins and it may be that some function of the poly A region itself i.e. timing of the addition or length, which together with the protein, results in selective transport. Therefore, it would be necessary to correlate the different polyadenylation rates of HnRNAs in the same system with the transport rates of the resulting mRNA molecules. The hypothesis that addition of poly A is sufficient

to ensure conservation and/or transport of HnRNA requires that this sequence is added in the nucleus and transported together with its mRNA to the cytoplasm. This idea has recently been questioned, since kinetic experiments designed to quantitate the transport of poly A from the nucleus to the cytoplasm have indicated that some of the poly A turns over in the nucleus (214, 215). Thus, the role of poly A (or its associated protein) in selective preservation or transport has yet to be demonstrated.

Although a vast body of information on a wide variety of organisms exists, no clear picture has emerged of the control processes involved at the post-transcriptional level. The biggest obstacle to understanding these processes is the lack of purity of specific HnRNA, HnRNP and mRNA. This is due to the instability and small quantities of these complexes, which make them extremely difficult to isolate free from contaminants and artifacts. However, purification of these particles will be necessary in order to monitor specific molecules (proteins or RNA) with any degree of certainty and also to compare information from different systems as to the relative importance of the various levels of control which operate under different physiological conditions. Although the poly A tract has been invaluable in the isolation of certain mRNAs, and HnRNAs, it cannot be used for the purification of specific mRNAs, nor for the isolation of non-poly A containing RNA molecules. Several mRNAs have been isolated which lack poly A (216, 217) and, indeed, there may be HnRNAs which either temporarily or permanently lack this sequence.

Recently, viral-specific RNA has been isolated free from host RNA by hybridisation to viral DNA followed by purification of double-stranded nucleic acids from unreacted single strands (host RNA) by hydroxyapatite chromatography (218).

In specific instances, it has been possible to synthesise cDNA from purified mRNA and hybridisation of this cDNA with HnRNA may make it possible to analyse the various intermediates by isolation of the hybrids. A development of this sort may enable many of the outstanding questions on HnRNA processing and transport to be answered.

3. Translation of RNA in eukaryotic systems

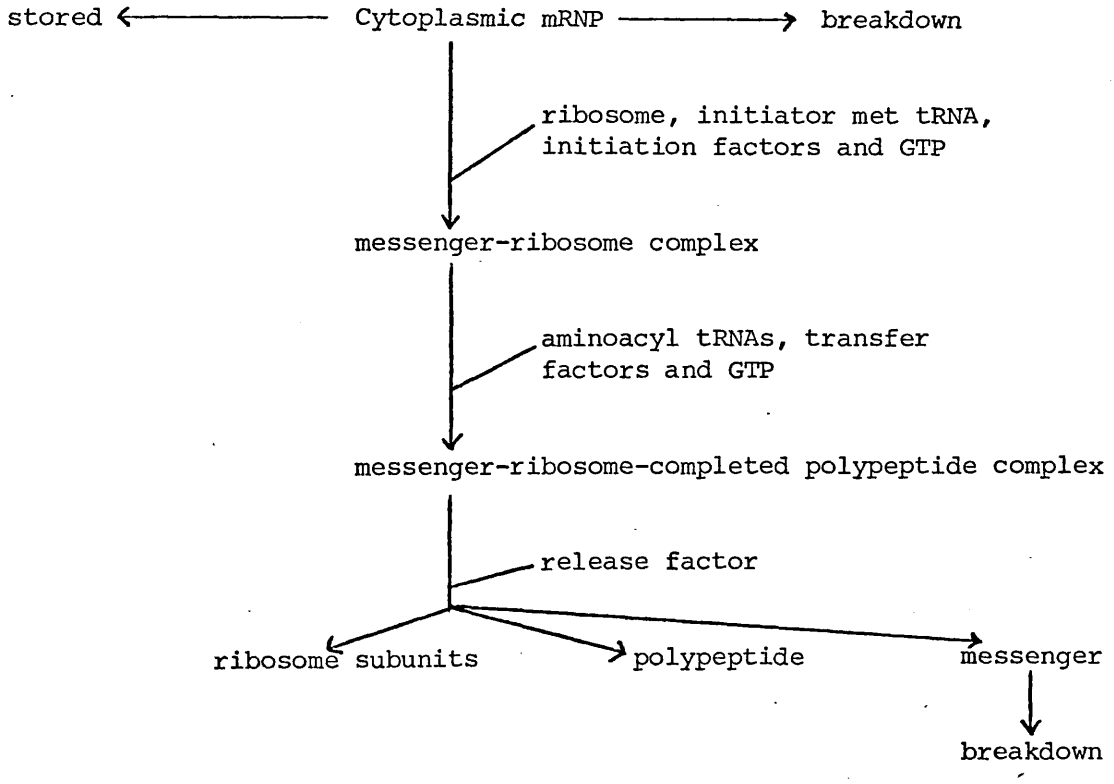
3.1. Cytoplasmic distribution of mRNA

Once the mRNA molecule reaches the cytoplasm, it may interact with ribosomes to initiate protein synthesis, or interact with some molecule

(or molecules) which causes the mRNA to be stored in an inactive form, stable to degradation, or may be degraded by nuclease action.

3.1.1. Uptake into polysomes

Studies on the mechanism of eukaryote protein synthesis have shown that, unlike mRNA production, there are great similarities to the bacterial mechanism. The fine points of the process have yet to be elucidated, but the bacterial (219 - 221) and mammalian (219) processes have been reviewed. Now that pure messengers and a wide variety of cell-free systems are available, the reconstitution of the various steps involved should prove fairly rapid. A brief summary is presented below.



3.1.2. Uptake into inactive complexes

The concept of stored mRNA is based on the fact that fertilisation of sea urchin eggs causes a burst of protein synthetic activity without concommitant RNA synthesis (222 - 224). This implies that mRNA existing prior to fertilisation is utilised. The pre-existence of mRNA has been confirmed by showing that RNA prepared from the post-ribosomal supernatant of unfertilised eggs stimulated cell-free synthesis of histones (225). Further proof has come from the demonstration by competitive

hybridisation of histone mRNA sequences in RNA from the post-ribosomal supernatant of unfertilised eggs and embryonic polysomal RNA (226). Having shown that unexpressed mRNA is stored in the unfertilised egg, the nature of the factor(s) responsible for storage remain to be defined. By centrifugation of post-ribosomal supernatant from unfertilised eggs and localisation of the fraction containing the histone mRNA sequence, it has been shown that this fraction contained a 20S RNP particle (226). Thus, it appears that protein is reponsible for the inactivity of the mRNA, since translation in the cell-free system proved that the mRNA itself was not defective (assuming that subtle control mechanisms present in the mRNA are not over-ridden in the cell-free system). The proteins and the precise mechanism for unmasking the mRNA at fertilisation have yet to be characterised. Other post-microsomal RNP particles have been isolated which may have similar storage functions (227 - 229), and stored mRNA has been implied in dry wheat embryos (230).

3.2. Control of cytoplasmic distribution of mRNA

It is generally assumed that unspecific degradative machinery exists in the cell and that a given mRNA will be degraded unless it is protected by translation or storage. Thus, the selective preservation of mRNAs depends on differential utilisation or storage. This in turn would predict the presence of mRNAs with different half-lives. Experiments designed to test this hypothesis utilise one of two approaches. The first follows the kinetics of labelling of mRNA in the approach to steady state, mRNA being defined by chemical (size, poly A content) or biological (cytoplasmic location, RNA synthesis at times of low rRNA synthesis) criteria (231 - 234). The second studies the decay of functional mRNA after further transcription has been stopped by actinomycin D (235, 236). This is accomplished by following decreases in either polysome quantity or protein synthetic ability or labelled polydisperse RNA.

Each of these approaches has disadvantages. The kinetic method requires that the specific activity of the precursor remains constant and that reincorporation of these precursors after degradation does not occur. Conclusions from experiments of this type are also limited by the criterion used e.g. that all mRNAs contain poly A stretches etc. Thus, it is advisable to repeat kinetic experiments using several different criteria to avoid problems of this type. On the other hand,

the actinomycin D approach suffers from the disadvantages of drug-induced side effects, such as inhibition of the initiation of protein synthesis or induction of changes in amino acid precursor pools (237 - 243). Another disadvantage is the assumption that there is a direct proportionality between mRNA concentration and translational productivity.

The major limitation of both these approaches is the inability to distinguish between the degradation of specific and total cellular mRNAs. Early studies of this type were restricted to bulk mRNA, since the methodology for isolation and characterisation of specific messengers had not been developed. The study of bulk mRNA half-life has the obvious disadvantage that a large number of different mRNA species with a continuum of half-lives may appear as one broad band, implying that an individual mRNA is present. Hopefully, the new methodology for mRNA purification will enable more rigorous studies to be performed. Preliminary work suggests that mRNAs from highly differentiated systems are very stable (244), which supports earlier studies indicating that differentiation-specific protein synthesis survives enucleation (245). Thus, a substantial diversity of half-lives exists, ranging from the relatively unstable histone mRNAs (246 - 248) to the extremely stable ovalbumin and globin mRNAs (249, 250). This diversity in turn implies that translation or storage of mRNAs is somehow controlled in a selective manner.

3.2.1. Uptake into polysomes

mRNA distribution could be regulated by the various components in the complicated mechanism of protein synthesis.

3.2.1.1. Initiation of protein synthesis

The differential translation of mRNAs may be effected by the availability and specificity of initiation factors. This assumes that different mRNA-initiation factor complexes possess different efficiencies with respect to initiation of protein synthesis. Evidence for or against this idea was slow to appear due to the lack of suitable cell free systems in which to test the effect of initiation factors on *in vitro* utilisation of heterologous messengers as well as the lack of suitable messengers to test. However, the relatively recent preparation of several cell-free systems and pure mRNAs has enabled considerable evidence

of a rather contradictory nature to accumulate. The translation of myosin mRNA in a reticulocyte cell-free system was stimulated by initiation factors from muscle, while corresponding factors from reticulocytes had no effect (251). The reverse was true for globin mRNA. This has been extended to the fractionation of muscle factors into two activities, one specifically stimulating the synthesis of myosin, the other of myoglobin (252). Also, EMC viral RNA and duck globin RNA were poorly translated in the rabbit reticulocyte system, possibly due to the absence of the correct initiation factors (253, 254, 161). These results imply both tissue and species specificity of initiation factors.

However, the fact that many mRNAs have now been successfully translated in reticulocyte, wheat germ and Krebs ascites lysates would appear to argue against a significant amount of specificity. It has been suggested that the ascites system, being undifferentiated, may not have specific control mechanisms and that subtle control processes may be over-ridden in _in vitro_ systems, which by their very nature are unphysiological. Thus, considerable interest has focussed upon the _X. laevis_ oocyte, which, in spite of being another undifferentiated system, nevertheless represents the only system capable of translating exogenous mRNA _in vivo_. The efficient translation of the mRNAs for globin (161, 162), α-crystallin (330), promellitin (331), and also of frog tadpole RNA (255) makes the idea of stringent specificity unlikely. However, it is possible that muscle cell factors represent an extreme case and that many other systems have initiation factors with a broader spectrum of efficiencies. This would theoretically enable cells to translate most mRNAs to a limited extent, whilst retaining a distinct preference for endogenous mRNA. Another alternative is that really specific factors exist, but that there are also unspecific factors which permit limited translation of heterologous messengers.

While initiation factors influence the selection of messengers in a positive way, it is also possible to envisage the existence of negative factors. Such factors have been isolated from erythroblast and chick muscle initiation factor 3 preparations and shown to be RNA (called tcRNA). Erythroblast and muscle tcRNA inhibited muscle protein and globin synthesis respectively, but had no effect on the translation of homologous mRNAs. The erythroblast tcRNA could also block the stimulatory effect of muscle initiation factor 3 (252).

The results above imply a level of specificity, but to what degree is at present unknown.

3.2.1.2. Elongation and/or termination of protein synthesis

Information on elongation is rather more sparse than on initiation. However, it has been shown that neither elongation nor termination rate is responsible for the differential translation of α and β globin mRNAs (256). This was accomplished by direct measurement of translation time and termination time. For the purposes of the analysis, α and β globin chains were analysed by studying the αT4, αT5, αT6, βT4, βT5, αT15 and βT15 peptides. These results suggest that elongation is not a rate-controlling step.

Several attempts have been made to explain the activation of maternal mRNA during embryogenesis in terms of an increase in the translational efficiency of the ribosomes. The results of experiments designed to investigate this hypothesis have been contradictory. Although a cell-free system isolated from fertilised sea urchin eggs synthesised endogenous proteins more efficiently than that isolated from unfertilised eggs, both showed equal efficiency in translating poly U (257). However, other experiments have suggested that the two types of ribosomes were indeed different, since high salt extracts of ribosomes from unfertilised and fertilised eggs exhibited different electrophoretic protein patterns. The salt extracts from unfertilised eggs contained an inhibitor, probably protein, which inhibited protein synthesis in a cell-free system prepared from gastrulae and which disappeared after fertilisation. It has therefore been suggested that this inhibitor is the factor responsible for the decreased protein synthetic activity of unfertilised egg ribosomes. The inhibitor is thought to interfere with elongation, since it prevented the disaggregation normally observed on incubation of reticulocyte lysate at 37°. However, it should be noted that these experiments do not rigorously exclude the possibility that initiation is the process involved (258).

It is also possible to envisage a situation where the rate of elongation is not simply decreased, but prevented altogether. This would result in the presence of inactive complexes of mRNA and ribosomes. Such complexes have been inferred from studies on sea urchin eggs. Ribosomal aggregates (> 80S), resistant to RNAse action, were isolated from unfertilised eggs. These results imply that aggregates

from unfertilised eggs contain a component which prevents RNAse action by masking the mRNA. This conclusion is supported by demonstration that treatment of ribosomal aggregates with trypsin sensitised them to RNAse attack (259). Similar results have been found for Ascaris eggs (260) and cultured chick embryo cells (261).

The availability of specific tRNA molecules or their respective tRNA synthetases could also regulate the rate of protein synthesis. Crude enzyme preparations from sea urchin embryos at different stages of differentiation have been used to charge yeast tRNA with radio-active amino acids. MAK column chromatography of these charged tRNA populations showed that the distribution obtained altered in both a qualitative and quantitative manner. The quantitative aspect was further shown using specific charging of tRNA with labelled valine. These results imply that the activity of aminoacyl tRNA synthetases alters in a selective manner during embryogenesis (262).

3.2.2. Uptake into inactive complexes

The 'masked' messenger theory mentioned above has also been illustrated in wheat seeds (263). It is possible that the presence of mRNP in the post ribosomal supernatant could be a common phenomenon. For example, globin mRNA has been found as an RNP particle in post-ribosomal supernatant of reticulocytes (227 - 229). However, the time period in which the messenger is not being translated may well vary, since there appears to be no pool of untranslated ovalbumin mRNA (264). Once the validity of stored mRNA is accepted, the question arises of how the stored messenger is reclaimed. If it is the association with protein which results in masking, then replacement of these proteins by others may induce a change which allows ribosome binding to occur (polysome-bound mRNA is thought to occur as an RNP particle). This theory can be tested by comparing the proteins of free and polysome-bound RNPs. Electrophoretic analysis of these proteins has been reported for duck erythroblasts (161). These results imply that the proteins are indeed different.

The presence of free mRNP does not necessarily suggest that a storage mechanism is operative, but may simply be the result of an equilibrium distribution between polysomes and the post-ribosomal supernatant due to lack of appropriate initiation factors. Differential initiation has been suggested for the excess of α globin

mRNP found in the post-ribosomal supernatant or rabbit reticulocytes (265). Whether or not the protein moiety of this RNP particle is of polysomal or cytoplasmic nature has yet to be determined.

4. Spermatogenesis as a suitable system for the study of protamine biosynthesis

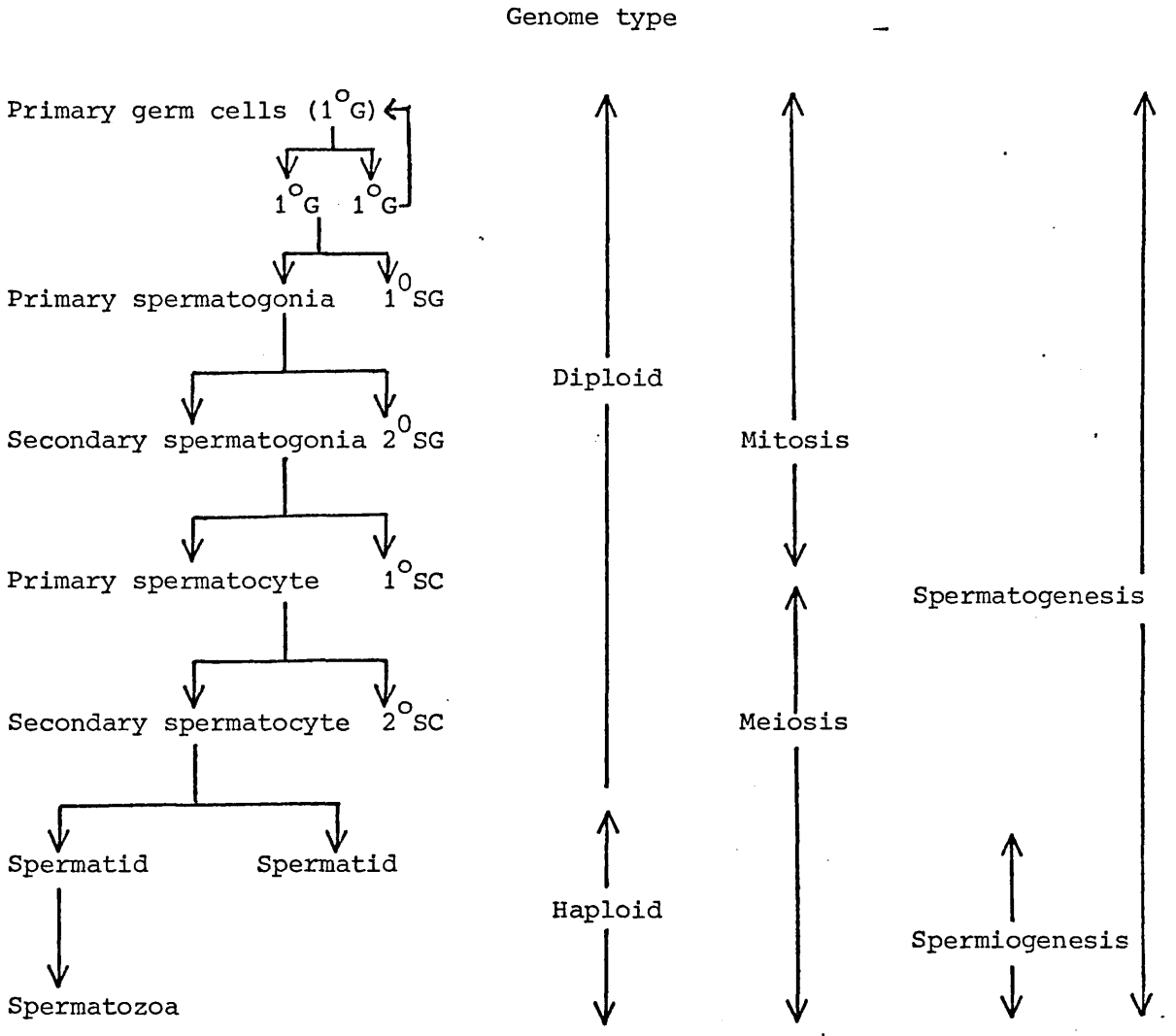
The maturation of diploid germ cells into haploid spermatozoa is called spermatogenesis. It is a complex process involving several different stages and has been the subject of much study. The precise sequence of events has been elucidated by histological and autoradiographical examination of testis development in humans (266), salmon (267 - 270) and herring (271) and by velocity sedimentation analysis of thymidine-labelled testis cells of varying maturity (272). A schematic representation is shown in Figure 4.1. As can be seen from the diagram, primary germ cells undergo a series of mitotic divisions, which culminate in the formation of primary spermatocytes; each primary spermatocyte then undergoes two meiotic divisions, forming firstly two diploid secondary spermatocytes and finally four haploid spermatids. Cell division ceases at this point and the spermatid cells mature into spermatozoa by a process called spermiogenesis.

Little is known about the mechanisms which initiate and regulate the maturation of testis cells, but it is thought that the pituitary gonadotropic hormones (luteinising hormone and follicle stimulating hormone) may be involved (273) and the subject has been reviewed for fish systems (274 - 276). It is known that immature fish can be induced to mature by injection with pituitary extracts obtained from mature salmon (277, 278). An alternative method is to adjust the photoperiod to which the fish are exposed. This stimulus is thought to act through the hypothalamus, which controls gonadotropin secretion (279). Fish can therefore be induced to mature by increasing the light exposure per day. Thus, it would appear that spermatogenesis is a hormonally-controlled event.

In studies on spermatogenesis, much interest has been focussed on the spermatid stage, since at this stage, profound changes occur in both morphology and the macromolecular composition of the cell. The cytoplasm is sloughed away, with concommitant decrease in the cellular RNA content (280 - 282). This has been measured by cytochemical and spectrophotometric methods. That this elimination of

Figure.4.1

Schematic representation of spermatogenesis



RNA is a specific process has been implied by study of the base composition of Oncorhyncus nerka total cellular RNA as a function of development. Mature testis RNA was much richer in guanosine than RNA from immature testis (283). This can be interpreted as an increase in the ratio of protamine mRNA to total RNA as development proceeds, since the mRNA coding for an arginine-rich protein would be expected to have a high G+C content. In addition to changes in RNA, the nuclear proteins were also affected. The histones characteristic of somatic cell nuclei were completely replaced by a new series of arginine-rich, highly basic proteins. The basicity of these proteins varies from species to species; those of intermediate basicity, found in mouse spermatozoa, are called arginine-rich histones, whilst the extremely basic proteins of salmonid and clupeid spermatozoa are called protamines. Evidence for the replacement of histones by more basic proteins has accumulated from several sources. Histochemical studies have shown that protamines appear in middle stage spermatids of Chinook salmon (O. tshawytscha) (268). Preferential incorporation of labelled arginine by middle stage mouse spermatids has been shown by autoradiographical studies, implying the synthesis of an arginine-rich protein (284). Velocity sedimentation separation of trout testis cells of varying maturity, followed by electrophoretic analysis of the labelled nuclear proteins demonstrated that radioactive protamine appeared in the nuclei of those cells which had reached the spermatid stage (285). Studies with the inhibitors actinomycin D, puromycin, chloramphenicol and cycloheximide have suggested that protamine appearing at the spermatid stage of trout testis cells is synthesised de novo using the classic mRNA-ribosome-tRNA mechanism and is not the result of a modification of histones already present (286). Electrophoretic analysis of histones as a function of development showed that these histones were replaced in a strict temporal sequence, which suggests that the process is under definite control (287).

In all cases, appearance of protamine or arginine-rich proteins was correlated with somatic histone disappearance. The possible mechanism for this replacement process has been studied in detail for trout testis cells (285, 288). Once the protamines had appeared, protein synthesis began to decrease. Since the interaction of protamine with DNA caused a definite contraction of the chromosome material (necessary to package the DNA into a small sperm cell), it may be that

this is sufficient to cause genome repression via steric hindrance to RNA polymerase. The ability of isolated chromatin to support *in vitro* synthesis of RNA by bacterial polymerase also decreased with increasing testis maturity (289). Further, RNA levels in *S. gairnerii* testis nuclei decrease as development proceeds and are virtually undetectable at the spermatid stage, when protamine appears in the nuclei (290). These observations imply that interaction with protamine represses the genome. Other functions for these proteins have been postulated, however, and the subject has been reviewed (291).

Recently, the implications of this process have been broadened by the discovery that whole protamine consists of a heterogeneous mixture of several components, differing from one another only in the number and distribution of neutral amino acids present. That these components were also observed in protamine from a single fish ruled out the possibility of genetic polymorphism (292). The analytical procedures used to detect these components have been adapted to their preparative scale production from whole salmine, iridine and clupeine (293). In addition, it has been shown that the relative amounts and rates of synthesis of the three salmine components alter as a function of development (294). Most progress has been made with fish systems, since fish testis cells mature in a much more synchronous fashion than their mammalian counterparts.

Summarising, protamines represent a well characterised group of proteins which are synthesised in relatively large quantities at a specific time in sperm maturation. Further, at least in trout, the differential synthesis of the three protamine components suggests a degree of independent control of their synthesis. Therefore, spermatogenesis appears to be an ideal system for the study of differentiation. The aims of this project were to study protamine biosynthesis in European herring (*C. harengus*). It was intended to:

1) isolate and characterise the protamine(s),
2) study protamine biosynthesis *in vivo*,
3) isolate and characterise the mRNA(s) for the protamine(s),
4) study protamine synthesis *in vitro* with particular reference to regulatory mechanisms.

During the course of these studies, the complete amino acid sequences of *C. harengus* protamines (295) and a comparable study on protamine mRNA(s) in trout testis (158) have been published.

MATERIALS AND METHODS

[^{14}C] arginine (342 mC/mmole) and [^{3}H] arginine (22C/mmole) were obtained from the Radiochemical Centre. Triton X100, formamide, Amberlite MB1 and SDS from BDH. Dithiothreitol, diethylpyrocarbonate, heparin, hepes, creatine phosphate, creatine phosphokinase, ATP, GTP, mercaptoethanol and E. coli alkaline phosphatase were obtained from Sigma. RNAse A was obtained from Miles, NCS from Amersham-Searle, oligo (dT) cellulose from Searle Products for Research. Acrylamide and N,N'bisacrylamide were obtained from Eastman Kodak and were recrystallised from chloroform and acetone respectively. CM-cellulose (CM52) was obtained from Whatman Ltd. Bio-Gel CM2 and BioRad AG11A8 were obtained from BioRad. All other chemicals were of the best available purity.

Herring testes were collected from the Blackwater estuary during the period October - February 1971 - 1974.

These testes were processed in one of two ways as soon as the fish were docked. They were either converted into cell suspensions for immediate incubation with radioactive precursors or were frozen on card ice, transported back to the laboratory and stored at -70^{o}C until required for RNA or protamine isolation.

The following abbreviations have been used for buffer compositions in the text:-

TMKS 0.05M tris, pH 7.6; 0.025M potassium chloride; 0.005M magnesium acetate; 0.25M sucrose.

TMNS As for TMKS buffer except that potassium chloride is replaced by sodium chloride.

NETS 0.1M sodium chloride; 0.001M EDTA; 0.01M tris, pH 7.4; 0.5% SDS.

ANE 0.01M sodium acetate; 0.01M sodium chloride; 0.001M EDTA.

TE 0.01M tris, pH 7.5; 0.005M EDTA.

Hepes 2-(N-2-hydroxyethylpiperazin-N'-yl)ethanesulphonic acid

All centrifugation procedures were carried out at 0^{o}C and all column chromatography at room temperature unless otherwise stated.

5. Protein methods

5.1 Radioactive labelling of cells

Fresh tissue was scissor-minced in 1.25 volumes (w/v) of TMKS buffer and homogenised by hand in a loose-fitting homogeniser (clearance 0.75 mm). The resulting suspension was strained through two layers of muslin and made up to 1.5 volumes with more TMKS buffer. Aliquots were removed and radioactive [^{14}C] arginine (342 µC/µmole) was added to a final concentration of 0.5 µC/ml. Incubation proceeded for 30 minutes at 14°C, then was stopped by addition of an equal volume of TMKS buffer containing 10^{-2}M [^{12}C] arginine and immediate freezing on card-ice. These incubations were stored at -70°C until required for isolation of basic proteins.

5.2 Isolation of basic proteins

5.2.1 Unlabelled

Frozen or fresh tissue was homogenised in two volumes (w/v) of TMKS buffer in a TriR homogeniser (clearance 0.25 mm) at 5,000 rpm for 45 seconds. The homogenate was centrifuged in the 8x50 MSE 18 rotor at 1,000g (R_{max}) for 10 minutes to collect the nuclei. The pellet was washed several times with TMKS buffer by resuspension and recentrifugation and finally suspended in 5 volumes (w/v) of 0.2M sulphuric acid by hand homogenisation (clearance 0.25 mm). After standing for 20 minutes at room temperature, the extract was centrifuged at 20,000g for 30 minutes in the 8x50 MSE 18 rotor. The supernatant was removed and stored at 0°C and the pellet re-extracted a further three times with the sulphuric acid. All four supernatants were pooled and precipitated with 3 volumes (v/v) of ethanol at -20°C overnight. The resultant precipitate was collected at 20,000g for 20 minutes in the 8x50 MSE 18 rotor, washed two times with ethanol and dried *in vacuo*.

5.2.2 Labelled

In vivo labelled protamine was isolated in the same way as unlabelled protamine except that the thawed incubations (prepared as in section 5.1) were used instead of tissue and the nuclei were more rigorously washed to eliminate as much free arginine as possible.

The final pellet (usually from 2 g of tissue) was dissolved in 4 ml of 0.2M sulphuric acid/10% sucrose and 0.2 ml aliquots electrophoresed on 20% acrylamide gels and counted as in section 5.3 below.

5.3 Electrophoresis of protamine labelled in vivo or in vitro

The electrophoresis system of Shepherd and Gurley (296) was used except that the gels measured 0.7 x 6.0 cm. These gels contained 20% acrylamide made up in 0.013M potassium acetate buffer, pH 2.9. The anode and cathode buffers were 0.3M valine, pH 4.0 and 0.3M glycine, pH 4.0, respectively. Labelled protamine was applied to each gel in 0.2 ml of 0.2M sulphuric acid containing 10% sucrose. Electrophoresis was carried out at 4 ma/tube for 2 hours. The gels were then removed from the tubes and the protamine bands visualised by surface denaturation with 10% TCA. This band (1 cm) was excised and cut into small pieces which were then digested in 3 ml of NCS/water (9:1) at 40°C for 16 hours. 15 ml of toluene scintillant was added and the vials counted after a further 3 hours at 40°C. The possibility of alkaline chemiluninescence (due to the presence of excess solubiliser) was found not to be a problem, since remeasurement of the vials over a period of 24 hours resulted in no change in the count rate. The blank consisted of an equal cylinder of gel containing unlabelled protamine and hence any residual chemiluminescence after incubation at 40°C should be accounted for.

5.4 Chromatography of basic proteins on CM-cellulose

The basic proteins obtained in section 5.2 were dissolved in 20 ml of deionised water and the pH adjusted to 5.0 with sodium hydroxide (1N). The sample was then applied to a 1 x 7 cm CM-cellulose column (H^+ form) at a flow rate of 12 ml/hour, and a temperature of 0-4°C. Unbound protein was removed by extensive washing (>200 ml) with deionised water at the same flow rate. Basic proteins (histones and protamines) were eluted with 0.2M hydrochloric acid (25 ml was usually sufficient to remove all of the sample). The acid was removed by passing the eluate through a 1 x 40 cm column of BioRad AG11A8 ion retardation resin at a flow rate of 40 ml/hour. The protamine was washed through with water whilst the acid remained attached to the column. The protein-containing eluate was lyophilised. After each use, the column was regenerated as described in the BioRad Technical Bulletin (300).

5.5 Chromatography of basic proteins on P-10

Histones and protamines were dissolved in 2 ml of 0.2M acetic acid, applied to a 2 x 30 cm BioRad P-10 column and eluted with 0.2M acetic acid at a flow rate of 20 ml/hour. 5 ml fractions were collected and the effluent monitored at 230 nm. This relatively unspecific and insensitive wavelength is necessary since protamine contains no aromatic residues and therefore does not absorb at 280 nm. The protamine fractions were pooled, deacidified and lyophilised as in section 5.4.

N.B. Occasionally protein samples were dissolved in 2 ml of 0.5M tris buffer, pH 7.6, containing *E. coli* alkaline phosphatase (1 mg). The solutions were incubated at 30°C for 3 hours, cooled and applied to the P-10 column.

5.6 Separation of the three protamine components

5.6.1 Chromatography on BioGel CM2

The method of Ando and Watanabe was used (293). Whole protamine from section 5.5 was dissolved in eluting buffer (or where dephosphorylation was required, 0.5M tris buffer, pH 7.6). The sample was applied to a 0.9 x 130 cm column of CM2 and eluted with 1.5M sodium chloride in 0.05M sodium acetate buffer, pH 5.8 at a flow rate of 2.5 ml/hour. 5 ml fractions were collected and then monitored by measuring the absorbance at 230 nm or by Sakaguchi determination on 1 ml aliquots (298).

5.6.2 Chromatography on CM-cellulose

5.6.2.1 Gradient chromatography

The method of Ling *et al.* was used (299), with or without the enzymatic dephosphorylation step. When dephosphorylation was used, the incubation was carried out as described in section 5.5; then 6 ml of deionised water was added and the sample applied to a 1.1 x 40 cm column of CM-cellulose which had previously been equilibrated in 0.01M lithium acetate buffer, pH 5.0. Protamines were eluted with a 700 ml linear gradient (0.75M - 1.3M lithium chloride in 0.01M lithium acetate buffer, pH 5.0) at a flow rate of 15 ml/hour. Effluents were monitored as in section 5.6.1.

5.6.2.2 Stepwise chromatography

The samples were applied as in section 5.6.2.1, but the degradation products and non-protamine proteins were removed by elution with 0.75M lithium chloride in 0.01M lithium acetate buffer, pH 5.0, at a flow rate of 15 ml/hour. The column was then eluted in a stepwise manner, firstly with 0.95M lithium chloride in buffer and secondly with 1.1M lithium chloride in buffer. Columns of variable length were used but all were of internal diameter 1 cm.

6. RNA methods

Extreme care was taken in order to minimise risk of degradation through RNAse action. All glassware used in RNA preparation was heat sterilised at 140°C for 12 hours. Solutions were sterilised in one of two ways:

(i) Sucrose solutions were made 0.1% in diethylpyrocarbonate (v/v) which immediately inactivates RNAse. The diethylpyrocarbonate was then removed by heating at 37°C overnight. The solutions were vigorously shaken to remove any carbon dioxide formed, then cooled to room temperature and the pH adjusted to the required value if necessary.

(ii) All other solutions were treated with 0.1% diethylpyrocarbonate as before but the decomposition was now effected by heating the solutions in a boiling water bath for 15 minutes prior to cooling and adjustment of pH.

6.1 Determination of optimum conditions for hypotonic lysis of testis cells

10 g of testis material was homogenised in 25 ml of TMN buffer, strained through four layers of muslin and made up to 30 ml with TMN buffer. This homogenate was left at 0°C for 10, 20, 30, and 40 minutes. At each time, two 3 ml aliquots were removed and centrifuged at 40,000g for 10 minutes in the 8x50 MSE 18 rotor. The supernatants were tested for absorbance at 260 nm, for RNA by the orcinol reaction (297), and for DNA by the diphenylamine reaction (301). The colorimetric methods were used after precipitation of nucleic acids with 5% TCA. The RNA precipitate was dissolved in water and the DNA extracted with 0.5N PCA at 70°C for 20 minutes prior to assay.

6.2 RNAse assay

This assay is based on the principle that RNAse action reduces the chain length of RNA molecules below the minimum necessary to allow precipitation of these molecules from solution by a denaturing agent. The extent of nucleolytic degradation can therefore be correlated with the nucleotide concentration remaining in solution after the addition of a precipitating agent (302).

The assay mixture contained:-

0.5 ml of buffer TMN containing 2 mg of RNA

+

0.5 ml of postmitochondrial supernatant (400,000g minutes)

OR

0.5 ml TMN buffer + 0.1% diethylpyrocarbonate (decomposed by heat)

A series of assay mixtures were incubated at 0^{o}, 20^{o} or 37^{o}C for 10 minutes (the reactions were shown to be linear up to this time). Then 0.2 ml of uranyl acetate (0.75% in 25% PCA) was added to each and the reaction mixtures allowed to stand in an ice bath for 30 minutes. They were then centrifuged for 15 minutes at top speed in an MSE bench centrifuge. The supernatants were diluted to give suitable readings at 260 nm. Pancreatic RNAse standards were run concurrently with the experimental tubes.

N.B. Repeat of this assay using a detergent-treated supernatant produced essentially similar results: therefore the RNAse content of the supernatant does not appear to be increased by the action of Triton X100.

6.3 RNA isolation by hypotonic lysis of testis cells

Scissor-minced testis material was hand homogenised in 2.5 volumes (w/v) of TMN buffer (homogeniser clearance 0.75 mm). The homogenate was strained through four layers of muslin and made up to three volumes with TMN buffer. After sitting at 0^{o}C for 20 minutes (as determined in section 6.1), it was centrifuged at 20,000g for 20 minutes in the 8x50 MSE 18 rotor. The supernatant was removed and made 2% in Triton X100 (v/v) After standing at 0^{o}C for 10 minutes, it was recentrifuged at 20,000g for 20 minutes. The supernatant was again removed and centrifuged at 100,000g for 2 hours in the 8x50 MSE 65 rotor. The microsomal pellets thus obtained were rinsed with homogenising buffer and resuspended in

NETS buffer to an E_{260} nm value of approximately 20 units/ml. After incubation at 37°C for 5 minutes, two volumes of ANE buffer-saturated phenol/chloroform (1:1 w/v) were added and the mixture shaken at room temperature for 10 minutes. The phases were separated at 5,000g for 10 minutes in the 6x100 MSE 18 rotor. The aqueous phase, which contained a large amount of denatured protein, was re-extracted firstly with two volumes of chloroform (to compress the interphase) and then with phenol/chloroform until no interphase protein remained. 0.1 volume of 1M sodium chloride and two volumes of ethanol were added and the mixture stored at -20°C overnight. The crude microsomal RNA precipitate was collected by centrifugation at 40,000g for 10 minutes in the 8x50 MSE rotor, washed two times with ethanol:0.2M sodium chloride (2:1 v/v) and dried under nitrogen.

6.4 RNA isolation by homogenisation in buffered sucrose

Frozen testis was homogenised in two volumes of TMNS buffer (w/v) with or without 900 µg/ml of heparin (TriR homogeniser, 0.25 mm clearance, 5,000 rpm for 45 seconds). The resulting homogenate was centrifuged at 20,000g for 15 minutes in the 8x50 MSE 18 rotor. The supernatant thus obtained was made 2% in Triton X100 (v/v). After standing at 0°C for 10 minutes, it was recentrifuged at 20,000g for 15 minutes. The supernatant was further processed as in section 6.3.

6.5 Salt fractionation of testis RNA

Crude microsomal RNA was dissolved in TE buffer and solid sodium chloride added to 2.5M. After four hours at 0°C, the precipitate (containing high molecular weight RNA) was collected at 10,000g for 10 minutes in the 8x50 MSE 18 rotor (303). The supernatant (containing low molecular weight RNA) was removed and kept and the precipitate was redissolved in TE buffer. RNA was precipitated from the original supernatant and from the redissolved precipitate by addition of 0.2 volumes of 1M sodium chloride and 2 volumes of ethanol and storing at -20°C overnight. The precipitates were washed and dried as above.
N.B. When polysomes were isolated in the presence of heparin, excess heparin was found in the RNA preparation. This contaminant partitions into the low molecular weight RNA fraction and precludes testing of this RNA in a cell-free system since heparin is a powerful inhibitor of *in vitro* protein synthesis (304 - 307). Thus, in the initial sequence of

experiments to find out the distribution of protamine messenger RNA activity between the various fractions, no heparin was used.

6.6 Purification of high molecular weight RNA on oligo (dT) cellulose

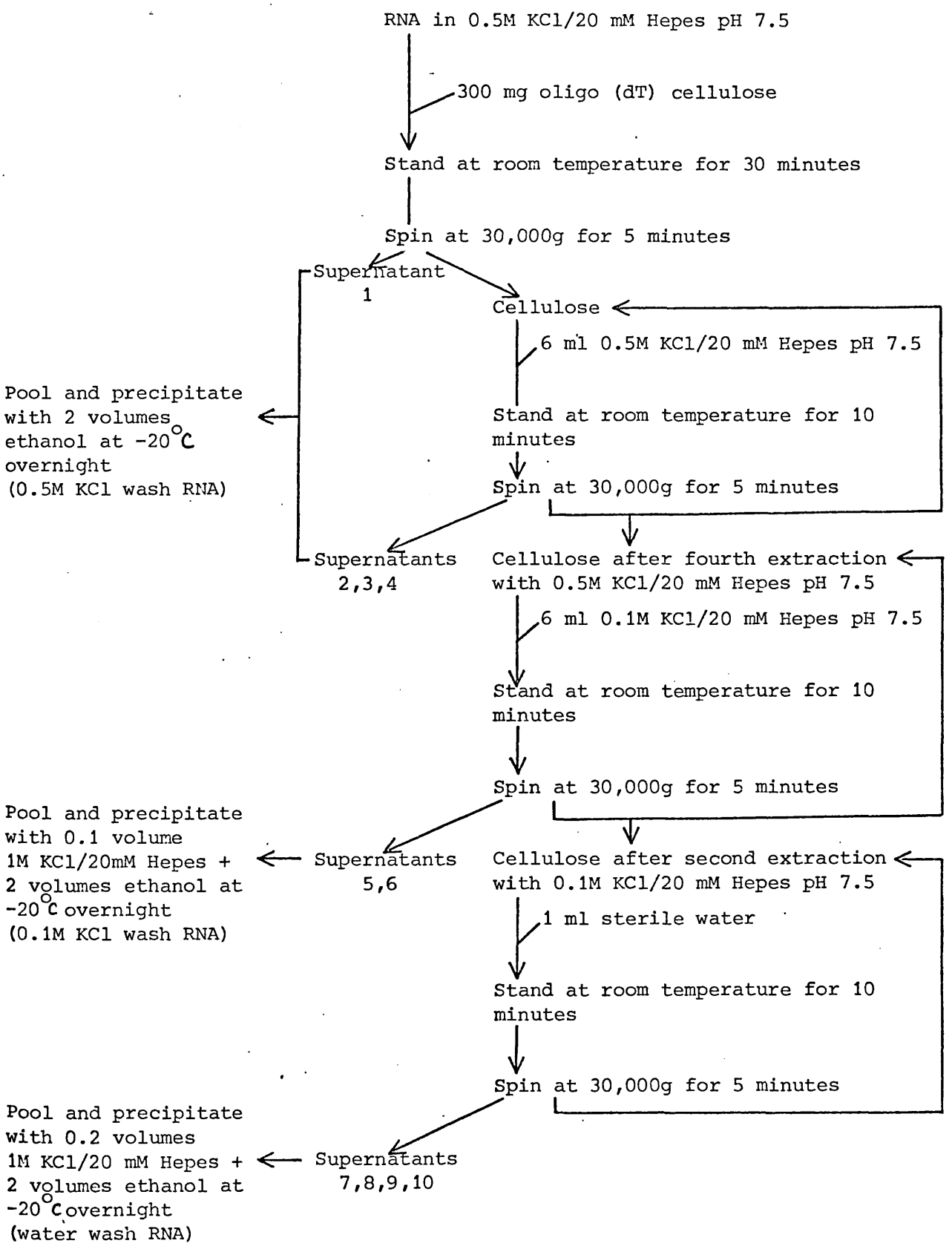
The high molecular weight RNA (200 E_{260}nm units) was dissolved in 4.6 ml of 0.5M potassium chloride, 0.02M Hepes, pH 7.5, and stirred into 0.3 g of oligo (dT) cellulose at 20°C. After 30 minutes, the cellulose was collected at 30,000g for 5 minutes in the 8x50 MSE 18 rotor. The supernatant (containing non-poly A RNA) was removed and the cellulose was re-extracted three times for 10 minutes with 6 ml of starting buffer. This was usually sufficient to bring the E_{260}nm readings of the washings to zero. The cellulose was then extracted two times with 6 ml of 0.1M potassium chloride, 0.02M Hepes, pH 7.5 to elute RNA containing short poly A stretches. RNA containing longer poly A stretches was eluted by extraction with four 1 ml aliquots of sterile water. The supernatants from each phase of the washing cycle were combined to give three final solutions (see Figure 6.1). The 0.5M potassium chloride solution was taken for ethanol precipitation without further addition. The other two solutions were made 0.2M in potassium chloride before addition of 2 volumes of ethanol. After standing at -20°C overnight, the resulting precipitates were collected, washed and dried as before. Each fraction was dissolved in sterile water and tested for mRNA activity in a cell-free system derived from commercial wheat germ (153), as described in section 6.9.2.

6.7 Sucrose gradient analysis of formamide-treated RNA

Formamide (25 ml) was deionised by stirring with 2 g of mixed bed resin (Amberlite Resin MB1) for two hours. The resin was removed by centrifugation on an MSE minor bench centrifuge at top speed for 15 minutes. The RNA sample to be analysed was dissolved in 50 µl of deionised formamide, then diluted to 500 µl with 0.01M Hepes, pH 7.5, 0.001M EDTA. 10 µg [^{3}H] labelled *E. coli* rRNA (500,000 cpm/µg RNA) was added and the solution layered over an 8.5 ml linear sucrose gradient (5-20% w/v). The gradient was centrifuged at 100,000g for 18 hours at 1°C, 0.5 ml of water layered on top and then fractionated into 12 drop fractions after puncturing the tube. Even-numbered tubes were made 0.2M in sodium chloride, 0.2 E_{260}nm units of carrier *E. coli* rRNA (unlabelled) were added, then RNA was precipitated by the addition of

Figure 6.1

Purification of high molecular weight microsomal RNA on oligo (dT) cellulose



2 volumes of ethanol (-20°C overnight). The precipitates were collected by centrifugation at 13,000g for 30 minutes in a clinical centrifuge. The supernatants were removed and each tube carefully dried with a stream of nitrogen. Each precipitate was redissolved in 5 µl of sterile water and assayed for mRNA activity in a 50 µl wheat germ cell-free incubation. 40 µl aliquots were removed from the odd-numbered fractions, added to 6.6 ml of scintillant and counted.

Scintillant composition:

PPO	4 g/l }	7 volumes
POPOP	0.1 g/l } in toluene	
Triton X100		6 volumes
Water		1.3 volumes

6.8 Sucrose gradient analysis of non-formamide-treated RNA

The method differs from section 6.7 in that the RNA sample was dissolved in 0.5 ml of 0.01M Hepes, pH 7.5, 0.001M EDTA, 2% sucrose (w/v) before layering onto the gradient.

6.9 In vitro systems for analysis of mRNA activity

6.9.1 Rabbit reticulocyte system

Rabbit reticulocyte lysates were prepared according to the method of Lingrel (308) except that the blood was obtained from the ear instead of by cardiac puncture. The components of the cell-free incubation were stored as stock solutions, which were added together when required. The master mix (solution E of Lingrel) was made from these stock solutions by adding them in the proportions shown below:

Solution 1.	Ammonium acetate (2.5M) Tris buffer, pH 7.4 (0.25M) Magnesium acetate (0.05M)	2 volumes
Solution 2.	ATP (0.05M) GTP (0.01M) Phosphocreatine (0.75M)	1 volume
Solution 3.	Phosphocreatine kinase (3 mg/ml)	1 volume

Solution 4.					
	Ala	(2.5 mM)	Phe	(2.0 mM)	
	Arg	(0.60 mM)	Pro	(1.75 mM)	
	Asp	(3.55 mM)	Ser	(2.0 mM)	
	Aspn	(2.5 mM)	Thr	(2.15 mM)	
	Cys	(0.5 mM)	Try	(0.365 mM)	
	Glu	(5.0 mM)	Tyr	(1.05 mM)	1 volume
	Gly	(6.5 mM)	Val	(5.0 mM)	
	Hist	(3.0 mM)			
	Ile	(0.375 mM)			
	Leu	(5.0 mM)			
	Lys	(2.25 mM)			
	Met	(0.425 mM)			

From this solution, the incubation cocktail was prepared by adding the following in order:-

Master mix	5 volumes
Phenol red (1 mM)	1 volume
Ammonium hydroxide	1 volume
Haemin (300 µM)	4 volumes
[^{3}H]arginine (60 µC/ml) (22C/mM)	5 volumes

N.B. Since the stock ammonium hydroxide solution varied in strength as a function of time, sufficient was added to produce a standard colour change in the Phenol Red i.e. to pH 7.5. In those cases where less than 20 µl was required the volume was made up to 20 µl with water.

5x150 µl incubations were set up, each one containing 1 µC of [^{3}H] arginine (22 C/mMole) and variable amounts of testis microsomal RNA, prepared by the hypotonic lysis method. The proportions used were as follows:

Cocktail	8 volumes
Lysate	10 volumes
RNA in 5 mM tris buffer, pH 7.5	7 volumes

After 2 hours incubation at 25°C, two 50 µl aliquots were removed from each incubation mixture and frozen in capillary tubes for analysis. The aliquots were applied onto CM-cellulose columns (0.5x3 cm), equilibrated in 0.02M sodium acetate buffer, pH 5.2. Each column was washed successively with 7 ml of 0.02M sodium acetate buffer, pH 5.2, 5 ml of 0.15M sodium chloride, 6 ml of 0.65M lithium chloride in 0.01M lithium acetate buffer, pH 5.2, and 6 ml of 0.1M hydrochloric acid. The elution was performed at 0-4°C. 1 ml fractions were collected and 0.1 ml aliquots from each fraction were mixed with 1 ml toluene/Triton scintillant (2:1 v/v) and counted in a Beckman LS 100 scintillation counter. Efficiency determinations were made by the external standard method and the total dpm in each peak determined. (These corrections were necessary as the fractions containing the globin were a pronounced red colour, whilst the other fractions were colourless. Since red solutions are known to quench very strongly this results in large differences in counting efficiency).

6.9.2 Wheat germ system

Wheat germ cell-free system was prepared according to the method of Roberts and Paterson (153). The components of the cell-free incubation were stored as stock solutions, which were added together when required. The master mix was made from these solutions by adding equal volumes of the following in order:-

Solution 1. Hepes (150 mM), dithiothreitol (15 mM), creatine phosphate (100 mM)

Solution 2. Creatine phosphokinase (500 µg/ml)

Solution 3. ATP (12.5 mM), GTP (2.5 mM) (previously neutralised)

Solution 4. Unlabelled amino acids (375 µM)

Solution 5. [^{14}C] arginine (68 µC/ml) (342 µC/µmole)

This produced a master mix of the following composition:-

Hepes, pH 7.6	30 mM
Dithiothreitol	5 mM
Creatine phosphate	20 mM
Creatine phosphokinase	100 µg/ml
ATP	2.5 mM
GTP	0.5 mM
[^{14}C] arginine	13.6 µC/ml
Ala, Asp, Aspn, Cys Glu, Gly, Hist, Ile Leu, Lys, Met, Phe Pro, Ser, Thr, Tyr Val	75 µM

The K^+/Mg^{++} solution had the following composition:-

K^+ = 400 mM Mg^{++} = 16 mM

The incubation mixture contained:-

Master mix	1 volume
Lysate	1 volume
K^+/Mg^{++} solution	0.25 volume
RNA solution	0.25 volume

Incubation was allowed to proceed for 2 hours at 25°C, when further processing occurred as described in sections 6.10.1 and 6.10.2.

6.10 Estimation of radioactive incorporation into protein synthesised in wheat germ in vitro system

6.10.1 Incorporation into pH2 TCA/tungstate-insoluble material

50 µl incubations were used. At the end of the incubation, two 20 µl aliquots were removed and spotted onto 2.1 cm discs of Whatman 3 MM paper and dropped into TCA/tungstate (10% TCA/0.25% sodium tungstate, pH 2). The discs were boiled for 15 minutes in this solution, then washed three times in fresh TCA/tungstate, once in ethanol, once in ethanol/ether (3:1 v/v) and once in ether. After drying the discs were counted in 5 ml of toluene scintillant (5 g/l PPO/0.1 g/l POPOP).

6.10.2 Incorporation into protamine

500 µl incubations were used. At the end of the incubation, two 20 µl aliquots were removed and processed as in the previous section. The remaining 460 µl were made 10 mM in EDTA and 20 µg/ml in RNAse A by adding an equal volume of 20 mM EDTA/40 µg/ml RNAse A. The mixture was incubated for 15 minutes at 37°C, then 920 µl of cold 0.4M sulphuric acid containing 0.5 mg of carrier protamine was added. Extraction of basic proteins was allowed to proceed for 30 minutes at 0°C. The debris was collected at 10,000g for 10 minutes in an 8x50 MSE 18 rotor and re-extracted two times with 1.5 ml cold 0.2M sulphuric acid. All three supernatants were combined and precipitated with 4 volumes of 90% ethanol at -20°C overnight. The precipitate was collected at 40,000g for 10 minutes, washed two times with 0.2M sulphuric acid:ethanol (1:4 v/v) two times with ethanol and dried in vacuo. It was then dissolved in 0.7 ml of 0.2M sulphuric acid containing 0.1 mg protamine and 10% sucrose. 0.2 ml aliquots of this solution were electrophoresed on 20% polyacrylamide gels and counted as described in section 5.3.

RESULTS AND DISCUSSION

7. Protamine biosynthesis

In order to study protamine biosynthesis and its regulation, it was first of all necessary to examine the timing of protamine synthesis as a function of development.

7.1 Determination of the timing of protamine biosynthesis

Testis material was classified into varying stages of maturity according to the Hjorst maturity scale, which is shown in Figure 7.1 (270). The distribution of these stages as a function of time can be seen in Figure 7.2. (This data was kindly supplied by the Ministry of Agriculture, Fisheries and Food). As can be seen from the figure, there is a rough correlation between fishing time and testis maturity. It should also be noted that quantities of testis at the early stages of maturity are strictly limited for several reasons. Firstly, the size of a testis at stages I - III is small (<2g). Further, these early stages only represent a significant fraction of the total distribution during the first month of the breeding season, a period when the total average catch of fish is small. This makes difficult the large scale isolation and analysis of both protamine and its mRNA as a function of development and represents a serious drawback to studies with the herring system.

To examine timing of protamine synthesis, whole cell suspensions were prepared from a standard weight of testis of varying maturity and incubated with radioactive arginine as described in section 5.1. The nuclear basic proteins were acid extracted and analysed by electrophoresis on polyacrylamide gels. Since the criterion for comparison between the stages is based on a standard amount of testis in the original incubation, it was necessary to establish that the extraction procedure was quantitative and that any unavoidable losses were consistent. An analysis of the quantitation procedure is presented in Appendix I.

The results of the electrophoretic analysis are shown in Figures 7.3 and 7.4. Figure 7.3 shows that protamine was just detectable in herring testis at stage II and that the amount present increased with time, reaching a maximum at stage VI. The increase in protamine was also accompanied by the disappearance of histones, thus confirming the

Figure 7.1

HERRING MATURITY STAGES

I Virgin herring. Gonads very small, threadlike, 2 - 3 mm broad. Ovaries wine red. Testes whitish or grey-brown.

II Virgin herring with small sexual organs. The height of ovaries and testes about 3 - 8 mm. Eggs not visible to naked eye but can be seen with magnifying glass. Ovaries a bright red colour. Testes a reddish-grey colour.

III Gonads occupying about half of the ventral cavity. Breadth of sexual organs between 1 and 2 cm. Eggs small but can be distinguished with naked eye. Ovaries orange. Testes reddish-grey, or greyish.

IV Gonads almost as long as body cavity. Eggs larger, varying in size, opaque. Ovaries orange or pale yellow. Testes whitish.

V Gonads fill body cavity. Eggs large, round, some transparent. Ovaries yellowish. Testes milk-white. Eggs and sperm do not flow, but sperm can be extruded by pressure.

VI Ripe gonads. Eggs transparent. Testes white. Eggs and sperm flow freely.

VII 1 Spent herring. Gonads baggy and bloodshot. Ovaries empty or containing only a few residual eggs. Testes may contain remains of sperm.

VII 2 Recovering spent: Ovaries and testes firmer and slightly larger than virgin herring, but no residual eggs or milt present. Ovaries colourless or wine coloured. Testes reddish-grey or greyish.

VIII Recovering spent. Ovaries and testes firm and larger than virgin herring in stage II. Eggs not visible to naked eye. Walls of gonads striated, blood vessels prominant. Gonads wine red colour. (This stage passes into stage III).

Figure 7.2

Distribution of herring maturity stages as a function of time during a typical breeding season in the Blackwater estuary

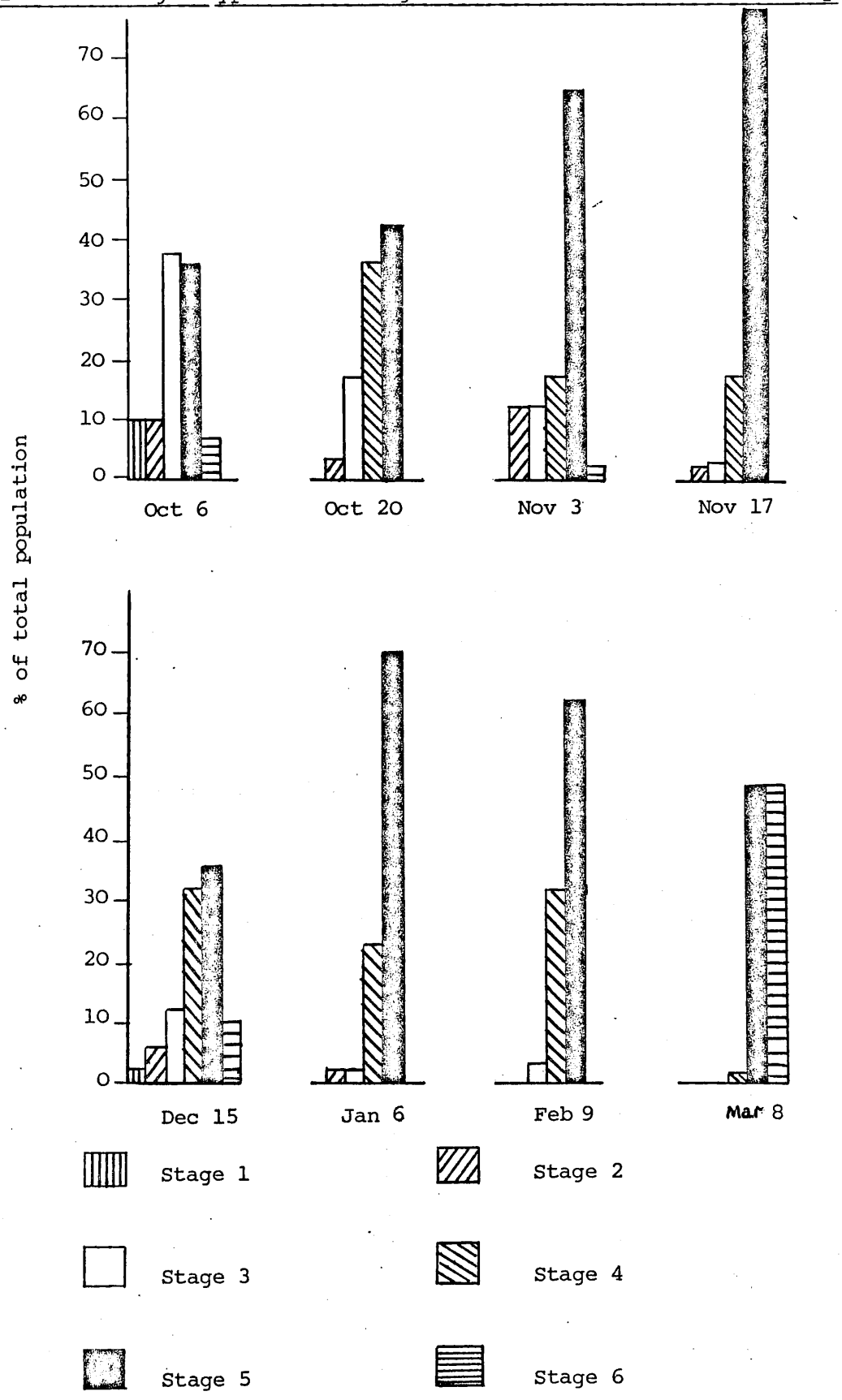
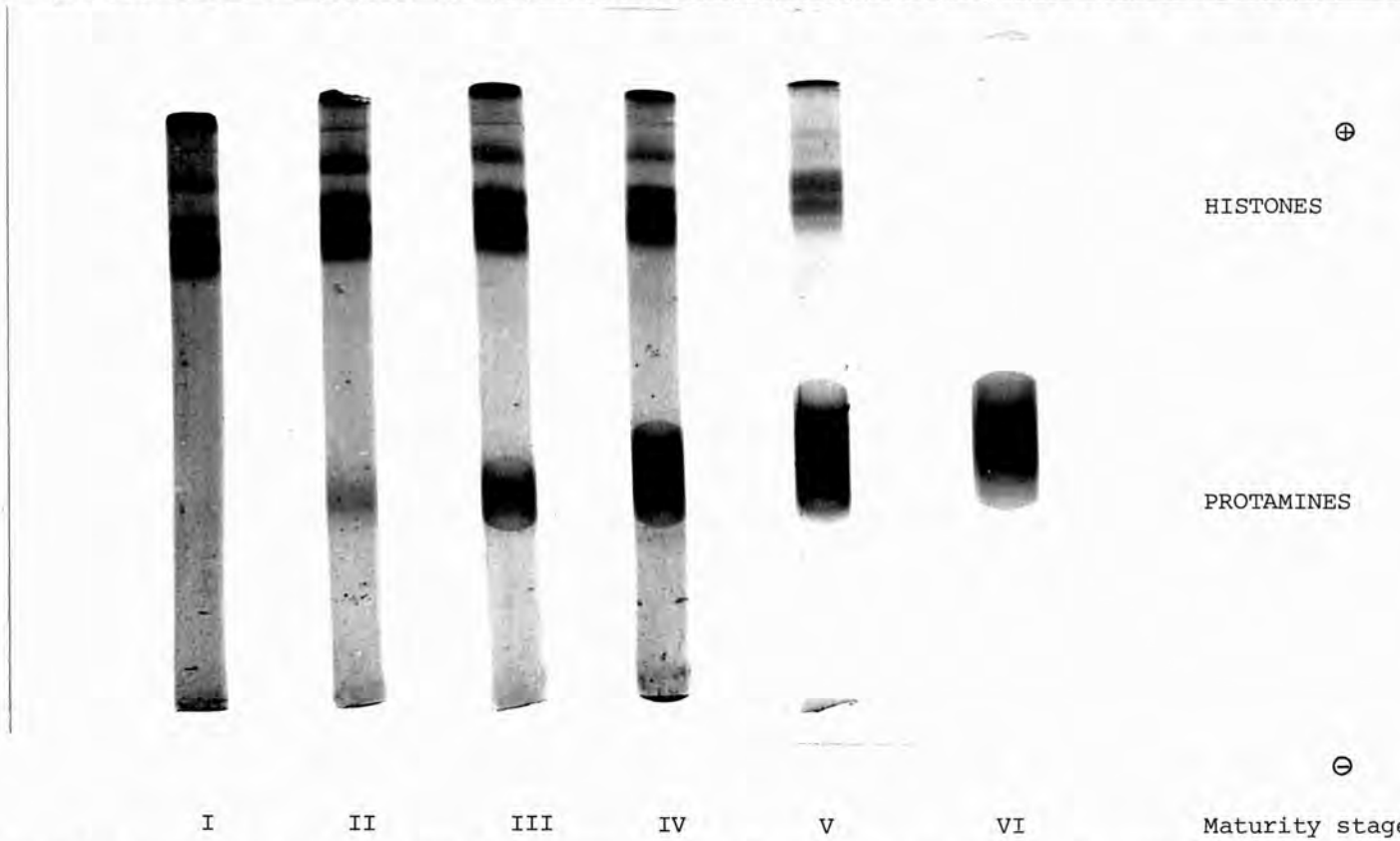


Figure 7.3

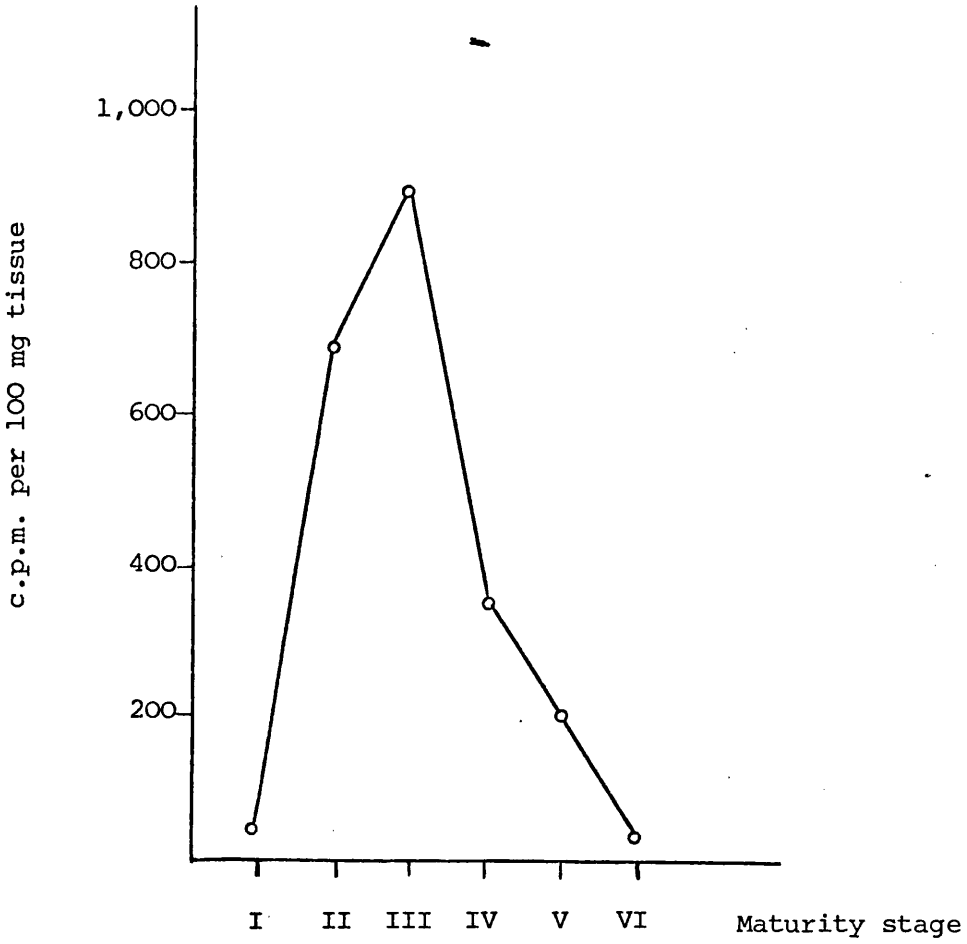
Polyacrylamide gel electrophoretic patterns of basic proteins isolated from herring testis of varying maturity



Radioactive proteins were prepared from 2g of herring testis of varying maturity as described in sections 5.1 and 5.2. These proteins were electrophoresed as in section 5.3, and placed in 10%TCA for at least 1 hour, Coomassie Blue was added to the TCA to a final concentration of 0.1% and the gels stained overnight. No destaining was necessary.

Figure 7.4

In vivo incorporation of [^{14}C]arginine into protamine by testis cells of varying maturity stages



Radioactive proteins were prepared from 2g of herring testis of varying maturity as described in sections 5.1 and 5.2. These proteins were electrophoresed and counted as in section 5.3.

sequence of events already demonstrated for several other systems (see section 4). Examination of protamine synthesis (Figure 7.4) shows that the peak of radioactive incorporation into protamine occurred in the intermediate stages (II - IV). Although this figure implies that stage III is the stage of maximal protamine synthesis, it should be remembered that classification of testis material by visual inspection is not particularly easy and assignment of stages to gonads which fall between the groups is somewhat arbitrary. Under these circumstances, the peak may alter with different samples.

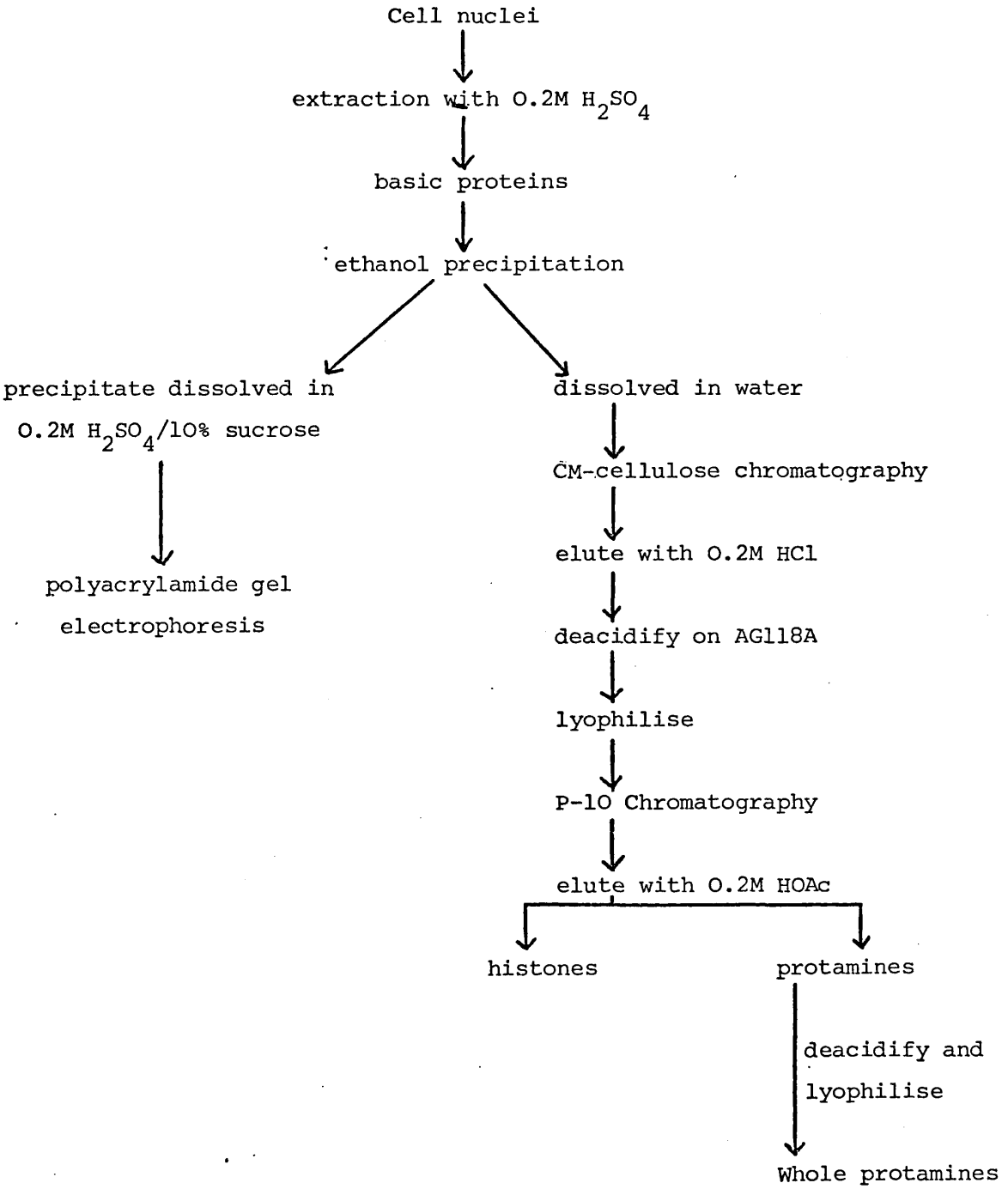
Since the level of protamine synthesis was extremely low for stages I and VI and only slightly higher for stage V, stages II - IV were chosen as suitable both for the isolation of mRNA and for the study of protamine biosynthesis. Stage VI material was used for the initial characterisation of the methodology for protamine analysis.

7.2 Isolation of protamine

A scheme for protamine extraction and isolation is shown in Figure 7.5. This procedure is based on the work of Ling (309). It was considered that direct lyophilisation of acidic solutions would cause damage to the equipment and therefore various methods for deacidification were examined. The methods of gel exclusion chromatography, precipitation from hydrochloric acid solutions and dialysis were not suitable because of the large volumes of CM-cellulose column effluents, the loss of material during precipitation and the small size of the protamine, respectively. Deacidification with anion-exchange resins also proved unsatisfactory since despite repeated pretreatment with acid, leaching out of impurities from the resin into the effluent could not be eliminated. Utilisation of an ion-retardation resin, AG11A8, finally proved successful in quantitatively removing the acid without contamination of the protamine. The ion retardation resin retains inorganic ions whilst allowing organic molecules to pass through unaffected. Since all but a small percentage of the resin-bound exchangeable groups are self-absorbed, little loss of sample should occur and recoveries of the order of 90% were routinely obtained.

Figure 7.5

Schematic representation of methods for the isolation and purification of protamine



7.3 Protamine component analysis

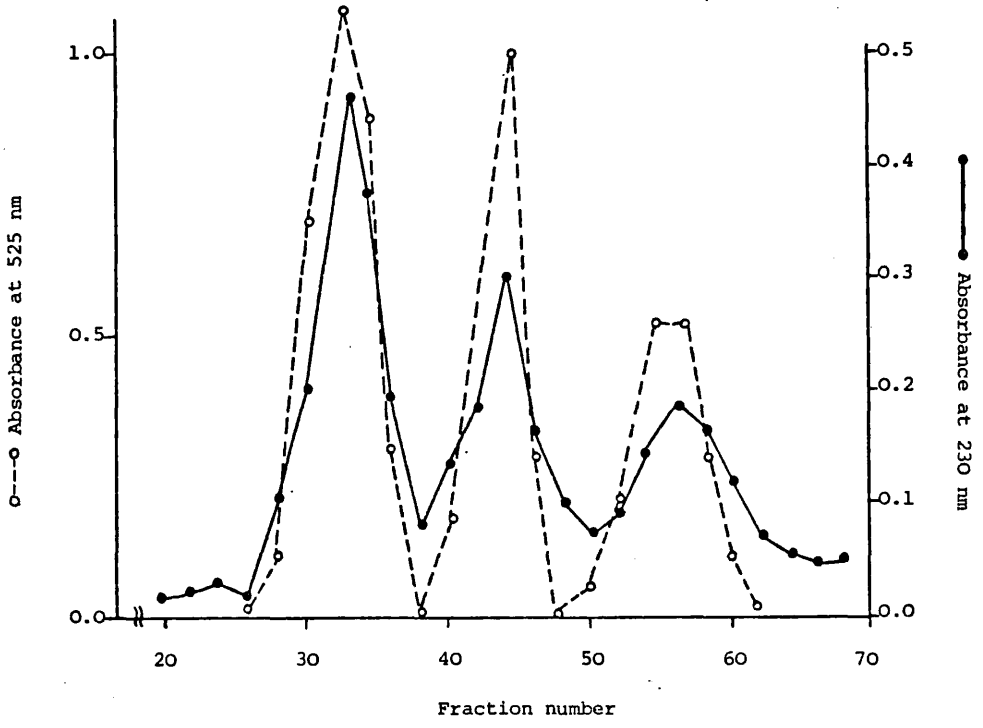
Two methods were available for this analysis: method 1, starting buffer elution of protein from BioGel CM2 according to the method of Ando (293); method 2, gradient chromatography on CM-cellulose according to the method of Ling et al. (309). Method 1 was shown to give excellent separation when a sample of mature protamine was applied (Figure 7.6). Protamine from European herring therefore appears to be composed of three components. This result has recently been confirmed and the amino acid compositions of the components determined (295).

The separation obtained by chromatography of protamine on CM-cellulose is shown in Figure 7.7 and is similar to that published for trout testis protamine (310). However, analysis of the area under the peaks obtained using methods 1 and 2 shows a marked difference. The figures, expressed as a percentage of input protamine, can be seen in Table 7.1. It is interesting that the addition of peaks 1 and 2 in the CM2 diagram produced a value similar to peak 2 in the CM-cellulose pattern, whilst peak 1 in the latter is completely absent from the profile obtained on CM2. It would appear that these two methods utilise different properties for separation, although both CM2 and CM-cellulose are recognised as ion-exchange materials. We are inclined to think that the resolution obtained on CM2 was more efficient than that obtained with CM-cellulose and that the small peak 1 observed in the latter case was due to degradation products. This would mean that the main peak observed with method 2 was in fact a combination of the first two peaks of method 1 as implied by the figures presented above.

Comparison of the amino acid compositions of trout testis protamine components after chromatography on CM-Sephadex C25 and CM-cellulose has shown that the figures are different. Since the protamine applied in both cases was isolated from the same species of fish, the observed difference must be due to a change in the distribution of the protamine components among the various peaks (294). These results would seem to support the hypothesis presented above and amino acid analysis of the various peaks would enable definitive conclusions to be drawn, although this was not attempted.

Figure 7.6

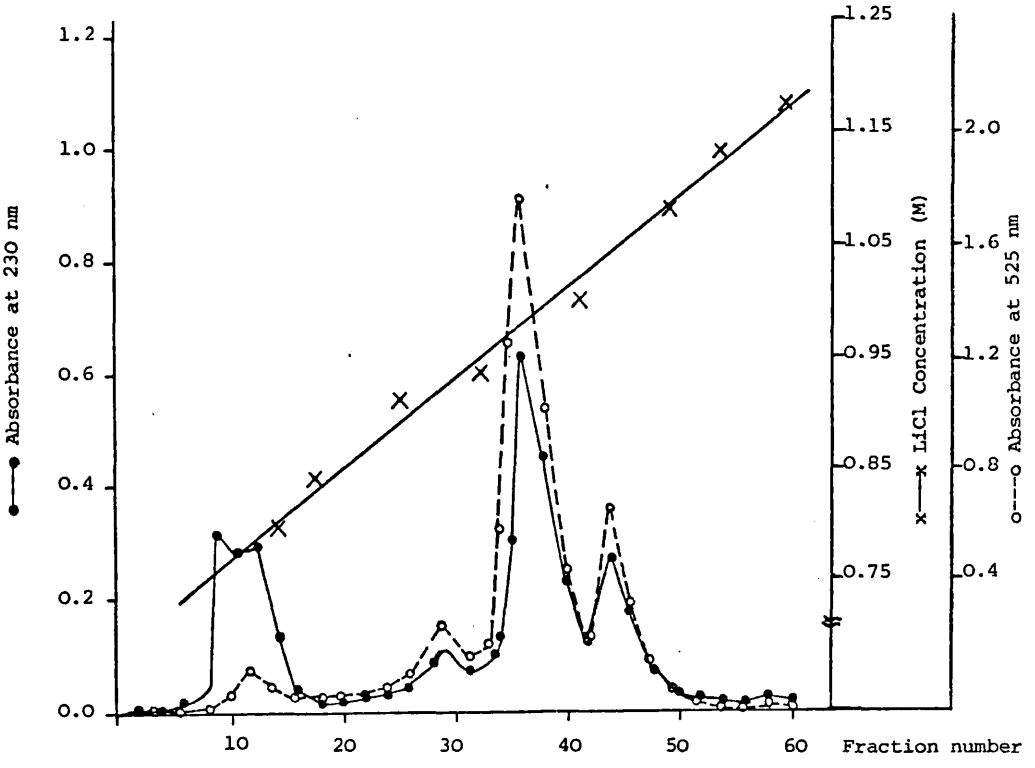
Starting buffer chromatography of protamine on BioGel CM2



Column dimensions	=	0.9 x 130 cm
Sample added	=	31.5 E_{230nm} units (28.6 mg)
Flow rate	=	2.5 ml/hour
Buffer	=	1.5M sodium chloride in 0.05M acetate buffer, pH 5.8
Fraction volume	=	5 ml

Figure 7.7

Linear gradient chromatography of protamine on CM-cellulose



Column dimensions	=	1.1 x 40 cm
Sample added	=	17.7 E_{230nm} units (16.1 mg)
Flow rate	=	15 ml/hour
Buffer	=	700 ml linear gradient, 0.75 - 1.3 M lithium chloride in 0.01 M lithium acetate buffer, pH 5.0
Fraction volume	=	5 ml

One fact that clearly emerges from the above results is that standardisation of conditions is of great importance, as the separation criteria are clearly very subtle; small changes in operating conditions may have quite serious consequences, especially when measuring radioactive components, since labelled arginine is a very unspecific way of recognising these components.

Method 1 is obviously better for resolving the three herring protamine components and is potentially a suitable method for analysing in vivo labelled protamine components as a function of development. Components of this type are characterised by low specific activity, especially when isolated from the later stages. Thus, relatively large amounts of protein must be processed in order to detect the radioactivity and long experimental turn-around times are therefore unavoidable. A somewhat different situation arises when analysing in vitro labelled components, since although the basic analytical procedures remain the same, they must be adapted to the study of small quantities of highly labelled material and to large numbers of samples. However, the extreme length of the column and the slow flow rate of method 1 resulted in a turn-around time of more than one week per experiment and large amounts of material were also required for easy detection. This method was therefore clearly unsuitable for analysing large numbers of in vitro labelled protamine samples. Gradient elution from CM-cellulose, on the other hand, was a much faster technique, but had less resolving power. It also suffered from the disadvantage of requiring gradient construction equipment, which again precluded simultaneous analysis of multiple samples. Thus, it was decided to design a hybrid method which would hopefully combine the fast flow characteristics of CM-cellulose with the resolving power of single buffer elution (infinitely shallow gradient) and which would circumvent the necessity for specialised equipment. The protamine sample was applied in acetate buffer, pH 5.0 and unbound material and degradation products were washed through with 0.75M lithium chloride in buffer. Protamines were eluted by 0.95M lithium chloride in buffer. 0.95M lithium chloride was chosen because this is the concentration at which protamine is just washed from the column in gradient elution. Resolution in this type of chromatography depends on differential absorption and desorption along the length of the column and is therefore significantly affected by column length, long, slender columns

being more useful than short, fat ones. A series of columns of increasing length but constant diameter were tried to see whether the resolution could be improved. Only two of the three components were resolved in each case, and the resolution appeared to be independent of column length for the sample and experimental conditions used. A typical elution profile is shown in Figure 7.8. The distribution of the two peaks as a percentage of the total is similar to that observed during gradient elution above (Table 7.1).

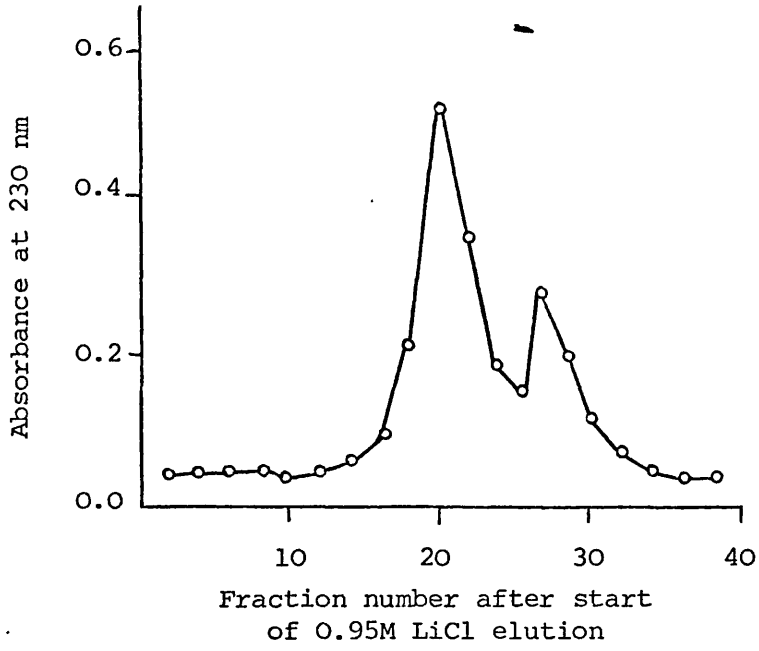
Thus, under the conditions used for these experiments, it appeared that stepwise elution had no more resolving power than gradient elution. Therefore, for analysis of *in vitro* labelled components, acceptance of inferior resolving power seemed unavoidable.

The situation was further complicated by the fact that the protamine may exist *in vivo* in phosphorylated and non-phosphorylated forms, depending on its time of synthesis. It has been shown that newly synthesised trout protamine is phosphorylated on its serine residues and is gradually dephosphorylated. This phosphorylation results in a decrease in the net charge of the molecule, causing early elution from the ion-exchange column. Under these conditions, protamine which normally elutes with peak 3 is found in peak 1, i.e. a spurious distribution results (310). That phosphorylation also occurs *in vitro* has been implied in analysis of protamine produced in the Krebs ascites cell-free system (158). It has been suggested that the phosphorylation is due to a cAMP-activated protein kinase (158). This is a ubiquitous enzyme which has been shown to catalyse the esterification of added protamine (311).

These studies suggest that it is necessary to remove the phosphate groups in both *in vitro* and *in vivo* protamine samples in order to circumvent unnecessary interpreteative difficulties. This has been accomplished with trout protamine by incubation with *E. coli* alkaline phosphatase prior to the separation of the protamine components (309). This method was attempted with herring protamine, the mixture being applied to a CM2 column at the end of the incubation. Under these conditions, however, the original three protamine peaks were not present (Figure 7.9). It was thought that the presence of the enzyme, or the tris buffer, or both may have altered the protamine-CM2 interaction. It was therefore decided to move the incubation back a step in the process of purification to the point immediately before P-10 chromatography.

Figure 7.8

Stepwise elution chromatography of protamine on CM-cellulose



Column dimensions	=	1.0 x 65 cm
Sample added	=	22.5 E_{230nm} units (20.5 mg)
Flow rate	=	15 ml/hour
Fraction volume	=	5 ml

The column was washed firstly with 0.75M lithium chloride in lithium acetate buffer, pH 5.0 till the E_{230nm} approached zero, then with 0.95M lithium chloride in buffer.

Table 7.1

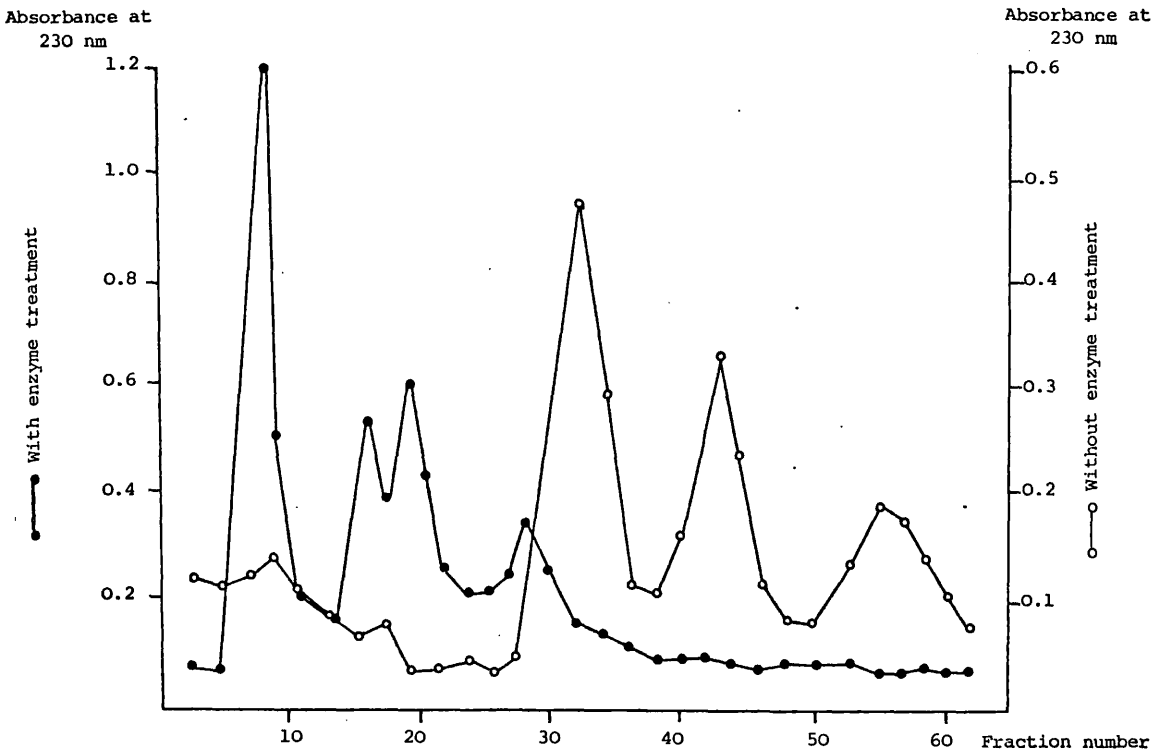
Summary of protamine component distribution obtained with different techniques

	% of total $E_{230_{nm}}$ in each peak.		
	I	II	III
BioGel CM 2	41.12	34.14	24.74
CM-cellulose (gradient)	6.25	71.63	22.12
CM-cellulose (stepwise)	77.6	22.3	-

Data obtained from Figures 7.6, 7.7 and 7.8

Figure 7.9

Effect of prior treatment with E. coli alkaline phosphatase on the chromatography of protamine on BioGel CM2



Sample applied = 31.5 $E_{230\ nm}$ units (28.6 mg)

Conditions as in Figure 7.6. When enzyme treatment was used the protamine was dissolved in 1 ml of 0.5M tris, pH 7.6 containing 1 mg of enzyme, incubated at 30° for 3 hours, cooled and applied to the column. Elution was carried out as in Figure 7.6.

It was hoped that this would remove these molecules. Pilot experiments showed that the enzyme appeared at the void volume of the column, before the protamine, and that most of the buffer eluted after the protamine (the tail end of the protamine peak was discarded to eliminate any residual contamination by tris).

The protamine sample thus obtained was chromatographed on CM2. As can be seen from Figure 7.10, the required separation was still not obtained. The elution profiles shown in Figures 7.9 and 7.10 are very similar, which implies that the loss of resolution was due to a change in some property of the protamine itself. Analogous experiments with linear gradient chromatography of enzymatically dephosphorylated protamine also resulted in premature and distorted elution (Figure 7.11).

These results are difficult to explain, especially since the method has been successfully applied to trout testis protamine component analysis (158). Control experiments have eliminated several possible explanations i.e. presence of tris, enzyme or ammonium sulphate, or application of sample in a high pH buffer. Thus, despite considerable effort, no obvious solution to the problems could be found and the analysis of herring protamine components, labelled both *in vitro* and *in vivo*, remains to be accomplished.

8. Protamine mRNA

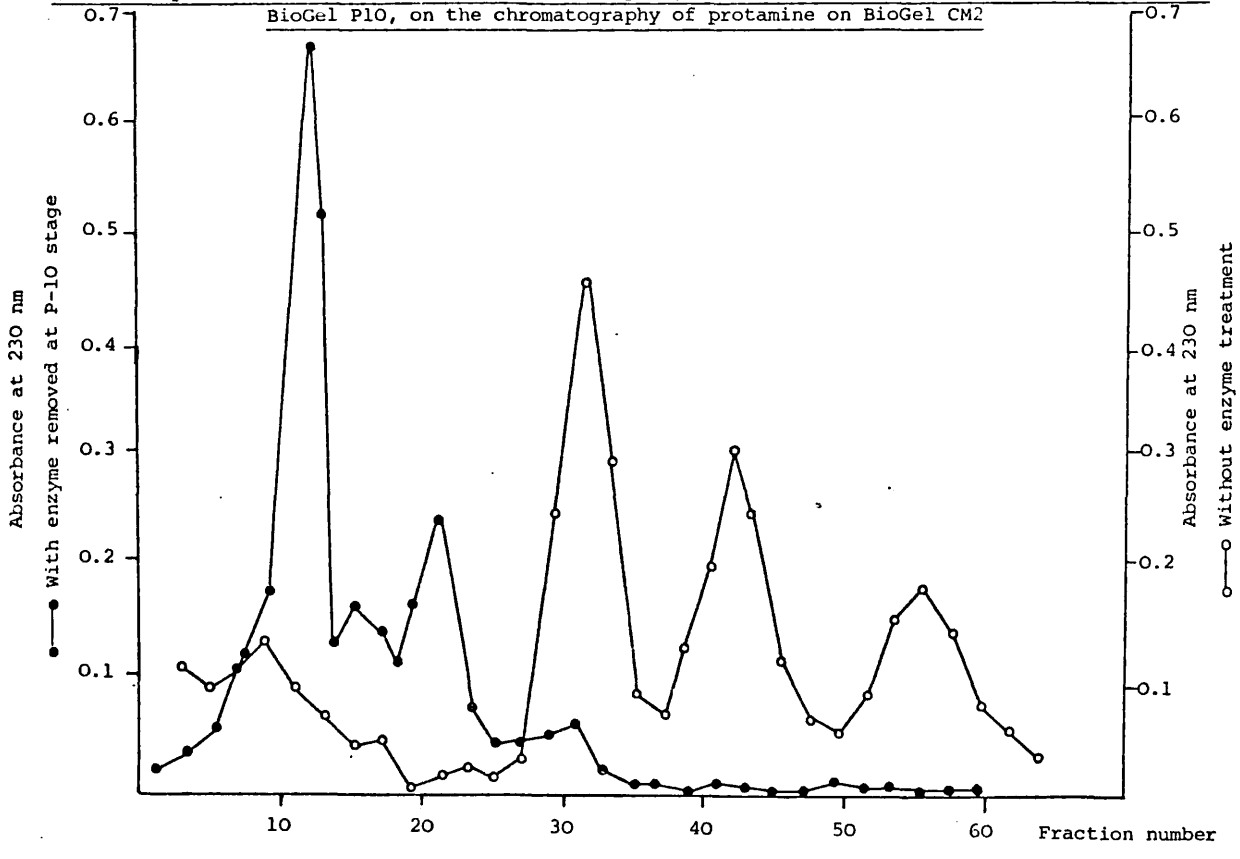
These studies can be divided into two sections, isolation of mRNA from testis material and characterisation of protamine mRNA.

8.1 mRNA isolation

mRNA can be obtained either from total cellular RNA or from polysomal RNA. Each of the two sources has advantages and disadvantages. Isolation from total RNA minimises loss through degradative or physical processes but results in a product heavily contaminated with DNA and non-messenger RNA, which must be removed. This presents a major problem in the case of testis cells, which contain enormous amounts of DNA relative to the cell volume. The DNA cannot easily be removed by the usual method of enzymic digestion, since the fragments produced are of a comparable size to that of a mRNA which codes for a protein of approximately 33 amino acids. Utilisation of polysomal RNA as the source

Figure 7.10

Effect of prior treatment with E. coli alkaline phosphatase, followed by removal of the enzyme on BioGel P10, on the chromatography of protamine on BioGel CM2

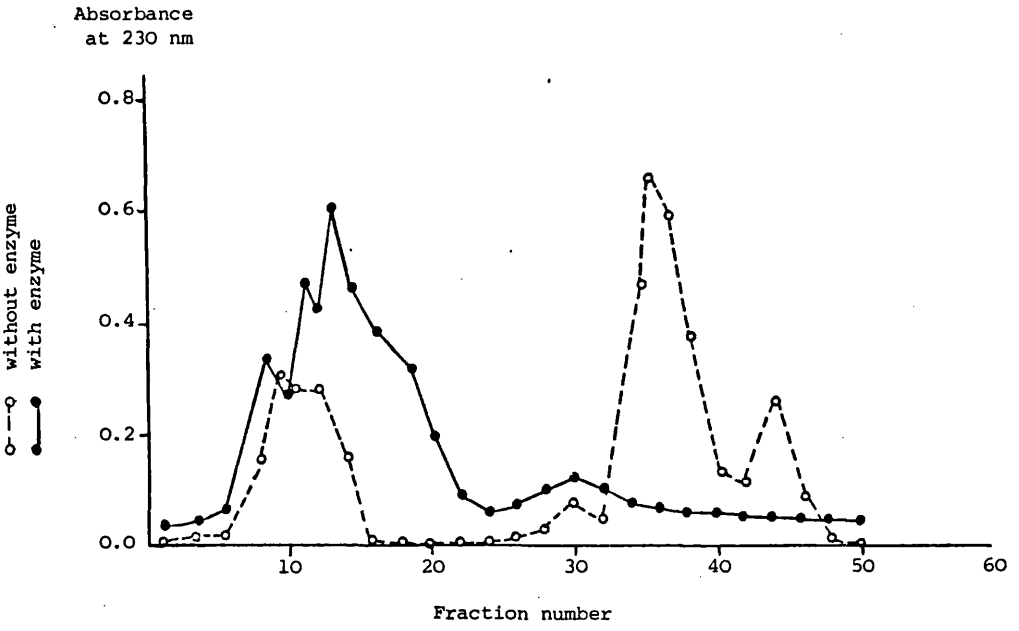


Sample applied = 18.5 $E_{230\ nm}$ units (16.9 mg).

Basic proteins were dissolved in 2 ml of 0.5M tris buffer, pH 7.6 containing 1 mg of enzyme, incubated at 30° for 3 hours, cooled and applied to the P-10 column. The protamine fraction obtained was processed as in section 5.5 and then chromatographed as in Figure 7.6.

Figure 7.11

Effect of pretreatment with E. coli alkaline phosphatase on the elution of protamine on CM-cellulose



Column dimensions = 1.1 x 40 cm
Sample added = 24 E_{230nm} units (21.8 mg)

The protamine was dissolved in 2 ml of 0.5M tris, pH 7.6, containing 1 mg of enzyme and incubated at 30° for 3 hours. The incubation mixture was cooled, 6 ml of deionised water added, and applied to the column. Elution conditions were as described in Figure 7.7.

takes advantage of a purer starting material, but introduces the possibility of degradation of the polysomes during their isolation. This method also results in a low yield of polysomal RNA, since testis cells have a much smaller cytoplasmic volume than other cells and it is not easy to get a good cell breakage rate without simultaneously rupturing nuclei and releasing chromatin into the medium.

After due consideration of the factors involved, it was decided to try the whole cell method first. However, this approach proved problematical since contact between testis cells and SDS (necessary to dissociate nucleic acid-protein complexes) caused immediate gelling to a point where the viscosity of the medium made further processing impossible. It was thought that perhaps the cell membrane contained some component which caused the gelling. Several attempts were made to determine the nature of such a component, in order to remove it. These can be summarised as follows: careful washing of the cells prior to adding the detergent, in case the component dissociated under these conditions; washing of the cells with buffered non-ionic detergent (Triton X100) which would dissolve membrane-type components; tryptic digests, and lysozyme digests, to remove protein and polysaccharide components respectively. None of these treatments proved successful, however, suggesting that perhaps the gelling was caused by something inside the cells. This could be explained by the extremely high DNA content of these cells, which would result in an extremely viscous solution once released into the medium by the action of SDS. In this case dilution of the medium would alleviate the problem to some extent, but this produces an aqueous phase of unmanageable proportions for subsequent phenol extraction. Digestion by DNAse is out of the question for the reasons described above. It was therefore decided that the whole cell approach should be abandoned as unsuitable for this particular system and attention was focussed on production of polysomes.

Of the several methods available for cell rupture, homogenisation in buffered sucrose was selected as suitable, due to the relative mildness of the treatment. However, in contrast to results reported by Dixon's group (312), this procedure resulted in a breakage rate too small to be of use. Thus, alternative methods had to be found. One possibility, hypotonic lysis, proved suitable and preliminary experiments were carried out to determine the optimal lysis time i.e. maximum

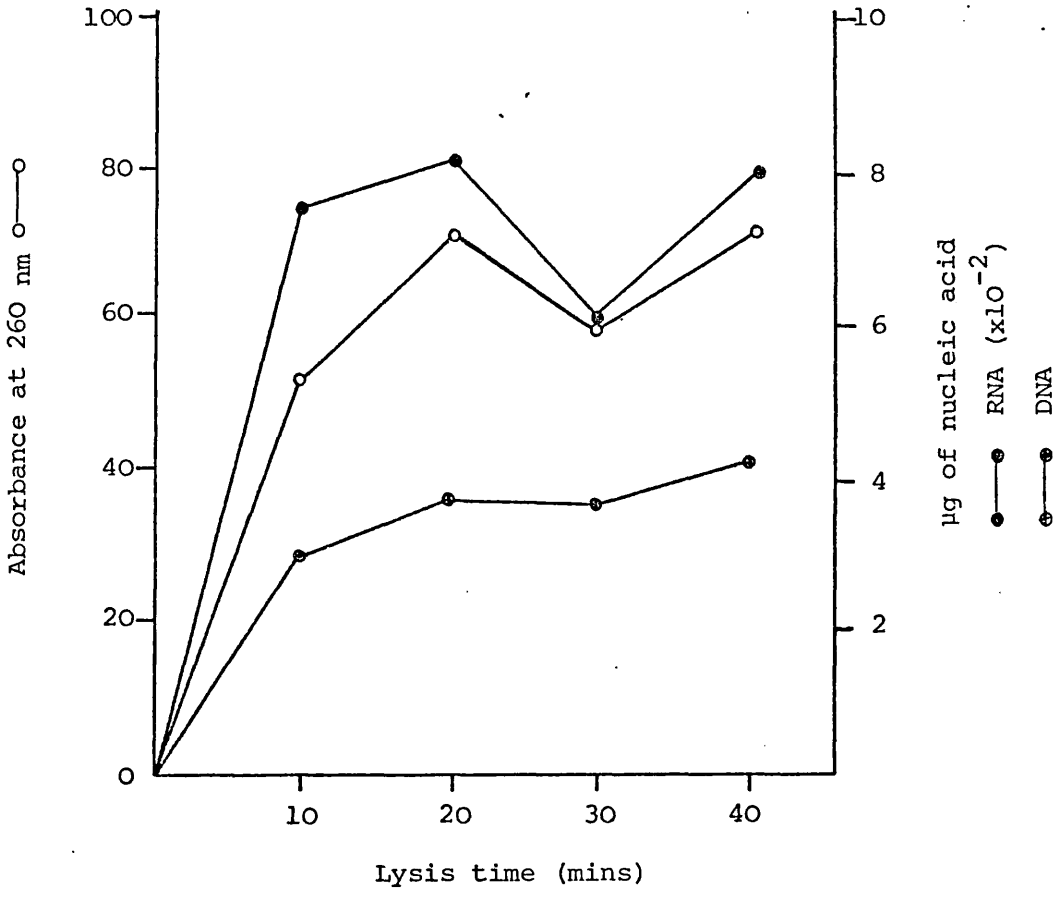
RNA yield with the minimum of DNA contamination from nuclear rupture. Figure 8.1 shows RNA and DNA estimations on the postmitochondrial supernatants after varying periods of cell lysis. The 20 minute time was eventually decided upon. However, the nature of the method necessarily raised the problem of lysosomal rupture, with concomitant release of RNAse. Thus the postmitochondrial supernatant from a 20 minute-lysed preparation was analysed for RNAse activity. It was found that enzyme activity at the temperature used for cell rupture ($0^{o}C$) was undetectable by the method used. (Figure 8.2).

Microsomes were sedimented from the postmitochondrial supernatant and resuspended in buffer and extracted with phenol/chloroform according to the method of Perry (this method allows retention of any poly A stretches which may be present in the messenger population) (313). For the preparation of RNA, phenol/chloroform extraction was chosen in preference to dissociation of the microsomes with EDTA and subsequent sucrose gradient fractionation for two reasons: direct deproteinisation minimises the risk of RNAse degradation, which is the big disadvantage of the EDTA method; since protamine contains approximately 33 amino acid residues, then the minimum number of nucleotides necessary to code for it would be 99, which means that a putative protamine messenger would sediment at the top of a sucrose gradient, along with contaminant proteins.

The RNA thus obtained was tested for messenger activity using the rabbit reticulocyte cell-free system. It was originally thought that this system would be very useful, since it contains very little RNAse and produces principally one endogenous protein (globin), which is easily separated from protamine. It has also been shown that the rabbit reticulocyte system will translate a given mRNA many times (314). Thus, an appreciable amount of protein will be produced by a limited amount of mRNA. The observation that globin contains very little arginine per molecule, whilst protamine is 66% arginine, means that utilisation of arginine as radioactive precursor should result in a highly differential labelling level per molecule which operates in favour of the protamine. The system is not without problems, however, since the relatively easy assay for messenger activity i.e. incorporation of radioactive precursors into TCA-insoluble material, is not possible since rabbit reticulocytes show a marked preference for translating endogenous mRNA (globin). This results in a high background

Figure 8.1

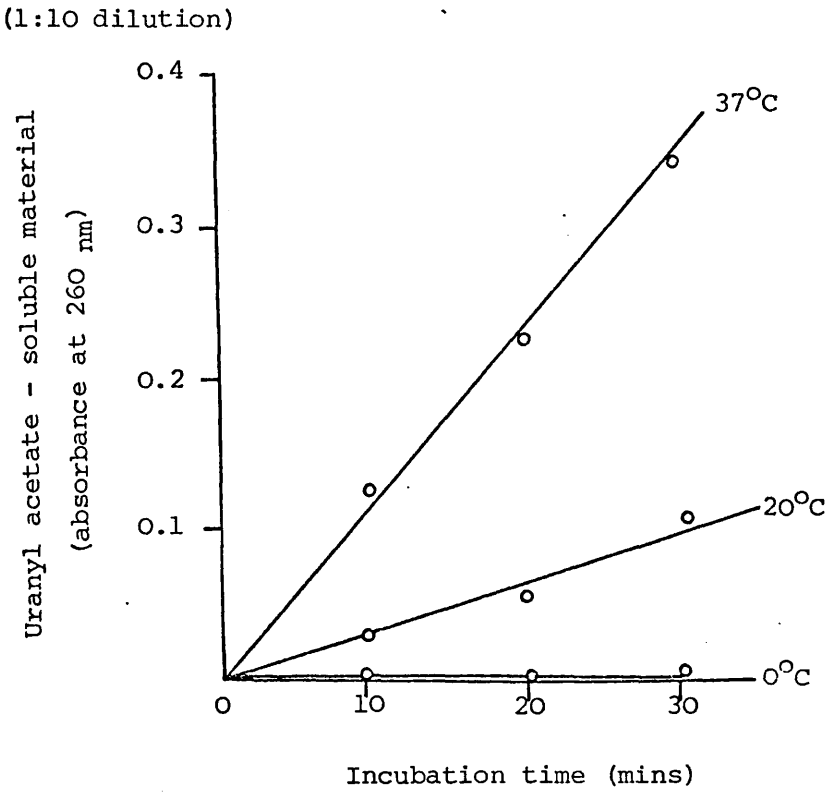
Effect of lysis time on the distribution of nucleic acids in the post-mitochondrial supernatant of hypotonically-lysed testis cells



1 g of cells were lysed in 3 volumes (w/v) of TMN buffer for the times stated. The lysates were centrifuged at 40,000g for 10 minutes and the supernatants thus obtained tested for absorbance at 260 nm, DNA and RNA.

Figure 8.2

RNAse determination in post-mitochondrial supernatant of hypotonically-lysed testis cells at various temperatures



Testis cells were lysed for 20 minutes in TMN buffer. Post-mitochondrial supernatants were prepared by centrifugation at 40,000g for 10 minutes. 0.5 ml aliquots of these supernatants were analysed for RNA activity as described in section 6.2.

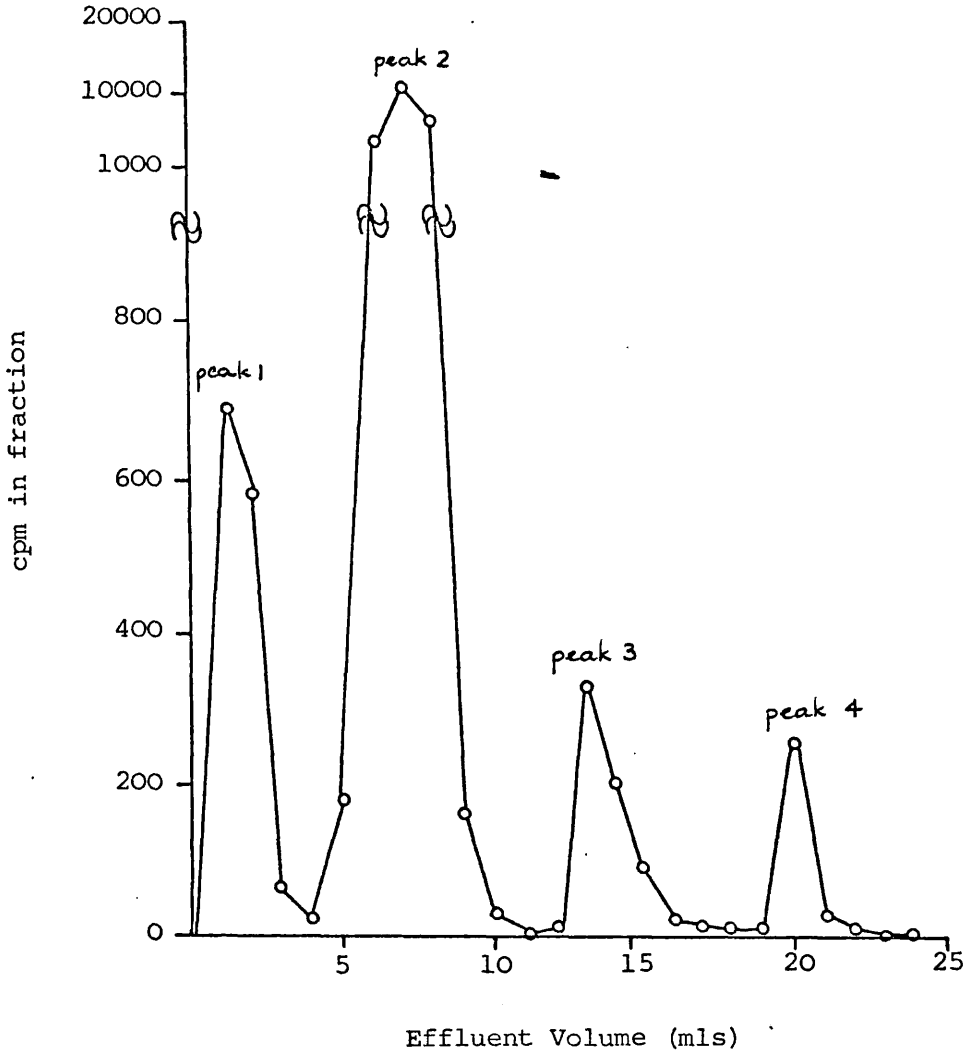
incorporation of radioactivity. The observed stimulation by endogenous RNA is frequently undetectable and, in fact, exogenous RNA has been shown to repress the entire system. Therefore, it is necessary to do some form of product analysis every time an assay is attempted.

Trout protamine synthesised under the direction of protamine mRNA in a reticulocyte cell-free system has been separated from the endogenous product, globin, and also from excess arginine by chromatography on CM-cellulose (158). Increasing amounts of herring testis microsomal RNA were added to the reticulocyte cell-free system and the product analysis carried out in an identical manner. A typical elution profile is shown in Figure 8.3 and is similar to that obtained for trout protamine mRNA. Studies with the trout mRNA showed that peaks 1 and 2 contain non-bound proteins and excess arginine, peak 3 contains globin and peak 4 contains protamine. In experiments with herring testis RNA, radioactivity was always obtained in peak 4, even when the blank incubation containing no RNA was chromatographed. This radioactivity may represent a leakage from either the free arginine or globin peaks. The radioactivity obtained in peaks 3 and 4 was determined as a function of increasing testis RNA input to the cell-free system and the results are presented in Table 8.1. As can be seen, at no time was the reticulocyte system stimulated to incorporate radioactivity into peak 4 in excess of that observed in the blank, implying that protamine was not being synthesised. Further, as the amount of input RNA increased the radioactivity in both peaks 3 and 4 decreased. This could be explained by the presence of an inhibitor in the RNA preparation which had a unspecific, repressive effect on total protein synthesis.

There are several other possible explanations for the above results. The input RNA could have been degraded. Since the levels of RNAse were estimated and found to be low at the temperature used for RNA isolation, this is unlikely. However, the RNAse assay involved precipitation of RNA with uranyl acetate in perchloric acid; this procedure may not have been accurate enough to detect a single break in RNA, which nonetheless had destroyed messenger activity whilst not producing RNA fragments small enough to remain in solution on addition of the precipitating agent. Recently a much more sensitive (and

Figure 8.3

Chromatography of rabbit reticulocyte cell-free system on CM-cellulose



50 µl aliquots from rabbit reticulocyte cell-free incubations were applied to CM-cellulose columns, eluted and counted as in section 6.9.1.

Table 8.1

Relationship between input of testis RNA and protamine synthesised in a rabbit reticulocyte lysate

µg input RNA	Total d.p.m. in globin peak	Total d.p.m. in protamine peak
0.0	28,182	11,010
3.5	24,442	10,739
7.0	23,397	8,950
10.5	18,781	8,409
17.5	16,666	6,051
24.5	17,909	5,970

50 µl aliquots from rabbit reticulocyte cell-free incubations were applied to CM-cellulose columns and eluted as described in section 6.9.1. The radioactivity obtained in the globin and protamine peaks was determined and the values corrected for any quenching by application of the external standard method of efficiency determination.

consequently much more laborious) assay has been developed in which a known amount of specific mRNA activity was added to a preparation of chromatin suspected of containing RNAse. The loss of mRNA activity after contact with the chromatin was quantitated by RNA isolation and in vitro translation. This may well be the standard method of the future (315).

Another possible explanation is based on the fact that certain mRNAs compete badly with endogenous globin mRNA for ribosomes in the rabbit reticulocyte lysate (EMC and duck globin mRNAs). This results in a low translation efficiency for the exogenous RNA in comparison with the rabbit globin mRNA and its is possible that this also occurs with protamine mRNA. Since the RNA sample tested above was also impure, the resulting stimulation may have been too small to be detected. Nevertheless, trout testis RNA has been shown to direct the synthesis of protamine in the rabbit reticulocyte system (158).

The last alternative is that the source tissue, which had been stored for some months, was deteriorating.

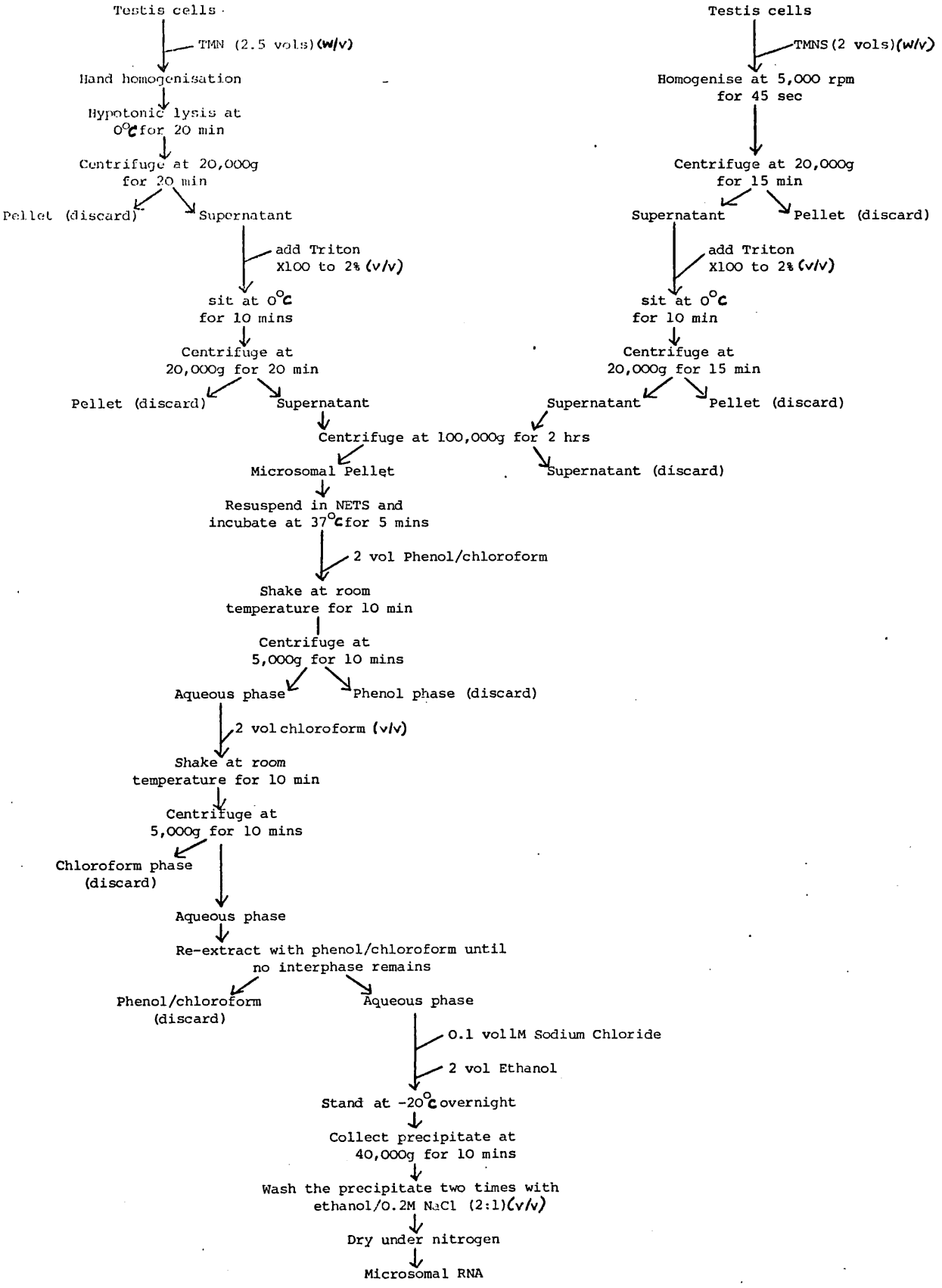
In an attempt to eliminate the possibility of degradation occurring in the tissue prior to isolation of RNA, or during the isolation itself, RNA was prepared from a completely fresh batch of testis. The hypothesis that protamine mRNA might not be translated efficiently in the reticulocyte lysate was circumvented by testing the new RNA preparation in the wheat germ cell-free system. This system has been shown to synthesise several proteins under the direction of the appropriate eukaryotic mRNAs (153, 316). Use of this system involves preincubation of the lysate to remove a large proportion of endogenous messenger activity, thus allowing exogenous mRNA to compete more efficiently for ribosomes and produce a larger stimulation over background.

Quite by chance, it was found that the new batch of tissue could be ruptured by homogenisation in buffered sucrose. Thus, all RNA preparations were made in this way and heparin was also added to inhibit RNAse activity.

Microsomal RNA was prepared from a microsomal pellet in the same way as used previously (for a schematic representation of both methods see Fig. 8.4). Increasing amounts of this RNA were added to the wheat germ system and the pH2 TCA-tungstate insoluble radioactivity was estimated (pH2 TCA-tungstate is the reagent developed by Gardner et al. (314) to quantitatively precipitate protamine, which is known to be partially soluble in TCA, the protein precipitant most commonly used). A linear

Figure 8.4

Isolation of microsomal RNA from herring testis



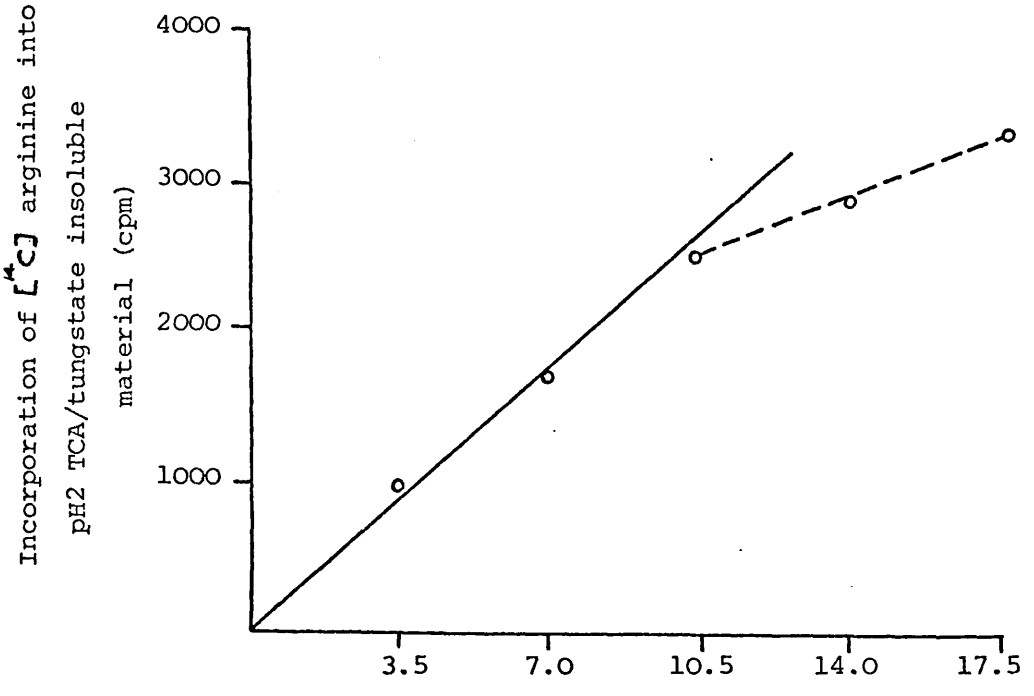
stimulation was observed up to 10.5 µg of RNA added per 50 µl incubation (Fig. 8.5). However, this stimulation is quite low (approximately twice background). In the analogous Krebs ascites system, it has been shown that rRNA can produce an apparent stimulation of this order of magnitude, simply by protecting endogenous mRNA from degradation (318). It was therefore decided to eliminate the possibility of a similar occurrence being responsible for the observed stimulation of protein synthesis under the direction of testis RNA. A suitable system to test this is found in the rRNA from E. coli. It is known that bacterial messengers are not well translated in eukaryotic cell-free systems and so the presence in the rRNA of contaminant mRNAs should not cause a stimulation of the cell-free system. Although a slight stimulation was observed when the wheat germ lysate was incubated with this RNA, the specific activity found was considerably less than that of the testis RNA (Table 8.2). Thus, it appears that the testis RNA causes a real stimulation of protein synthesis, although the results do not prove that the product is protamine. The preparation of RNA used in these experiments was also excessively impure, containing a vast amount of rRNA and tRNA, and so a further purification was necessary before definitive statements about products could be made.

8.2 Characterisation of protamine mRNA

Since RNA can be separated into different size classes by differential salt precipitation, this procedure was used to prepare two fractions, one containing predominantly high molecular weight RNA, the other containing low molecular weight RNA. Since protamine is such a small protein, it was expected that its mRNA would also be small. The corresponding mRNA from trout testis has been shown to have a sedimentation coefficient of 4-6S (158). Both high and low molecular weight fractions were tested in vitro. In this experiment, however, a more specific product analysis was attempted in addition to acid-insoluble radioactivity data. Since protamine has an unusually small size and high basicity, it can be separated from most other proteins by electrophoresis on 20% polyacrylamide gels. This concentration of acrylamide excludes many other proteins and will also separate protamine from histones. It is therefore possible to eliminate virtually every endogenous protein from that portion of the gel to which carrier protamine

Figure 8.5

Relationship between pH2 TCA/tungstate-insoluble radioactivity and amount of testis RNA added to the wheat germ cell-free system



50µl incubations were set up. At the end of the incubation duplicate 20µl aliquots were removed and analysed for pH2 TCA/tungstate - insoluble radioactivity as described in the materials and methods section 6.10.1.

migrates. With this product analysis, it was necessary to eliminate the possibility that free arginine was being estimated due to comigration with protamine, since the protamine band moves quite close to the ion front, as can be shown by electrophoresis of carrier protamine and the basic dye Pyronin Y. Thus, several blank incubations without added mRNA preparations were used as a source of basic proteins after addition of carrier protamines. These proteins were electrophoresed and the protamine band visualised, excised and counted. In all cases, very little radioactivity was found (average value 150 cpm). Thus, it appears that free arginine does not interfere with analysis of radioactive protamine by this method. When incubations containing herring testis salt-fractionated RNA were analysed in this way, significant amounts of radioactivity were found in the protamine region of the gel (Table 8.2).

Examination of Table 8.2 shows that, contrary to expectation, removal of rRNA from the low molecular weight fraction did not result in a dramatic increase in specific activity in that fraction. The reason for this can be seen by looking at the total messenger activity distribution. The majority of the activity was found in the high molecular weight fraction. There are two possible explanations for this result, depending on whether the apparent large size observed for protamine messenger is real or artifactual. If it is real, then the mRNA either has very extensive regions of non-coding RNA of the type observed in lens crystallin mRNA, or is polycistronic i.e. the individual protamine messengers are joined together serially to form one larger molecule. The latter possibility is not very likely, since in general eukaryotic mRNAs appear to be monocistronic (319 - 321). However, both possibilities must be considered. If the result is artifactual, this could have arisen by aggregation of the RNA during isolation. mRNAs from several organisms (e.g. ovalbumin) have been shown to aggregate under certain conditions, usually the presence of high salt concentrations, which is known to encourage formation of base-pairing. Aggregation could either occur via mRNA-mRNA or mRNA-rRNA interaction. If the CGX codon for arginine appears extensively in protamine mRNA in preference to the AG^{A}_{G} codons, then a considerable amount of self-complementarity would occur, making mRNA-mRNA interations possible. The high G+C content of rRNA would also favour mRNA-rRNA complex formation. Experiments designed to determine whether or not protamine mRNA is of high molecular

Table 8.2

Messenger activities in RNA fractions isolated from herring testis

RNA fraction	Radioactivity in acid-insoluble fraction (c.p.m.)		Radioactivity in protamine (c.p.m.)	
	c.p.m./µg of RNA	Total c.p.m. in RNA fraction	c.p.m./µg of RNA	Total c.p.m. in RNA fraction
Total	135	1,080,000	-	-
Low molecular weight	64	31,360	81	39,445
High molecular weight	91	782,600	116	993,300
E. coli rRNA	24	-	-	-

weight were carried out and will be described later.

One interesting property of many eukaryotic mRNAs is the possession of a stretch of polyadenylate residues at the 3' end. This has enabled substantial purification from rRNA, which does not contain this sequence. However, it has been shown that the other group of basic chromosomal proteins, the histones, have mRNAs which do not contain this sequence (322, 323). It was therefore of interest to see whether protamine mRNA contains this stretch, both from the abstract theoretical viewpoint and from the immediate practical necessity of separating the messengers from contaminant rRNA present in the high molecular weight fraction.

As was mentioned above, formation of base pairs i.e. hybridisation, is favoured by the presence of raised salt levels. Under these conditions (0.5 M potassium chloride), the poly A stretches present in many mRNAs can be induced to hybridise with complementary oligonucleotides (oligo (dT)) which have been immobilised by attachment to cellulose. RNA molecules not containing poly A of sufficient length to hybridise are eluted with excess application salt solution. The ionic strength of the medium is gradually reduced by washing firstly with 0.1 M potassium chloride in buffer, then with buffer (or water) alone. The poly A-containing RNA is eluted by the last solution i.e. when the ionic strength of the medium is low enough to render the hybridisation untenable.

High molecular weight RNA was applied to oligo (dT) cellulose, chromatographed as in section 6.6. and the resulting fractions tested for messenger activity. The results shown in Table 8.3 demonstrate the presence of messenger activity in all fractions. (Presumably the RNA eluted by the 0.1 M potassium chloride represents those molecules which were incompletely washed away by the application salt solution and which may have contained poly A sequences capable of only limited binding). The greater majority of the messenger activity occurred in the non-poly A fraction, with a small percentage in the poly A fraction. The presence of poly A-containing RNA in high molecular weight testis RNA has also been confirmed by column affinity chromatography on oligo (dT) cellulose (Table 8.4). The method of Aviv and Leder (143) was used, except that tris buffer was replaced by Hepes buffer. In this case, much more activity adhered to the column than when the suspension method of chromatography was used. This disparity in the results obtained using different

Table 8.3

Messenger activities in high molecular weight testis RNA fractions after suspension affinity chromatography on oligo (dT) cellulose

RNA fraction	Radioactivity in acid-insoluble fraction (c.p.m.)		Radioactivity in protamine (c.p.m.)	
	c.p.m./µg of RNA	Total c.p.m. in RNA fraction	c.p.m./µg of RNA	Total c.p.m. in RNA fraction
Poly A (-) (500 mM KCl)	176	1,602,500	217	1,977,100
Poly A (-) (100 mM KCl)	1,500	30,000	3,086	61,735
Poly A (+) (0 mM KCl)	1,142	20,000	2,006	35,110

Acid-insoluble fraction is the fraction that is insoluble in pH2 trichloroacetic acid-tungstate reagent.

Table 8.4

Messenger activities in high molecular weight testis RNA fractions after column chromatography on oligo (dT) cellulose

RNA fraction	Radioactivity in acid-insoluble fraction (c.p.m.)		Radioactivity in protamine (c.p.m.)	
	c.p.m./µg of RNA	Total c.p.m. in RNA fraction	c.p.m./µg of RNA	Total c.p.m. in RNA fraction
Poly A (-) (500 mM KCl)	292	1,259,396	284	1,222,736
Poly A (-) (100 mM KCl)	275	482,350	217	386,757
Poly A (+) (0 mM KCl)	3,471	208,260	6,664	399,840

Acid insoluble-fraction is the fraction that is insoluble in pH2 trichloroacetic acid-tungstate reagent.

chromatographic techniques may possibly be explained by increased binding efficiency of the column method when compared with the suspension method.

The presence of high molecular weight testis RNA in poly A (+) and poly A (-) forms was a little unexpected, both by analogy with histone mRNAs (which do not contain poly A at all) and with many other mRNAs (which appear to exist entirely in the poly A (+) form). Cases where a given mRNA existed in poly (+) and poly A (-) forms were therefore rare, the only other systems exhibiting this type of distribution being that for trout testis protamine (158), ewe α-casein (170), HeLa cell (216) and sea urchin mRNAs (217).

However, before it could be stated definitely that herring protamine mRNA exists in two forms, with and without poly A, control experiments were necessary to exclude two alternative explanations for the results obtained above i.e. overloading of the oligo (dT) cellulose and degradation of the poly A-containing mRNA during isolation. Overloading was tested by applying increasing amounts of high molecular weight RNA to a constant amount of oligo (dT) cellulose and measuring the messenger activity which hybridised as a function of input RNA (Table 8.5). It can be seen that the proportion remains relatively constant and since the minimum and maximum loading values occur on either side of that used to obtain the data in Table 8.3, it would appear that overloading of the oligo (dT) cellulose is not the reason for the appearance of mRNA activity in the poly A (-) fraction. Degradation is a much more difficult problem to prove or disprove, since the quantity of poly A (+) mRNA is such a small percentage of the total activity. It would need only a small proportion of the poly A (-) RNA to remain (due to incomplete washing) to double the yield in the poly A (+) fraction. However, when RNA isolation and analysis is accomplished without the usual precautions against RNAse action e.g. heparin addition, sterilisation of all glassware and solutions etc., the yield of poly A (+) RNA is never significantly less than that obtained when these precautions were taken. Also, the yield of poly A (+) mRNA was surprisingly constant over a large number of preparations, something not expected if the RNA was broken down unspecifically by necleolytic attack. Thus, it would appear that degradation is not responsible for the large amount of poly A (-) RNA, although this has not been rigorously proven.

Table 8.5

Examination of the capacity of oligo (dT) cellulose to selectively retain poly A (+) RNA from increasing amounts of testis RNA

Quantity of RNA applied to column (mg)	2.5	5.0	6.5
Messenger activity bound by column (cpm)	18,500	33,500	49,000
Messenger activity bound per mg input RNA	7,400	6,700	7,538

Increasing quantities of high molecular weight RNA were added to 150 mg of oligo (dT) cellulose and affinity chromatography performed as in section 6.6. The poly A (+) RNA was isolated from the water eluate by precipitation. The washed and dried precipitate was dissolved in 0.1 ml of sterile water. 2µl aliquots of this solution were assayed for messenger activity as described in section 6.10.1 and the figures corrected to the activity in the whole fraction.

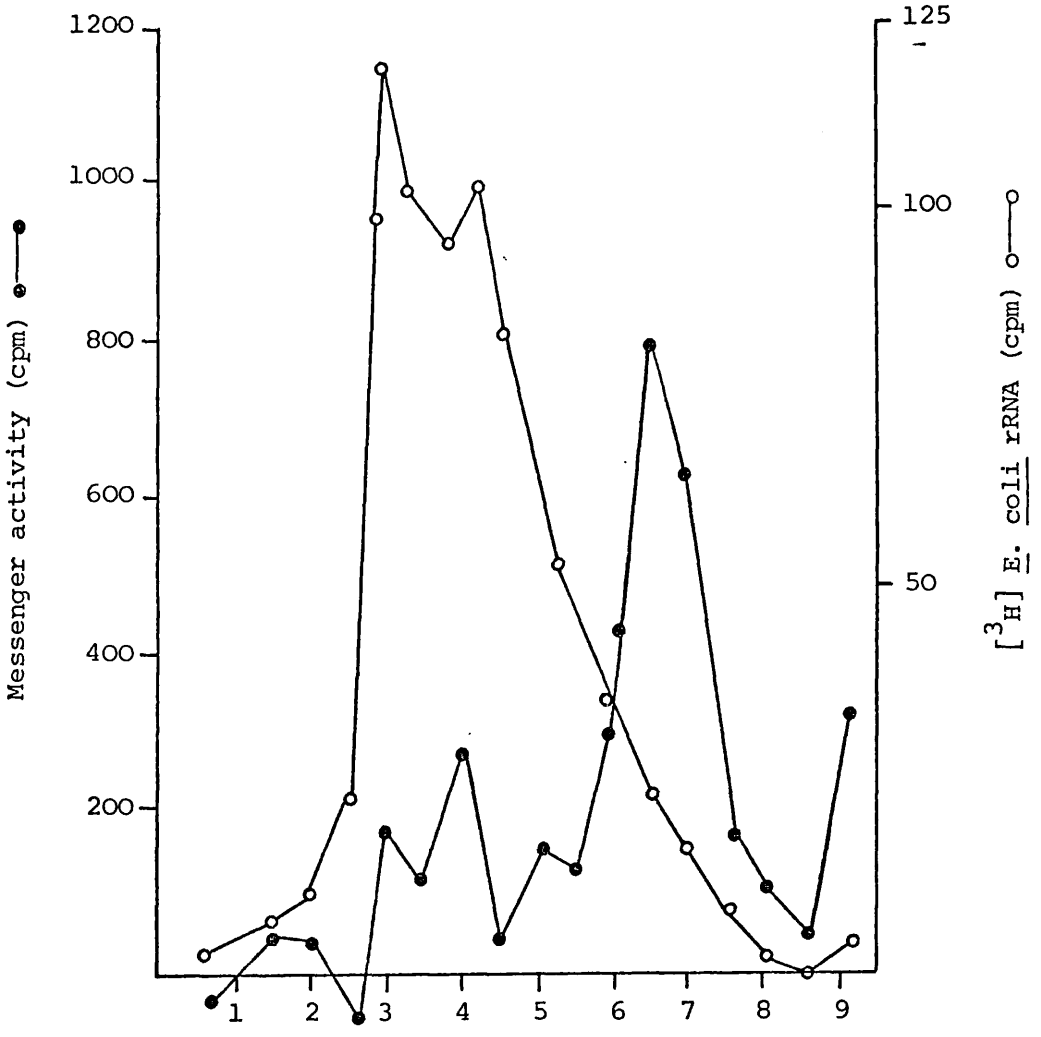
Thus it is tentatively concluded that protamine mRNA from herring testis does exist in a polyadenylated and non-polyadenylated form. It is possible to confirm the presence of poly A and to quantitate the amount present by hybridisation of the mRNA with radioactive oligo U, followed by separation and analysis of any duplexes formed, although this has not been attempted.

Returning to the determination of the size of protamine mRNAs, both poly A (+) and poly A (-) high molecular weight RNAs were analysed on sucrose gradients, both before and after treatment with formamide (a denaturing agent which breaks down any RNA aggregates). The preliminary experiments to set up centrifugation conditions are described in Appendix II. The results of these analyses are shown in Figures 8.6 to 8.9. These gradients were run in low salt buffer in an attempt to prevent dissociated aggregates from reforming and because high salt buffer would have caused the RNA to precipitate out during the long period of centrifugation required. As can be seen, most of the messenger activity appeared at the top of the gradient, both in the presence and absence of formamide, which implies that low ionic strength and formamide will both dissociate the high molecular weight RNA and thus the presence of mRNA activity in the high molecular weight fraction would appear to be artifactual. Artifactual or not, this apparent distribution of activity has practical utility, as it enables easy separation of the mRNA from any excess contaminant heparin. The presence of heparin in any RNA preparation causes severe inhibition of protein synthesis on addition to a cell-free system and therefore would make subsequent analysis difficult.

Thus, both poly A (+) and poly A (-) protamine mRNAs appear to exist in a low molecular weight form, in agreement with results found for trout protamine mRNA.

Figure 8.6

Sucrose gradient analysis of poly A (+) RNA from herring testis



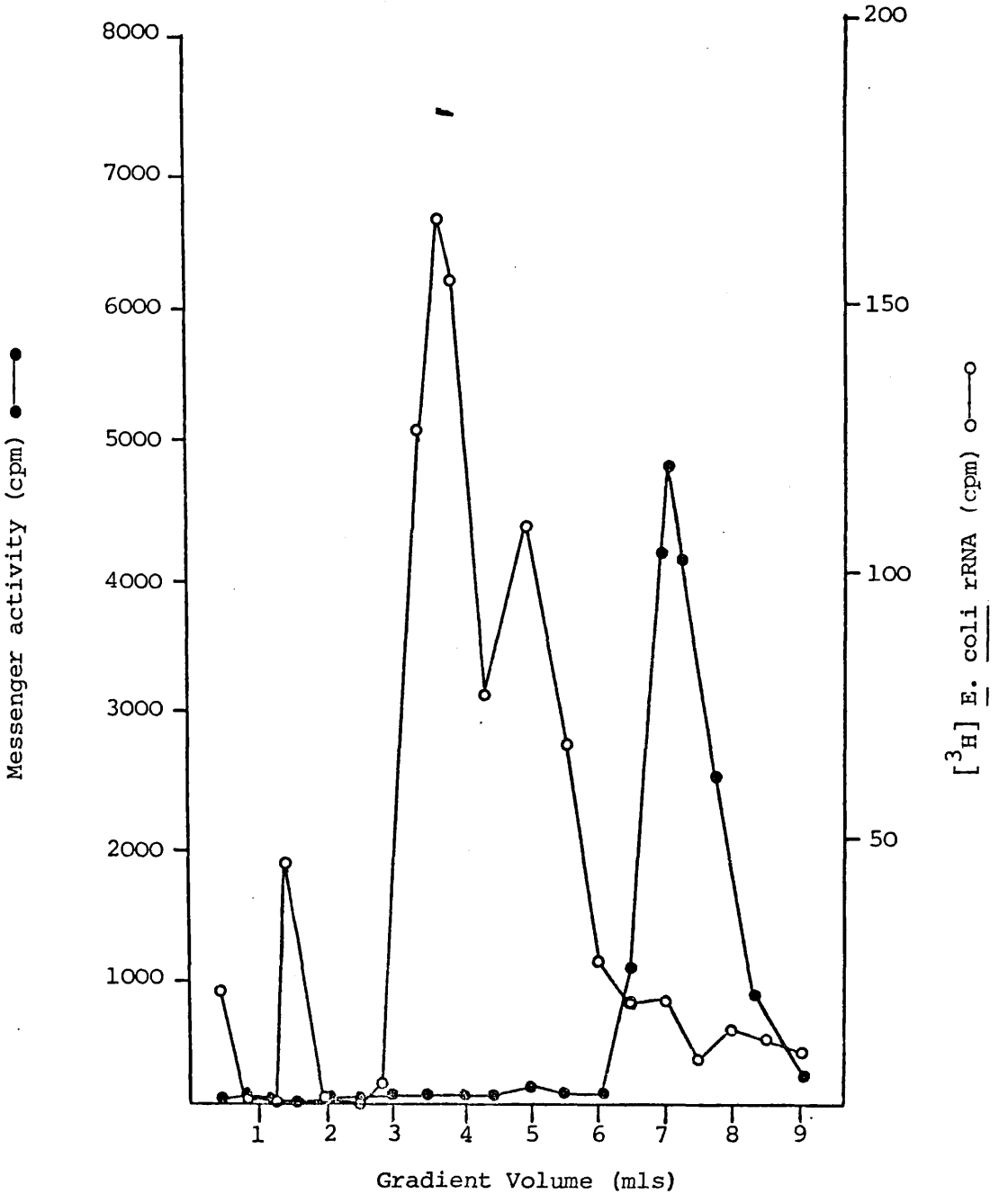
Number of µg of RNA applied = 12.2

Gradient = 8.5 ml linear 5 - 20% (w/v) sucrose

Centrifugation Conditions = 100,00g for 18 hours at 1°C

Figure 8.7

Sucrose gradient analysis of formamide-treated poly A (+) RNA from herring testis

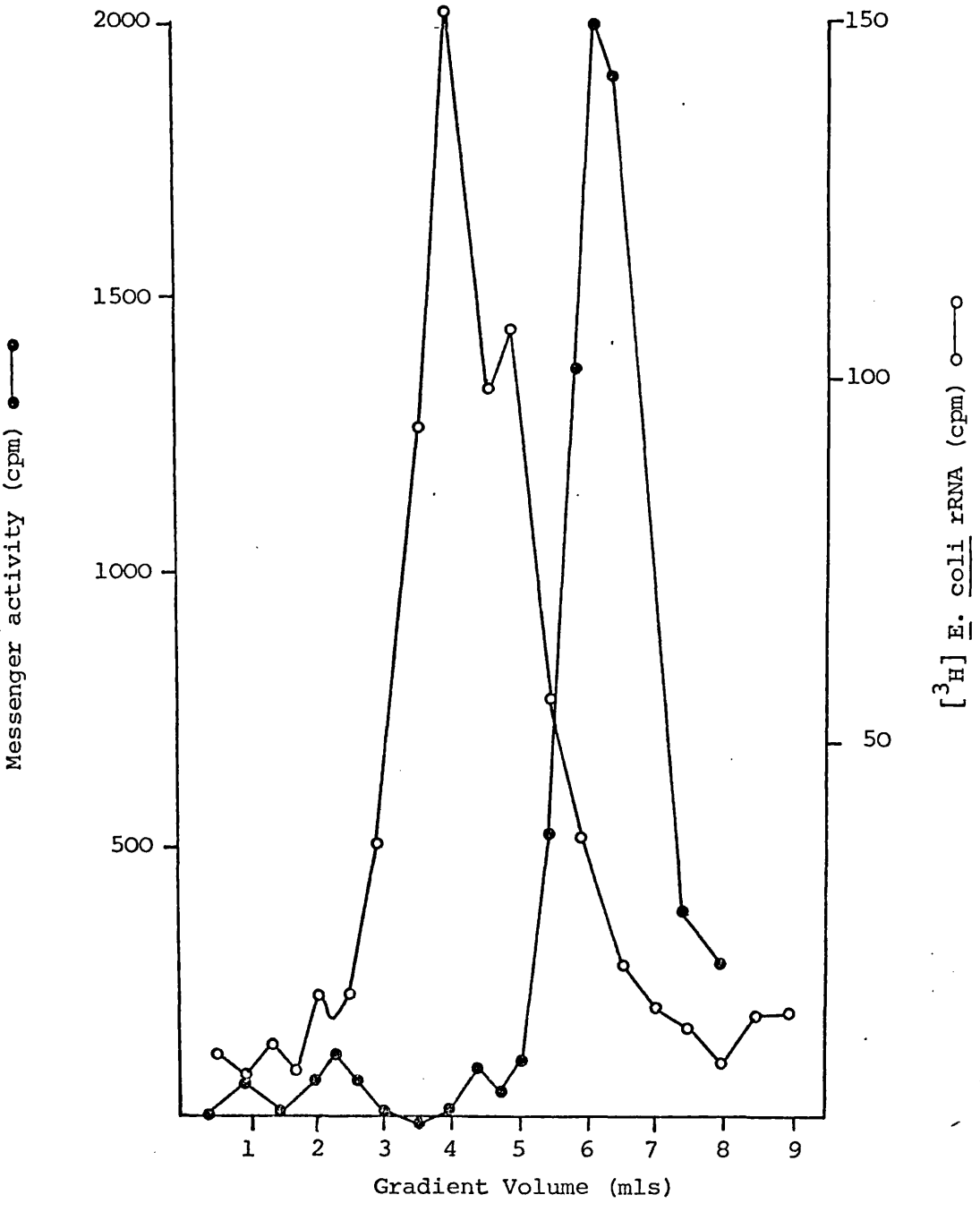


Number of µg of RNA applied = 175

Other conditions as in Figure 8.6

Figure 8.8

Sucrose gradient analysis of poly A (-) RNA from herring testis

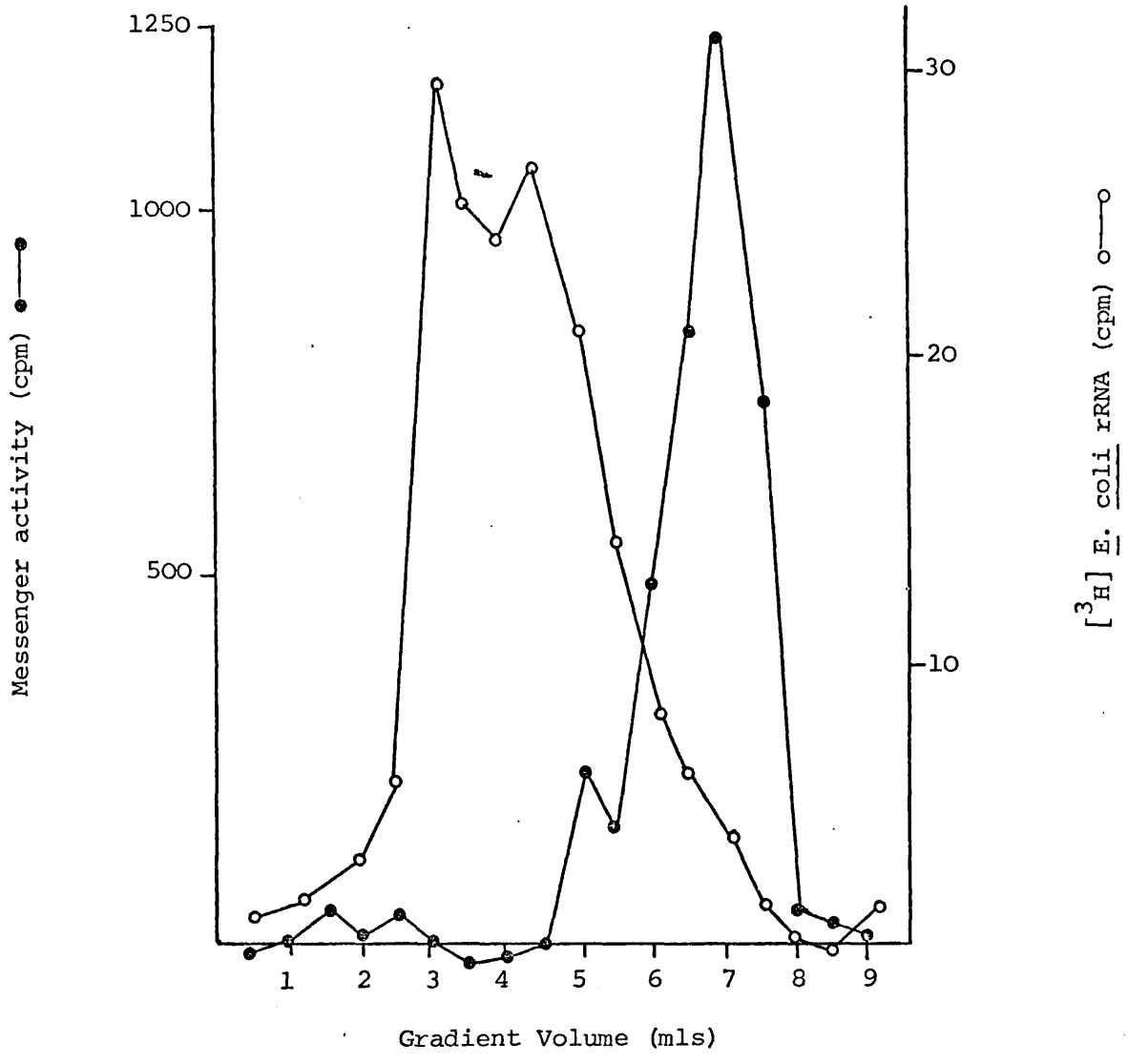


Number of µg of RNA applied = 910

Other conditions as in Figure 8.6

Figure 8.9

Sucrose gradient analysis of formamide-treated poly A (-) RNA from herring testis



Number of µg of RNA applied = 910

Other conditions as in Figure 8.6

SUMMARY

The original objectives were to study the biosynthesis of protamine(s) and the regulation of this process. It was intended that the following would be carried out:-

i) Isolation and characterisation of protamine(s) from *Clupea harengus*.

ii) Examination of protamine biosynthesis *in vivo*.

iii) Isolation and characterisation of the mRNA(s) for the protamine(s).

iv) Examination of protamine mRNA(s) distribution as a function of development with particular reference to regulatory mechanisms.

The studies presented here have demonstrated that protamine from *Clupea harengus* consists of at least three components, a finding recently confirmed by other workers, who also elucidated the amino acid sequence of the components (295). The timing of protamine biosynthesis was also determined and it was shown that protamine is synthesised at a particular period in the maturation sequence of the testis cell. Unfortunately, no success was obtained in attempts to extend these studies to the monitoring of individual component synthesis as a function of development. Despite considerable effort, a suitable method for analysing dephosphorylated protamine components could not be found. This was disappointing, as it had been hoped that differential synthesis of the three components may have been demonstrated, similar to the situation demonstrated in trout (299). Had this been the case, this would have implied that not only is protamine synthesis started at a specific point in time, but that a degree of independent control is exerted over individual component synthesis. In common with other hormonally-controlled systems, the large scale committment to protamine synthesis may well be an example of control at the transcriptional level. However, independent control of individual component synthesis may also be due to post-transcriptional or translational control.

Thus, the mRNA(s) for these components were isolated and characterised as a first step to studies on possible control mechanisms. It was found that protamine mRNA is of low molecular weight (about 4S) and that in the microsomes it exists in two forms, one containing a poly A sequence, the other not.

The small size of the protamine messenger means that it is probably monocistronic, in common with most other known mRNAs. Therefore, the protamine mRNA is probably composed of three discrete mRNAs, each coding for one of the protamine components, although this has yet to be proven. If this were true, it would be very interesting to study these mRNAs as a function of development to see whether the differential rate of protamine synthesis could be correlated with a similar differential in the amounts of individual component mRNAs present. In order to accomplish this objective, it would be necessary to identify and quantitate each individual component mRNA in a series of RNA samples isolated from testes at different stages of maturity. Two approaches are available for such studies, involving characterisation by function or by structure.

Characterisation by function would involve translation in a cell-free system and quantitation of the product. However, this method makes one very important assumption, that all mRNAs are translated with equal efficiency by a given cell-free system. Although this is known to be untrue for unrelated mRNAs e.g. EMC and globin mRNAs, it has hitherto been assumed that closely related mRNAs, like the protamine mRNAs and also α and β globin mRNAs, should have equivalent translational productivity. The fragility of this assumption has recently been demonstrated by studies with α and β globin mRNAs. These studies have shown that the relative amounts of α and β globin produced from a given sample of globin mRNA are critically dependent on several parameters i.e. ionic conditions (324), the amount of mRNA present (325, 326), the presence of suitable initiation factors (327), and the concentration of other cell-free incubation ingredients (328). Thus, extreme care must be taken in extrapolation from product synthesised to mRNA added due to the difficulty of eliminating the possibility of differential mRNA activation. As such, a functional analysis of the relative proportions of protamine mRNAs is of little value and a study of chemical or physical properties, i.e. structure of the molecules, may be more precise.

One possibility would be to separate the individual mRNAs. Until relatively recently, this has not been possible, but the increasing sophistication of available techniques has now enabled the separation of the mRNAs for globins and histones. However, since mRNAs are present in such small quantities and with protamine mRNAs, at least, it is not possible to label them to high specific activities, it is unlikely that the relative concentrations of related mRNAs could be obtained by direct measurement. Thus, a more indirect method of quantitation must be found. If a component mRNA of sufficient purity could be prepared, it should be possible to make a cDNA copy using reverse transcriptase. This could then be utilised to quantitate the amount of its complementary mRNA in a given sample. This method has the attraction that only very small amounts of purified mRNA are necessary to make a cDNA copy and that the hybridisation reaction is very sensitive. It suffers, however, from several drawbacks. The length of time required for efficient hybridisation depends on the stringency of base pairing required i.e. detection of completely matched sequences demands long hybridisation times. Since the type of experiment proposed above requires that the cDNA probes be able to differentiate effectively between the three protamine mRNAs, which will all be very similar in structure, it follows that long hybridisation times or large input of mRNA may be required. The difficulties associated with experiments of this type have been discussed in the introduction. However, experiments to examine the specificity of hybridisation of α and β globin cDNA with the corresponding mRNAs from human and rabbit cells have shown that α globin cDNAs can differentiate between α globin mRNAs from human and rabbit cells (329) and β globin cDNA can differentiate between the two β globin mRNAs. Thus, it may be possible to detect one protamine component mRNA in the presence of the other two. The relative concentrations of each protamine component mRNA in a given sample could then be estimated.

If this approach were successful, analysis of the relative concentrations of each component mRNA as a function of development should then be possible and the correlation with the observed in vivo pattern of component synthesis may be made. However, success in this correlation procedure would yield no information on the mechanisms by

which the differential had been implemented. If the control were exerted at the transcriptional level alone, then different amounts of component mRNAs would be produced as a function of development. To examine this possibility, it would be necessary to study individual component mRNA distributions in the cytoplasm as a whole. If similar distributions were observed to those obtained for microsomal mRNAs, then transcriptional control would appear to be a dominant factor. If this were not the case, however, then some form of translational control must also be operative.

Translational control can be exerted in either of two ways (or a combination of both). Either the uptake of a given mRNA into polysomes or its translational productivity could be regulated. These functions may be dependent in some way on the possession of a poly A sequence. The presence of poly A (+) RNA in protamine mRNA may be important in this respect and re-examination of the distribution studies above may yield useful information.

Thus, the testis system appears, in theory at least, to offer many advantages for the study of differentiation. The problems encountered arose primarily from the difficulty of obtaining quantities of tissue in a fresh condition, especially in the early part of the breeding season, and also from the unavailability of an assay for protamine components. However, sufficiently encouraging results have been obtained that if this assay could be devised, the project should support further studies.

Quite apart from the developmental studies described, protamine mRNA is an attractive messenger to study because its small size makes it one of the few molecules of its type to be amenable to sequencing studies. As such, it certainly merits further attention.

Appendix I

Quantitation of the acid extraction and estimation of protamine

Quantitation of the procedure involves examination of the following steps:

(1) application of the sample to the polyacrylamide gel and counting of the separated protamine;

(2) precipitation of the extract with ethanol to remove the majority of the labelled arginine present;

(3) extraction of the cells with 0.2M sulphuric acid.

(1) Quantitation of the gel electrophoretic prccedure

A cell suspension was incubated with [^{14}C] arginine, extracted with 5 volumes of 0.2M sulphuric acid and basic proteins were prepared as described in sections 5.1., 5.2. and 5.3. In order to determine the effect of the protein loading on both the ability of labelled protein to enter the gel and on the counting technique, increasing amounts of basic proteins were electrophoresed and counted as in section 5.3. The results are given in Table I.

Table I

Quantitation of the detection of radioactive protein in 20% acrylamide gels

Ratio of the volume of radioactive protein solution to the final volume applied to the gel.	cpm in gel	
1:4	157 156	156.5
1:2	239 236	237.5
3:4	314 306	310
1:1	400 409	404.5

As can be seen from the table, a linear relationship was obtained between protein loading and cpm recovered from the gel. In this experiment, the maximum protein loading gave a protamine band which when stained appeared of a comparable density to 200 µg of standard protamine. Thus, whenever a sample of basic protein was electrophoresed and found to exceed this upper limit, the determination was repeated using a more dilute solution and a correction factor applied to make all results equivalent to a standard volume of acid extract. The fact that the dilution series yields a straight line would imply that all, (or a reproducible amount), of the protamine in a given precipitate of basic proteins dissolves and enters the gel under the conditions involved. If this were not the case, then the results would depart from linearity at higher protein loadings and the determination of radioactivity would be underestimated.

(2) Quantitation of the precipitation of basic proteins

Aliquots of an acid extract of labelled cells were precipitated, redissolved in acid and counted directly. The radioactivity observed in each case agreed to within 5% counting error.

(3) Quantitation of the extraction of cells

A cell suspension prepared from 2 g of testis tissue was extracted with 5 volumes (w/v) of 0.2M sulphuric acid. The debris was removed by centrifugation and re-extracted three times. Each fraction was concentrated by ethanol precipitation, dried and dissolved in a standard volume of 0.2M sulphuric acid. 0.2 ml aliquots of these solutions were electrophoresed and counted. The results are presented in Table II.

Table II

Quantitation of the [^{14}C] labelled protamines obtained after successive acid extractions

	cpm in gel	
1st extraction	716, 746	731
2nd extraction	170, 212	191
3rd extraction	60, 56	58
4th extraction	24, 32	28
		1008 total

Since the fourth extract contained only 2.8% of the total, a minimum of four extractions was taken as standard procedure.

The reproducability of the whole method was demonstrated by quantitation of protamine prepared from duplicate incubations from the same testis cell preparation. The results are shown in Table III.

Table III

Quantitation of protamine prepared from duplicate incubations

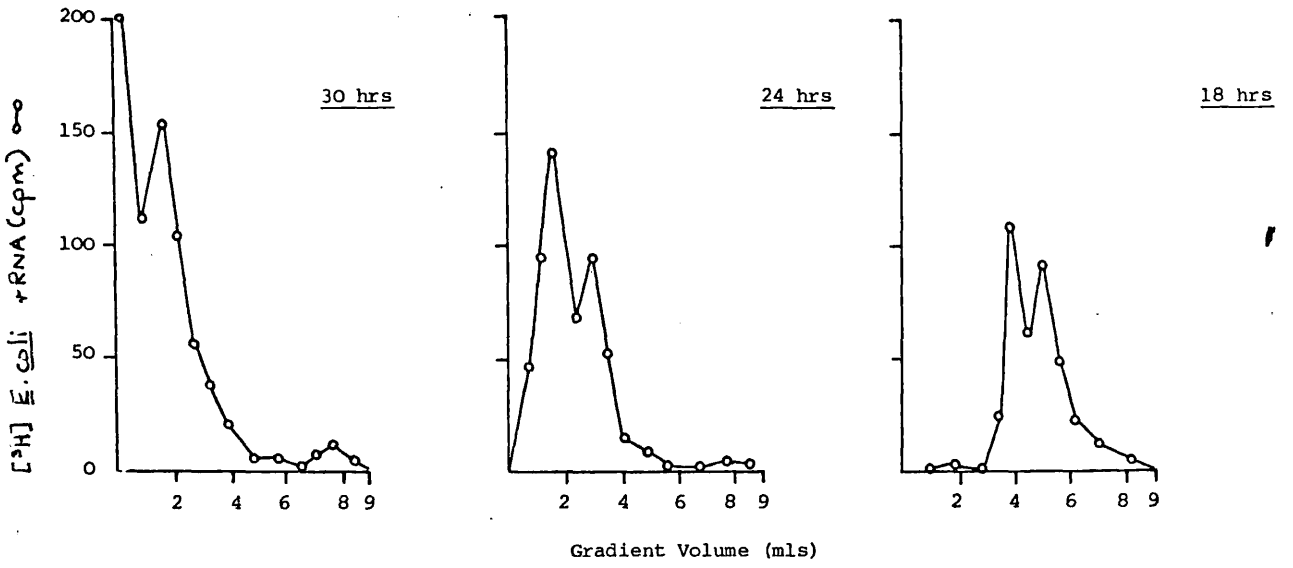
Incubation 1		Incubation 2
1401	Gel 1 (cpm)	1527
1381	Gel 2 (cpm)	1490
1321	Gel 3 (cpm)	1505
1367	Average value (cpm)	1506

Average incubation value = 1437cpm ± 5%

The method of acid extraction, precipitation and electrophoretic analysis therefore appears to be quantitative.

Appendix II

Determination of optimal centrifugation time for RNA analysis on sucrose gradients



In order to determine the optimal time for resolution of E. coli rRNA by sucrose gradient analysis, sucrose gradients were prepared and analysed as described in section 6.8. The gradients were centrifuged at 100,000g for the times shown. 18 hrs was chosen as the best time for general purpose analysis of RNA.

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