CONTROL OF ANTIBODY BIOSYNTHESIS

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To my father, Harry P. Mills

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ABBREVIATIONS

Abbreviations used in this thesis are as laid down in the Biochemical Journal Instructions to Authors (revised, 1976) with the following additions:-

a) General

A-site	Amino acyl-site
DATD	Diallyltartardiamide
DMSO	Dimethyl sulphoxide
DTT	Dithiothreitol
EGTA	Ethyleneglycol-bis-(β aminoethylether) NN' tetraacetic acid
Hepes	N-2-Hydroxyethylpiperazine N'-2-ethanesulphonic acid
hnRNA	Heterogeneous nuclear RNA
MDL	Messenger-dependent lysate
NP-40	Nonidet P-40 (Non-ionic detergent)
PBS-A	Phosphate buffered saline (no Ca^{2+} or Mg^{2+})
poly(A) RNA	RNA species containing poly(A) sequences at their 3' termini-functionally equivalent to mRNA in this study
PPO	2,5 diphenyloxazole
P-site	Peptidyl-site
SAH	S-adenosylhomocysteine
SAM	S-adenosyl methionine
SDS	Sodium dodecyl sulphate
Temed	NN N'N' tetramethylethylenediamine
TMV	Tobacco mosaic virus

b) Serological

Ig	Immunoglobulin
H	Ig heavy chain
L	Ig light chain
γ_{2A}, α	Classes of Ig H chains
k, λ_2	Classes of Ig L chains
pre-L	Precursor to Ig L chain
pre-H	Precursor to Ig H chain
GARIg	Goat anti-rabbit Ig
NorIg	Normal rabbit Ig
RAMIg	Rabbit anti-mouse Ig

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SUMMARY

The postulated mechanism whereby secreted proteins are transferred across cellular membranes involves the synthesis of a hydrophobic amino acid sequence at the N-terminus of the protein molecule which triggers the binding of polysomes synthesizing such proteins to the membrane of the endoplasmic reticulum. To determine the presence of such 'precursor' sequences on secreted proteins, it is necessary to translate the appropriate mRNA in a cell-free translation system since the precursor sequence may be cleaved rapidly *in vivo*. Precursors to several secreted proteins have been identified, including immunoglobulin light chain, but no conclusive evidence for a precursor to immunoglobulin heavy chain has yet been published.

The aim of this work was to study the primary translation products of mouse immunoglobulin heavy and light chain mRNAs in a wheat germ cell-free system. The wheat germ system efficiently synthesizes immunoglobulin light chain but more difficulty is experienced in the synthesis of immunoglobulin heavy chain. This phenomenon appears to be the result of ribonuclease activity. The conditions of cell-free translation have therefore been optimized to minimize the effect of mRNA degradation.

Immunoglobulin heavy chain was identified by immunoprecipitation and subjected to analysis by peptide mapping and amino acid sequence determination. The presence of a precursor peptide could not be determined by peptide mapping due to inadequate resolution of the peptide fragments. A preliminary amino acid sequence of the N-terminal region of heavy chain synthesized *in vitro* is, however, indicative of a precursor molecule since the sequence

contains several hydrophobic residues and does not contain any of the invariant residues common to the known mouse heavy chain subgroups.

1. INTRODUCTION

1.1 EUKARYOTIC MESSENGER RNA

Since the formulation of the messenger RNA theory (Jacob and Monod, 1961), messenger RNA has been shown to display great size heterogeneity with a base composition similar to DNA. This early work was carried out in prokaryotic systems but has since been extended to eukaryotes. Eukaryotic messenger RNA represents only a small percentage of the total cellular RNA and its lack of any known distinguishing physical features hindered its characterization. In the last ten years, however, methods for the isolation of messenger RNA have been developed which have enabled its partial characterization.

Much of the initial study of eukaryotic messenger RNA was carried out using polysomes from reticulocytes. Such polysomes, if treated with SDS, showed the occurrence of both a minor 9S peak and an 18S ribosomal peak (Chantrenne et al., 1967). Treatment of the polysomes with ribonuclease not only fragmented the polysomes but resulted in the loss of the 9S component. From this result, it was concluded that this 9S component was the chain responsible for holding the ribosomes together.

The development of mammalian cell-free systems later showed the capacity of this 9S component to induce the synthesis of globin in a heterologous cell-free translation system (Lockard and Lingrel, 1969). The 9S component was also shown to be the only reticulocyte component capable of inducing globin synthesis when injected into frog oocytes (Lane et al., 1971)

1.1.1 Presence of Poly(A) in Messenger RNA

The most conspicuous structural feature of messenger RNA (mRNA) is a polyadenylate sequence at the 3' end of the molecule. In 1960, a poly(A) synthesizing system was discovered in isolated mammalian nuclei (Edmonds and Abrams, 1960). The actual association of poly(A) with high molecular weight RNA was demonstrated by Edmonds and Caramela, nine years later. They observed that poly(T), bound to a cellulose matrix, was capable of binding a portion of heterogeneous nuclear RNA (Edmonds and Caramela, 1969). The following year, Lim and Canellakis showed the presence of a ribonuclease-resistant adenosine-rich cluster of about 50-70 nucleotides in a 10S globin messenger RNA preparation (Lim and Canellakis, 1970). An earlier estimate of globin mRNA size had already suggested the presence of extra non-coding sequences at the ends of the message (Labrie, 1969). Other workers subsequently confirmed this observation and demonstrated that the poly(A) segment was present in RNA preparations which served as a template for protein synthesis (Lanyon et al., 1972; Morrison et al., 1972; Stevens and Williamson, 1972).

Further studies on poly(A) RNA indicated that the poly(A) sequence was located at the 3' end of the mRNA molecule (Soreq et al., 1974) and that it was not transcribed but added post-transcriptionally (Darnell et al., 1973; Perry et al., 1974). A nuclear enzyme has, in fact, been isolated and purified from calf thymus and shown to have poly(A) polymerase activity (Winters and Edmonds, 1973).

Of several species of messenger RNA that have now been studied, histone messenger RNA is the only well-defined species known to lack this polyadenylate sequence (Adesnik and Darnell, 1972). Thus, the question arises

as to the function of this particular segment of the message. The translational capacity of a message, over a short time interval, does not seem to be affected by enzymatic deadenylation of the native mRNA (Huez et al., 1974; Soreq et al., 1974; Williamson et al., 1974). However, over a longer time period, a significant difference is observed in the rates of translation of native mRNA and poly(A)-free mRNA (Soreq et al., 1974; Huez et al., 1974) which may be due to a reduction in functional stability (Huez et al., 1974) or to a lowered efficiency of ribosome recycling in the poly(A)-free species (Soreq et al., 1974). Doel and Carey have also presented evidence that the poly(A) sequence at the 3' end of ovalbumin mRNA has an effect on its translational efficiency, suggesting that deadenylated mRNA has a lowered efficiency of reinitiation (Doel and Carey, 1976). The poly(A) segment has also been complexed with poly(U) to form a triple stranded complex (mRNA: $poly(U)_2$) and translated quite adequately in a cell-free protein synthesizing system, providing evidence that poly(A) in mRNA does not contribute to secondary structure necessary for protein synthesis (Fernandez and Darnell, 1974).

It has already been mentioned that the poly(A) segment may be responsible for the stability of the mRNA. One feature of the poly(A) sequence which may be related to this is its diminishing size with increasing time. It has been demonstrated that once mRNA molecules reach the cytoplasm, the attached poly(A) sequences get continually shorter with age (Sheiness and Darnell, 1973; Gorski et al., 1975). However, this time-dependent shortening does not seem to be hindered by preventing the mRNA from engaging in protein synthesis which contradicts any 'ticketing' theories similar to that of Sussman, 1970, (Sheiness and Darnell, 1973). The actual functior of the poly(A) sequence, therefore, remains unknown.

1.1.2 Other 3' Non-Coding Sequences

The above mentioned polyadenylate sequences do not constitute the entire 3'non-coding region. Sequence analysis of 52 nucleotides adjacent to the poly(A) sequence of rabbit β-globin mRNA has been carried out and striking sequence and structural homology shown to exist between it and mouse immunoglobulin light chain mRNA. It is suggested that such similarity indicates that this untranslated region has a common function such as the recognition of a part of this sequence, immediately adjacent to the poly(A) sequence, by poly(A) polymerase (Proudfoot and Brownlee, 1974). Furthermore, the sequence A-A-U-A-A-A, some 20 residues away from the 3' terminal poly(A) sequence, has been shown to be present in six different purified mRNA molecules from four different mammalian sources (Proudfoot and Brownlee, 1976). Further studies have shown that the human and rabbit 3' non-coding region sequences of β-globin mRNA are extensively homologous, with the exception of 39 extra nucleotides in the human mRNA (Proudfoot, 1977). Rabbit β-globin mRNA has now been completely sequenced (Maniatis et al., 1976; Proudfoot, 1977) and shown to be 589 nucleotides long, of which 148 are untranslated. Two-thirds of this non-coding sequence is at the 3' terminal end and therefore suggests a strong possibility of a significant functional role.

The function of the 3' non-coding sequences is, in fact, unknown although several suggestions have been put forward. These include the regulatory functions of translation, transport and degradation of mRNA. It has recently been suggested by Baralle, 1977, that the 5' and 3' non-coding sequences interact in such a way as to allow the 3' non-coding region to play a part in initiation of protein synthesis.

1.1.3 5' Terminal Sequences

In prokaryotes, the 5' end of a messenger RNA molecule has a triphosphorylated purine, pppN, corresponding to the residue which initiated primary transcription, unless the message has been processed by cleavage from a precursor. In the latter case, the mRNA would contain a 5'-monophosphate terminus (Cory and Adams, 1975). However, it has recently been discovered that most eukaryotic cellular and viral mRNAs have modified 5' termini.

This modification is in the form of a blocking group, a nucleoside with a methylated base, in 5'-5' pyrophosphate linkage with another 2'-O-methylated nucleoside which is in turn linked to either another such methylated nucleoside or to a non-methylated nucleoside by a conventional 3'-5' phosphodiester bond. This structure was determined by Rottman et al.,1974, and given the name 'cap'. 'Caps' have now been detected in several eukaryotic viral mRNAs, including vaccinia virus mRNA (Wei and Moss, 1975) and reovirus mRNA (Furuichi et al., 1975a). Exceptions include picornavirus and satellite tobacco necrosis virus mRNA (Shatkin, 1976). Polio virus mRNA, which is also included in this category (Nomoto et al., 1976), is now thought to be blocked in some other way - perhaps by a viral protein (Shatkin,1976). A large number of eukaryotic cellular mRNAs have also been studied and all found to have a 5' terminal cap. Such mRNAs studied include HeLa cell mRNA (Furuichi et al., 1975b; Wei et al., 1975), L-cell mRNA (Perry et al., 1975a) and myeloma mRNA (Adams and Cory, 1975). The proposed precursor of mRNA, heterogeneous nuclear RNA (hnRNA), has also been found to possess a 5'-capped terminus.

These blocked 5' termini of mRNA exist in two forms, namely cap I and cap II, with their respective structures m^7GpppX^mpYp and $m^7GpppX^mpY^mpZp$. Results indicate that cap II structures arise by a late methylation of a restricted class of molecules bearing cap I termini. Such methylations occur after the mRNA has entered the cytoplasm (Perry and Kelley, 1976).

There are two main theories as to the derivation of the cap on mRNA. Rottman et al., 1974, proposed that, if mRNA is cleaved from the 3' end of hnRNA, one function of 2'-O-methylated nucleotides would be a high protection of phosphodiester bonds, adjacent to the cleavage site, against nucleases requiring the formation of a 2'-3' cyclic intermediate for their activity. The 5' blocked terminus could then be added after cleavage. Alternatively, the 5' termini of the hnRNA may be conserved to form the 5' termini of mRNAs (Perry and Kelley, 1976).

The actual function of the cap is thought to be related to initiation of protein synthesis. Reovirus and vesicular stomatitis virus mRNAs have been synthesized in vitro by their virion-associated RNA polymerases in the presence of S-adenosyl methionine (SAM). This results in capped messages which are efficiently translated in a wheat germ protein-synthesizing system and unaffected by addition of SAM or S-adenosylhomocysteine (SAH), a methylation inhibitor. Viral mRNA, however, synthesized in the absence of SAM and translated in the presence of SAH exhibits a substantial decrease in ability to synthesize protein (Muthukrishnan et al., 1975). This suggests that 5' 7-methylguanosine is required for the translation of these viral messages. Furthermore, aurintricarboxylic acid, which inhibits polypeptide chain initiation, also prevents mRNA methylation by wheat germ extracts whereas sparsomycin, an inhibitor of elongation, does not. This suggests that mRNA methylation occurs at the initiation step of protein synthesis (Both et al.,1975). Hickey and co-workers have found that the

nucleotide $m^7G^{5'}$ p specifically inhibits translation of mRNAs with the sequence $m^7G^{5'}$ ppp but not of other mRNAs that do not have this group at the 5' terminus (Hickey et al., 1976). They have also shown that 7-methyl and 5' phosphate groups are essential for inhibition of protein synthesis, by using a variety of analogues (Hickey et al., 1977). There is also evidence for a role of 7-methylguanosine in the mechanism of initiation of protein synthesis, related to specific recognition of eukaryotic mRNA by initiation factor $IF\text{-}M_3$ (Shafritz et al., 1976). Such interdependence of cap and initiation factors has also been suggested by Lodish and Rose, 1977, who have shown that wheat germ extracts are much less efficient in translating mRNAs lacking the 7-methylguanosine residue than are reticulocyte extracts. Moreoever, $m^7G^{5'}$ p and $m^7G^{5'}$ ppp5'Am are potent inhibitors of translation of vesicular stomatitis viral mRNAs in a wheat germ protein-synthesizing system but have minimal effect on translation in a cell-free system derived from reticulocyte lysate. It is postulated that this discrepancy between the two systems reflects the absence of relatively low concentrations of appropriate initiation factors in the heterologous plant system and a more complete reliance on a factor which recognizes the 7-methylguanosine of at least heterologous mRNAs.

It is thought, therefore, that this peculiar 5' terminus, together with several extra non-coding nucleotides at the 5' end (Baralle, 1977), plays a part in the initiation of protein synthesis but more information is required before the exact mechanism is known.

1.1.4 Messenger RNA Precursor

The considered precursor of cytoplasmic RNA is the heterogeneous DNA-like RNA synthesized in the nucleoplasm (hnRNA) (Weinberg, 1973). It is considerably larger than mRNA, with a range in size from that of mRNA to

10-100 times longer (Lewin, 1975). A precise relationship between hnRNA and mRNA has been difficult to define since the former is rapidly turned over in the nucleus (Brawerman, 1974).

As previously mentioned, most eukaryotic mRNAs and some hnRNAs have a 3' polyadenylate sequence (Lewin, 1975). Evidence showing the presence of such a poly(A) sequence in both hnRNA and mRNA was suggestive that mRNA was processed from the 3' end of hnRNA. However, labelling of nuclear and cytoplasmic poly(A) RNA in mouse L-cells has shown that much of the nuclear poly(A) is not converted to cytoplasmic RNA but is degraded within the nucleus (Perry et al., 1974). Furthermore, some hnRNA molecules possess a 'cap' structure as described for poly(A) mRNA with sequence homology between mRNA and hnRNA at the 5' terminus (Perry et al., 1975b). A kinetic analysis of the labelling of the methylated components of mRNA and hnRNA indicates that the 5' terminal cap I structures of mRNA are derived from 5' terminal cap structures of hnRNA (Perry and Kelley, 1976). The evidence available is still not conclusive, however, for the formation of mRNA from either the 3' or 5' end of hnRNA.

The inhibition of RNA synthesis by drugs has also been used to obtain some understanding of the hnRNA-mRNA relationship (Darnell et al., 1973; Lewin, 1975). Messenger RNA has a sensitivity to actinomycin in the same range as hnRNA which suggests that either they are both synthesized by the same nucleoplasmic RNA polymerase or that mRNA is derived from hnRNA. On the other hand, cordycepin inhibits mRNA but not hnRNA synthesis. This is open to the interpretations either (1) that hnRNA is not the precursor to mRNA or (2) that the processing mechanism is inhibited (Lewin, 1975).

Further information on hnRNA has been gathered from experiments with cells

infected by DNA viruses. One example of such a virus is the oncogenic virus SV40. Cells transformed by this virus are known to contain viral DNA integrated into cellular DNA and to produce virus-specific RNA. Results indicate that there are large nuclear molecules and smaller polysomal molecules which contain RNA sequences complementary to SV40 DNA (Lindberg and Darnell, 1970). Hybridization experiments have also been carried out using cDNA prepared from purified eukaryotic mRNA of known coding function e.g. ovalbumin mRNA and globin mRNA. The results suggest the presence of mRNA sequences in hnRNA in all cases reviewed by Chan et al., 1977, with the exception of work carried out by McKnight and Schimke, 1975, on ovalbumin mRNA, who concluded from their results that there was no evidence that hnRNA was the precursor of mRNA.

The main objection to the above mentioned hybridization experiments is the lack of controls to ensure that the hnRNA is free of cytoplasmic RNA contamination (Chan et al., 1977). From the accumulated data, however, it appears that at least a small percentage of the hnRNA is the precursor to cytoplasmic mRNA.

1.2 ISOLATION OF MESSENGER RNA

The separation of individual mRNA species and the isolation of mRNA from ribosomal RNA has been hindered by the lack of sufficient distinguishing physical features. Only globin mRNA (Lanyon et al., 1972) and silk fibroin mRNA (Suzuki and Brown, 1972) can be readily separated from the other RNA components owing to unique size and high G-C content, respectively. Methods have now been developed, however, for the isolation of other eukaryotic mRNAs.

1.2.1 Preparation of Polyribosomes

Polyribosomes are normally isolated as the first step in most messenger RNA purifications. Generally, cells are lysed in the presence of magnesium, sucrose, heparin, as a ribonuclease inhibitor, and detergents (Schechter, 1973; Palacios et al., 1972). Depending on the type of cells or tissue used, the amounts of sucrose, heparin and detergent are varied. The homogenate is cleared of nuclei by centrifugation and the cytoplasmic supernatant layered over sucrose. Ultracentrifugation yields polyribosomes collected either as a layer or pellet.

A technique for the precipitation of ribosomes with magnesium was first reported by Takanami, 1960, and has been used recently instead of ultra-centrifugation for the precipitation of polysomes (Lee and Brawerman, 1971; Palmiter, 1974). This method has been shown to provide undergraded polysomes.

1.2.2 Phenol Extraction of RNA

Under specific conditions, it is possible to separate RNA from contaminating proteins by phenol extraction. The phenol extraction of messenger RNA from polyribosomes at neutral pH results not only in the loss of poly(A)-containing RNA from the aqueous phase but also appears to cause the cleavage of poly(A) from the messenger RNA (Perry et al., 1972). Messenger RNA has been found to occur as nucleoprotein complexes (Perry and Kelley, 1966), and it is these proteins which seem to be associated with loss and cleavage of poly(A)-containing RNA. When the extractions are performed at pH 9.0, however, this loss can be avoided or at least reduced. Interactions of the poly(A) segment in messenger RNA with denatured proteins causes the messenger RNA to be lost from the aqueous phase. High concentrations of neutral tris buffer supply monovalent cations which aggrevate the situation and should thus be avoided (Brawerman, 1974).

A valuable discussion of messenger RNA extraction at acid pH is given by Palmiter, 1974. At acid pH, ribonuclease activity is minimized; also, EDTA, used to dissociate the ribosomes, tends to precipitate in the presence of ethanol at alkaline pH whereas it can be used freely at acid pH. Optimal conditions for phenol extraction at both acid and alkaline pH have therefore been defined (Palmiter, 1974; Lee et al., 1971). These conditions have been optimized to minimize the fractionation of messenger RNA into the phenol phase. This can be done by avoiding a neutral pH, monovalent cations e.g. K^+,Na^+, and using a mixture of phenol:chloroform rather than phenol alone (Perry et al., 1972).

1.2.3 Isolation of Poly(A) RNA

The 3' poly(A) terminus of mRNA has proved most useful in the separation of messenger RNA from other RNA components. Several procedures are in fact available. A method for the preparation of oligo dT-cellulose has been described by Gilham, 1964. Oligo dT-cellulose is highly effective in selecting all molecules containing long poly(A) sequences, without binding RNA molecules known to lack them (Nakazato and Edmonds, 1972; Faust et al., 1973). Poly(A) RNA is bound to oligo dT-cellulose in the presence of high salt. Its elution in low salt buffer yields undegraded poly(A) RNA.

Adesnik et al., 1972, have described a method for the preparation and use of poly(U) sepharose. Polysomal RNA is passed over a column in high salt buffer, washed with the same, thus binding poly(A) RNA and providing a means of separation from contaminating ribosomal RNA. The poly(A) RNA can be eluted with a buffer containing 90% formamide.

Hybridization with polyuridylic acid followed by chromatography on hydroxylapatite for the separation of messenger RNA and heterogeneous

nuclear RNA molecules which contain polyadenylic acid has been described by Greenberg and Perry, 1972. Under favourable conditions (high salt and excess poly(U)), a triple helix can be formed with one strand of poly(A) and two strands of poly (U). The RNA is then adsorbed to hydroxylapatite and eluted with a gradient of phosphate buffer. Hydroxylapatite fractionates nucleic acids according to secondary structure, with double or triple stranded structures being bound more strongly than single stranded ones.

Nucleic acids can be made into insoluble gels by ultraviolet-induced cross-linkages. Using this information, poly(U) cellulose has been prepared (Britten, 1963) and used for the isolation of poly(A)-containing RNA (Philipson et al., 1971).

Nitrocellulose filters are also capable of binding poly(A) RNA selectively in the presence of high salt (Rosenfeld et al., 1972; Brawerman et al., 1972

1.2.4 Isolation of Individual Messenger RNA Species

The separation of individual mRNA species from the total mRNA population has been complicated by the size heterogeneity of the 3' poly(A) sequence of the individual mRNA species and the difficulty of separating RNA species of similar molecular weight.

One approach to this problem has been the immunological precipitation of specific protein-synthesizing polysomes by antibody to the protein. Ovalbumin-synthesizing polysomes have been isolated by direct immunoprecipitation by the addition of anti-ovalbumin, ovalbumin and finally a large excess of anti-ovalbumin (Palmiter et al., 1972). When attempting to isolate large amounts of polysomes, sequential addition of ovalbumin and anti-ovalbumin

has some disadvantages. This procedure involves a large excess of antibody and thus high levels of protein, increasing the chance of contamination with ribonuclease. An alternative method has been devised by Palacios et al., 1973. Ovalbumin-synthesizing polysomes were incubated with anti-ovalbumin antibody and then adsorbed to a matrix of glutaraldehyde-linked ovalbumin. The matrix was washed thoroughly to minimize non-specific binding prior to elution of the specific polysomes. A method which avoids the use of large amounts of pure antigen is indirect immunoprecipitation (Shapiro et al., 1974). Polysomes synthesizing ovalbumin or albumin were reacted with antibody to the nascent protein and this soluble complex precipitated by reaction with anti-antibody. The amount of protein added was minimized by using antisera purified by affinity chromatography.

Mueller-Lantzsch and Fan, 1976, have described a method for the immunoprecipitation of specific polysomes using inactivated Staphylococcus aureus. Polysomes synthesizing the major internal structural protein, p30, of Moloney murine leukemia virus were precipitated with anti-p30 antiserum in conjunction with inactivated Staphylococcus aureus. Staphylococcus aureus bears membrane-bound Protein A which has binding sites for the antibody. A greater percentage of the specific polysomes could be precipitated using Staphylococcus aureus than with an anti-antibody antiserum.

Isolation of individual mRNA species, after the preparation of polysomal or poly(A) RNA, involves separation of RNA by size fractionation. This can be achieved either by successive sucrose density gradient centrifugation (Mach et al., 1973), or by gel electrophoresis (Dolja et al., 1976).

All these methods have been applied most efficiently to the purification of major mRNA species coding for proteins which represent about fifty percent of the total cellular proteins.

1.2.5 Isolation of Immunoglobulin Heavy and Light Chain Messenger RNAs

Enrichment of heavy and light chain mRNAs can be achieved by isolating polysomal RNA from microsomes since immunoglobulins are secreted proteins and hence predominantly synthesized on membrane-bound ribosomes (Lisowska-Bernstein et al., 1970). Further purification can be carried out by sucrose density gradient centrifugation and the separation of poly(A) RNA from other RNA species. Mach et al., 1973, have purified light chain mRNA by this method but more difficulty is experienced in applying such methodology to heavy chain mRNA owing to its sedimentation coefficient of 17S which hinders its separation from 18S ribosomal RNA on sucrose density gradients. This difficulty has been avoided by Cowan et al., 1976, by isolating heavy chain mRNA from a cell line synthesizing a heavy chain of reduced size.

Immunoprecipitation of polysomes synthesizing heavy and light chains has been successfully achieved by Ono et al., 1977, and Schechter, 1973.

1.3 TRANSLATION OF EUKARYOTIC MESSENGER RNA

Translation of the messenger RNA code into protein requires the cooperation of ribosomal subunits, transfer RNA (tRNA) and several protein factors. The entire process of protein synthesis can be subdivided into the three stages of initiation, elongation and termination.

1.3.1 Initiation of Protein Synthesis

The first step in initiation is the GTP-dependent binding of the specific initiating tRNA molecule, met-$tRNA_f$, to the 40S ribosomal subunit. After the formation of this complex, the initiator met-$tRNA_f$ directs the binding of the mRNA (Darnbrough et al., 1973; Schreier and Staehelin, 1973). The mechanism of formation of the initiation complex appears to be the same for both mammalian and plant systems (Hunter et al., 1977).

The role of several protein factors in initiation has been reviewed by Weissbach and Ochoa, 1976. The GTP-dependent binding of the initiator met-$tRNA_f$ to the small ribosomal subunit is mediated by an initiation factor, eIF-2, while another initiation factor, eIF-3, assists the binding of the mRNA to this complex. Finally, the formation of the 80S.mRNA. met-$tRNA_f$ complex by addition of the 60S ribosomal subunit is coupled to GTP hydrolysis and may involve the recycling of the initiation factor eIF-2.

1.3.2 Polypeptide Elongation

Following the formation of the 80S-mRNA.met-$tRNA_f$ complex, the ribosome is ready to move along the messenger RNA so that the polypeptide chain can be elongated. The amino acids are added sequentially according to the code of the messenger RNA. As in the initiation process, protein factors are involved in elongation.

The first two elongation factors isolated were two enzyme fractions found to catalyse the transfer of phenylalanine from phenylalanine-tRNA to polyphenylalanine (Arlinghaus et al., 1963). These factors are now called elongation factors 1 and 2 (EF-1 and EF-2). EF-1 has been purified from a variety of sources, such as reticulocytes (McKeehan and Hardesty, 1969), wheat embryo (Golinska and Legocki, 1973) and Krebs ascites (Drews et al., 1974). EF-1 is involved in the bringing of amino acyl-tRNA to the amino acyl site on the 80S ribosomal complex. It has been shown to interact with GTP and amino acyl-tRNA to form a ternary complex, EF-1.amino acyl-tRNA.GTP (Richter, 1970), which is an intermediate in the binding of amino acyl-tRNA to the ribosomal amino acyl site.

Two species of EF-1 have been detected in wheat embryo: a heavy species

($EF\text{-}1_H$) with a molecular weight of 200,000 and a light species ($EF\text{-}1_L$) with a molecular weight of 50,000 which is converted from $EH\text{-}1_H$ by GTP or GDP. The conversion of $EF\text{-}1_H$ to $EF\text{-}1_L$ is faster in the presence of GDP than in the presence of GTP while the conversion of $EF\text{-}1_L$ to $EF\text{-}1_H$ is faster in the presence of GTP (Tarragō et al., 1973). A mechanism for the interaction of the heavy and light forms has been postulated by Moon et al., 1973. $EF\text{-}1_H$ can react with GTP to form an $EF\text{-}1_H$.GTP complex which forms a ternary complex with amino acyl-tRNA. In this complex, EF-1 exists in the light form. This complex can react with the ribosome resulting in the binding of the ribosome and GTP hydrolysis to yield EF-1.GDP. The actual mechanism of recycling $EF\text{-}1_L$.GDP to $EF\text{-}1_H$ or $EF\text{-}1_L$GTP is not known.

Once the amino acyl site (A-site) has been occupied, a peptide bond is formed between the amino acid on the A-site and the elongating nascent chain located on the peptidyl site (P-site). This reaction is catalysed by peptidyl transferase. After peptide bond formation, the nascent chain-tRNA-mRNA complex is attached to the ribosomal A-site. The translocation of this complex from the A-site to the P-site involves the factor, EF-2. This process enables the ribosome to move along the mRNA, one codon at a time but the details of this mechanism remain to be resolved (reviewed by Weissbach and Ochoa, 1976).

1.3.3 Termination of Protein Synthesis

After completion of the polypeotide chain, it is released from the ribosome mRNA complex. This event requires terminator codon recognition, involves protein release factor(s) and occurs on ribosomes. Three polyribonucleotid templates, poly(U,A), (U,A,G) and (U,G) have been found to stimulate peptid termination (Goldstein et al., 1970). The eukaryotic release factor has been purified from rabbit reticulocytes and liver (Goldstein et al., 1970; Beaudet and Caskey, 1971) and used in a formylmethionine release assay. Available information would seem to suggest that the initial step

in the termination event is GTP-dependent binding of the release factor to the aminoacyl site on the ribosome which contains a terminator codon. It is then thought that peptidyl transferase, which normally catalyses the transferance of peptidyl moieties from the peptidyl site on the ribosome to the aminoacyl site to form a new peptide, is involved in hydrolysing the completed peptide attached to a tRNA on the peptidyl site, thereby releasing it from the ribosome (reviewed by Weissbach and Ochoa, 1976).

1.3.4 Cell-Free Protein-Synthesizing Systems

Protein synthesis in the intact cell is a complicated process, making the role of single factors and the mechanisms of protein synthesis difficult to analyse. However, the development of *in vitro* protein synthesizing systems has enabled the study of several aspects of mRNA translation.

The earliest systems were cell-free liver extracts which contained mitochondria. The conditions for the incorporation of labelled amino acids were the same as those required for oxidative phosphorylation. In 1954, Zamecnik and Keller showed that active incorporation of amino acids could be obtained anaerobically in an extract from rat liver simplified by the removal of the mitochondria. This system was activated by the addition of ATP and of substrates such as phosphocreatine, for the continuous regeneration of ATP. At this point, four major components seemed necessary for active incorporation of amino acids: (1) an ATP generating system, (2) a non-dialyzable soluble cell fraction from rat liver, (3) a microsome-rich fraction and (4) amino acids.

It was further shown that if the active proteins in the soluble cell fraction were precipitated at pH 5.2 and added to the microsome fraction, on the addition of ATP and an ATP-generating system, only a slight incorporation of labelled amino acids could be observed. (The proteins precipitated at pH 5.2 are called the pH 5 enzymes). However, the

addition of GTP or GDP restored the activity of the system (Keller and Zamecnik, 1956).

Treatment of microsomes from Ehrlich ascites tumour with deoxycholate to free the lipoprotein gives ribonucleoprotein particles. These ribonucleoprotein particles (subsequently named ribosomes), were found to give active incorporation of labelled amino acids when combined with pH 5 enzymes, ATP and GTP (Littlefield and Keller, 1957).

The optimal requirements were more clearly outlined by Allen and Schweet in 1962. Their cell-free system consisted of ribosomes isolated from reticulocytes, a fraction called AS_{70} enzymes, soluble RNA,ATP and generating system, glutathione and magnesium chloride. The AS_{70} enzymes replaced the pH 5 enzymes and were more stable. They were prepared from the reticulocyte supernatant after removal of the ribosomes. The supernatant was precipitated with protamine sulphate from which complex the tRNA (soluble RNA) was isolated. The remaining supernatant was further precipiated with ammonium sulphate to yield the AS_{70} enzymes.

Using the above described fractionated cell-free system, cell-free protein synthesis was only one to two percent efficient as compared to synthesis in the intact cell. A comparison of different methods of cell-free synthesis suggested a failure of initiation to be the cause of inefficiency in the totally fractionated system (Lamfrom and Knopf, 1964). A cell-free system composed of salt, an energy generating system and total reticulocyte lysate gave ten to twenty-five percent amino acid incorporation into haemoglobin compared to intact cells but the system expired after ten minutes. Addition of haemin to the system improved the activity to better than sixty percent of intact cell synthesis for twenty minutes (Adamson et al., 1968.

The first definitive demonstration of protein synthesis in a mammalian cell-free system under the direction of a messenger RNA isolated from a different mammalian species was made by Lockard and Lingrel, 1969. A 9S fraction of mouse reticulocyte polysomes was purified and shown to direct the synthesis of mouse haemoglobin in a rabbit reticulocyte lysate cell-free system.

Several cell-free protein-synthesizing systems have now been developed from extracts of plant and animal cells and bacteria which have provided an invaluable tool in the study of protein synthesis. Cell-free systems have been prepared from bacteria by disrupting the cells and sedimenting the cell wall and debris. The low molecular weight components, such as amino acids, were then removed by dialysis (Nirenberg and Matthaei, 1961). Similar preparations can be made from eukaryotic cells, including Krebs II ascites cells (Mathews, 1972) and wheat germ (Roberts and Paterson, 1973).

Rabbit reticulocytes also yield an efficient cell-free protein-synthesizing system by a slightly different methodology (Adamson et al., 1968). Reticulocytes are obtained from rabbits which have been made anaemic by injection of phenylhydrazine. The cells are then lysed by osmotic shock and cleared of cellular debris. This system translates endogenous globin mRNA but is also capable of translating exogenous mRNAs very efficiently. A modification of this reticulocyte lysate preparation has recently been reported yielding a cell-free system with the efficiency of the normal reticulocyte lysate system but with no endogenous globin mRNA activity. The endogenous mRNA is degraded by a calcium-dependent nuclease which is subsequently inactivated by addition of EGTA (Pelham and Jackson, 1976).

Exogenous mRNA has also been translated in Xenopus laevis oocytes. This

system requires only small amounts of mRNA and can continue translation for long periods of time (Gurdon et al., 1971).

All the protein-synthesizing systems mentioned above are capable of faithful translation of exogenous mRNA. However, these systems vary in ease of preparation, endogenous mRNA activity and efficiency of translation. For the studies described in this thesis, a cell-free system prepared from wheat germ was chosen. The wheat germ system has been used to translate a variety of mRNAs (See Section 3.1). The system is easy to prepare, synthesizes proteins with good fidelity under optimal conditions and contains very low endogenous mRNA levels which facilitates the identification of the proteins synthesized by the exogenous mRNA. Further details of the wheat germ system will be discussed in the Experimental Section.

1.4 BIOSYNTHESIS OF SECRETED PROTEINS

In mammalian cells, polyribosomes can be divided into two classes: those bound to the membrane of the endoplasmic reticulum and those free in the cytoplasm. Cells synthesizing large amounts of protein for secretion contain a high proportion of membrane-bound ribosomes and it is now accepted that all secreted proteins are synthesized on these ribosomes while non-secreted proteins are generally synthesized on free ribosomes, although there are exceptions (reviewed by Shore and Tata, 1977).

The first evidence that proteins destined for secretion were synthesized by membrane-bound ribosomes was provided by Siekevitz and Palade, 1960, who, by cell labelling experiments, showed that the pancreatic enzyme, alphachymotrypsinogen, was synthesized on ribonucleoprotein particles attached to the membrane of the endoplasmic reticulum. They suggested that the appearance of the protein in other cell components was related

to intracellular transport and storage. Since then, under normal conditions, all secretory proteins studied have been shown to be synthesized on membrane-bound ribosomes including thyroglobulin (Vassart, 1972), collagen (Diegelmann et al., 1973) and albumin (Takagi and Ogata, 1968; Hicks et al., 1969). However, albumin synthesis in 5123 hepatoma is predominantly on free ribosomes but the synthesized albumin, in this case, is not secreted (Uenoyama and Ono, 1972). This suggests that not only are secreted proteins synthesized on membrane-bound polysomes but that non-exportable proteins are synthesized on free polysomes.

The functional differences between membrane-bound and free ribosomes are not quite distinct in that membrane-bound ribosomes have been found in cells with no known secretory function. In most cases, the bound polyribosomes are then involved in the synthesis of membrane or mitochondrial proteins (Bergeron et al., 1975; Bingham and Campbell, 1972). One exception to this is globin, a non-exported protein synthesized in reticulocytes. Approximately twenty percent of mouse cellular globin mRNAs have been shown to be associated with membrane-bound polysomes (Morrison and Lingrel, 1975).

1.4.1 Polysome-Membrane Interactions

There are three mechanisms proposed for ribosome-membrane interaction. Such mechanisms involve interactions between membrane and 60S ribosomal subunit, nascent chain and/or the 3' end of the mRNA.

1.4.1.1 Ribosome-Membrane Interaction

Some technical difficulties have hampered the study of ribosome subunit-membrane interactions since treatments used to remove the ribosomes can

result in degradation of the ribosomal subunits or the membrane. However, Adelman et al., 1973, have described the non-destructive release of ribosomes. These ribosomes fall into two categories: the 'loosely' bound which are removed by high salt treatment only and the 'tightly' bound which require puromycin and high salt treatment. From these observations, it was postulated that interactions disrupted by salt only represented the association of ribosome and membrane at early stages of protein synthesis prior to further anchorage by the nascent chain (Harrison et al., 1974).

From electron microscopic studies, further evidence of attachment of the 60S ribosomal subunit to the membrane has been obtained. Unwin, 1977, has shown by a three dimensional map of crystalline membrane-bound ribosomes that both large and small ribosomal subunits are adjacent to the membrane surface, attached to it by a part protruding from the large subunit.

1.4.1.2 Messenger RNA-Membrane Affinity

There is some evidence suggesting that messenger RNA has affinity for the membranes of the rough endoplasmic reticulum. After treatment of the rough endoplasmic reticulum with high ionic strength buffer and puromycin (Lande et al., 1975; Cardelli et al., 1976), about fifty percent of the mRNA was still associated with the membranes. Furthermore, after digestion with pancreatic ribonuclease, the 3' poly(A) segment of the mRNA remained attached (Lande et al., 1975). These results suggest that either the poly(A) segment itself or other untranslated sequences adjacent to the 3' end may play a role in polysome-membrane interaction. Since almost all mRNAs contain a poly(A)-rich 3' segment, the specificity of mRNA and membrane is more likely to be mediated by specific proteins associated with this part of the molecule or by RNA sequences distinguishing mRNAs coding for secreted and non-secreted proteins.

1.4.1.3 Nascent Chain-Membrane Interaction

The role of the nascent chain in polyribosome-membrane binding may be either primary or secondary to ribosome-membrane interaction. It has been suggested by Rosbach, 1972, that protein synthesis is initiated free in the cytoplasm and only after the protrusion of the nascent chain does the ribosome-mRNA complex bind to the membrane, the specificity being carried by the nascent chain. Harrison et al., 1974, postulated that the nascent chain played a secondary role in membrane-binding, increasing the interaction already achieved by the 60S ribosome subunit.

1.4.1.4 Segregation of Secreted and Non-Secreted Proteins

Although it appears that either the 60S ribosome subunit, the mRNA or the nascent chain can interact with the membrane, any theory must explain the differential binding of mRNAs synthesizing secreted and non-secreted proteins.

An attractive theory for the preferential synthesis of secreted proteins on membrane-bound polyribosomes was suggested by Milstein et al.,1972, and outlined in further detail in 1974 by Harrison et al.. A similar hypothesis has been proposed by Blobel and Dobberstein, 1975, termed the 'signal hypothesis' and involves the occurrence of a unique set of codons at the 5' end of the mRNA molecules coding for secreted proteins only. This codon sequence would in turn synthesize a unique N-terminal polypeptide chain triggering attachment to specific sites on the membrane. Thus, protein synthesis would be initiated in the cytoplasm but, on the appearance of the nascent chain of secreted proteins, membrane-binding would occur and the elongating polypeptide chain would be embedded in the membrane and finally vectorially discharged into the lumen of the endoplasmic reticulum.

Experimental evidence for this theory has been obtained by the translation in vitro of many mRNAs coding for secreted proteins, showing the existence

of primary translation products which contain extra amino-terminal sequence Proteins displaying this feature include pancreatic secretory proteins (Devillers-Thiery et al., 1975), parathyroid hormone (Kemper et al., 1972), insulin (Tager et al., 1975), glucagon (Tung and Zerega, 1971) and collagen (Benveniste et al., 1976; Harwood et al., 1975). Furthermore, sequence analysis of such precursor polypeptides has shown a high percentage of hydrophobic amino acid residues at the N-terminus which could facilitate membrane-nascent chain interaction (Devillers-Thiery et al., 1975). This extra polypeptide sequence is cleaved proteolytically in vivo prior to secretion (Blobel and Dobberstein, 1975). Processing of the precursor to fish proinsulin has been carried out in vitrc by synthesis in the presence of dog pancreatic microsomes (Shields and Blobel, 1977). Proteolyti processing of a chloroplast protein precursor has also been achieved in vitro to yield a polypeptide identical to the protein processed in vivo (Dobberstein et al., 1977).

Recent evidence indicates that the synthesis of such a unique nascent chain is not necessary for the secretion of all proteins. Palmiter et al., 1977, has shown that, although conalbumin, ovomucoid and lysozyme from hen oviduc are synthesized as precursor proteins, ovalbumin is not, suggesting two different secretion mechanisms in the same gland cells.

1.5. BIOSYNTHESIS OF IMMUNOGLOBULIN

Immunoglobulin is virtually the only secretory protein of mature plasma cells and most plasma cell tumours. The immunoglobulin molecule consists of four polypeptide chains, or multiples thereof, two heavy chains (50,000 to 70,000 mol.weight) and two light chains (23,000 mol.weight). Each polypeptide chain can be subdivided into a constant (C)-region and a variable (V)-region, the former including two-thirds of the molecule and

the latter, one-third. Five distinct classes of immunoglobulin have been defined according to the physical properties of the heavy chains. These classes are further subdivided by their serological specificity. The C-region amino acid sequence is conserved among heavy or light chains of the same subclass while the heavy and light chain V-region sequences are unique to each clone of immunoglobulin-secreting cells (reviewed by Bevan et al., 1972).

Studies on immunoglobulin-producing myeloma cells have shown that heavy and light chain polypeptide chains are synthesized independently on different classes of polyribosomes (Shapiro et al., 1966; Williamson and Askonas, 1967). These polyribosomes are predominantly membrane-bound (Lisowska-Bernstein et al., 1970) and the nascent polypeptide chains are vectorially released into the cisternae of the endoplasmic reticulum (Bevan, 1971).

Like the other secreted proteins studied, immunoglobulin light chains have been synthesized in vitro as precursor molecules, identified both by peptide mapping (Milstein et al., 1972; Mach et al., 1973) and sequence analysis (Burstein and Schechter, 1976). As in the case of dog pancreatic secretory proteins (Devillers-Thiery et al., 1975), the N-terminal precursor segment of mouse myeloma light chains contains a high percentage of hydrophobic amino acid residues (Schechter and Burstein, 1976).

The evidence that immunoglobulin heavy chains are also synthesized as precursor molecules is less conclusive. Comparisons of heavy chains synthesized in vitro and in vivo have provided contradictory results. Cowan and Milstein, 1973, have subjected MOPC 21 heavy chain, synthesized in vitro, to analysis by digestion with trypsin and subsequent chromatograph

of the peptide products. The digestion products of the cell-free synthesized heavy chain yielded an extra peptide compared to a digestion of the heavy chain synthesized *in vivo*. During the course of the studies described in this thesis, however, Bedard and Huang, 1977, demonstrated that peptide profiles generated by cyanogen bromide digestion of MOPC 315 heavy chain synthesized *in vivo* and *in vitro* were chromatographically indistinguishable. In contrast to this, preliminary sequence analysis of heavy chain synthesized *in vitro* has suggested the presence of an amino acid precursor segment of eighteen amino acids of MOPC 315 α-chain but little data concerning this is available at the moment (Schechter *et al.*, 1977). The existence of a precursor to heavy chain therefore still remains controversial and requires more analyses of heavy chains synthesized *in vitro* to clarify the issue.

1.5.1 Glycosylation of Immunoglobulin

As early as 1945, serum globulin was shown to contain carbohydrate residues (Meyer, 1945) and since this time much controversy has existed as to the role of glycosylation and its importance in secretion. Several investigators have proposed that the addition of carbohydrate is essential for the secretion of proteins in general (Eylar, 1965) and for the secretion of immunoglobulin (Melchers and Knopf, 1967). The addition of carbohydrate moieties occurs in several steps and is apparently initiated prior to termination of protein synthesis (Melchers and Knopf, 1967; Melchers, 1971a) Using 2-deoxy-D-glucose, an inhibitor of glycosylation, Melchers, 1973, has proposed that the attachment of sugar residues to IgG molecules located in the smooth membranes of the endoplasmic reticulum is not essential for secretion but that attachment of such residues in the rough endoplasmic reticulum is necessary for transport to the smooth membranes and, therefore, for secretion. In contrast to this result, Eagon and Heath, 1977, have shown that immunoglobulin light chains synthesized in the presence of

2-deoxy-glucose, and thus non-glycosylated, are still secreted but at 40% the rate of normal glycosylated light chains.

Further evidence indicating that glycosylation is not requisite for secretion, is the secretion of a non-glycosylated light chain from MOPC 41A mouse plasmacytoma cells (Melchers, 1971b). No carbohydrate residues have been detected on rat serum albumin although it, too, is a secreted protein (Peters et al., 1971). Weitzman and Scharff, 1976, have demonstrate the secretion of immunoglobulin heavy and light chains in the absence of carbohydrate attachment. A mutant cell line, M3.11, derived from the MPC-11 mouse myeloma, synthesizes abnormal heavy chains of which 30 to 50% are not glycosylated. Half the synthesized immunoglobulin is assembled normally and secreted as H_2L_2 molecules whereas the remaining immunoglobulin heavy and light chains are synthesized as half molecules, HL. These half molecules are of two types one with glycosylated and the other with non-glycosylated heavy chains. (The light chains are not glycosylated either in the parent or mutant line.) The whole molecule also contains non-glycosylated heavy chain but it is not known whether any of the complete H_2L_2 molecules are totally lacking carbohydrate.

This evidence suggests that, although carbohydrate addition may facilitate secretion, it is not essential. The possibility remains, however, that all secreted proteins are glycosylated but that, at some stage in the export of certain proteins, the carbohydrate residues are removed.

2. MATERIALS AND METHODS

2.1 MATERIALS

Unless otherwise mentioned, all materials were 'Analar' grade and supplied by BDH Chemicals Ltd., Dorset.

2.1.1 Cell Lines

Four murine myeloma tissue culture lines were used in the following studies. The cell line, 5563, was adapted to tissue culture growth, in our laboratory, by Dr. L. Fitzmaurice, from X5563 tumours grown in C3H/He mice. The tumour was obtained from the Salk Institute, La Jolla, California, USA. The cell line, Pl.17, was also obtained from the Salk Institute where it was adapted to tissue culture growth from the solid tumour, Adj-PC5, grown in BALB/c mice. Both these cell lines synthesize and secrete $IgG_{2A\kappa}$.

The myeloma cell line, MOPC 315, was obtained from Dr. M.D. Scharff and subcloned by Dr. T. Mosmann. MOPC 315.40, like the parent line, synthesizes and secretes both α heavy chains and λ_2 light chains. MOPC 315.26, a variant clone which synthesizes and secretes λ_2 light chains but not heavy chains, was selected by antiserum overlay (Coffino et al.,1970).

2.1.2 Cell Culture Materials

Cell culture materials were obtained as follows:-

RPMI 1640, dry powder	Gibco Bio-Cult, Ltd., Paisley, Scotland.
Foetal Calf Serum	Flow Laboratories Ltd., Irvine, Scotland.
Penicillin	Glaxo Pharmaceuticals, London.
Streptomycin	Glaxo Pharmaceuticals, London.

Tissue culture plastics were supplied by Falcon, Oxnard, California and Corning Glass Works, Corning, New York.

2.1.3 Antisera

Anti-5563 antiserum was prepared by immunisation of rabbits with 5563 myeloma protein purified by DEAE and G-200 Sephadex chromatography. Anti-λ_2 antiserum was also raised in rabbits against purified λ_2 light chains. This purification procedure included the isolation of MOPC 315 myeloma protein by DNP-Sepharose affinity chromatography followed by the separation of α and λ_2 chains on a G-100 Sephadex column in urea-formate buffer (6 M urea in 0.1 M formic acid, neutralised with 0.2 N NaOH). Thanks is given to Mrs. E. Blakely for the preparation of these antisera.

Goat anti-rabbit IgG (GARIg) antiserum was prepared by immunisation of goats with rabbit IgG. Anti-5563 and GARIg antibodies were a gift from Mr. P.A. Singer and were prepared by affinity chromatography on Sepharose-bound purified 5563 myeloma protein and rabbit IgG, respectively. Normal rabbit immunoglobulin (NorIg), also a gift from Mr. P.A. Singer, consisted of the IgG fraction of normal rabbit serum purified by G-200 Sephadex chromatography.

Rabbit IgG was supplied by Nordic Immunological Laboratories, Maidenhead, Berkshire.

2.1.4 Radiochemicals

All isotopically labelled compounds were supplied by the Radiochemical Centre, Amersham, Buckinghamshire.

Amino Acid	Specific Activity
L-(^{35}S) Methionine	>500 Ci/mmole
L-(4,5-^{3}H) Leucine	50-60 Ci/mmole
L-(4,5 (n)-^{3}H) Lysine	33 Ci/mmole
L-(4-^{3}H) Phenylalanine	12 Ci/mmole
L-(3,4 (n)-^{3}H) Proline	40 Ci/mmole
L-(3-^{3}H) Serine	29 Ci/mmole
L-(3,4 (n)-^{3}H) Valine	36 Ci/mmole

2.1.5 Photographic Supplies

Photographic materials were supplied by Kodak Limited, London with the exception of Kodak X-Omat-R film which was supplied by Kodak Canada Ltd., Ontario.

2.1.6 Wheat Germ

Different brands of wheat germ were obtained as follows:-

Texan wheat germ was a generous gift of Dr. J. Tata, National Institute of Medical Research, London.

Israeli wheat germ is a product of the Bar-Rav Mill, Tel Aviv and was kindly obtained by Dr. F. Richards.

St. Vincent St. wheat germ was purchased from The Health Food Shop, St. Vincent St., Glasgow.

General Mills wheat germ was a gift from Dr. J. Buxbaum, New York University and was supplied by the Kansas City Mill.

2.1.7 Liquid Scintillation Spectrometry Materials

Liquid scintillation spectrometry materials were supplied as follows:-

2,5 Diphenyloxazole (PPO)	Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire.
Toluene, AR grade	Rohm and Haas (U.K.) Ltd., Croydon.
Protosol	New England Nuclear, Boston, Massachusetts.

2.1.8 Other Materials

Apart from those materials listed above and those supplied by BHD Chemicals Ltd., Poole, Dorset, all other materials were supplied as follows:-

N-2-Hydroxyethylpiperazine N'-2-ethanesulphonic Acid (Hepes)	Sigma London Chemical Co. Ltd.,
Tris(hydrozymethyl)aminomethane (Trizma Base)	"
Cycloheximide	"
Spermidine, free base	"
Dimethyl Sulphoxide (DMSO)	"
N,N,N',N'-Tetramethyl-ethylenediamine (Temed)	"
Albumin, bovine serum	"
D, L Dithiothreitol (DTT)	"
Phosphocreatine (creatine phosphate)	"
Iodoacetic Acid, free acid	"
Deoxycholic Acid, sodium salt	"
Periodic Acid	"
2-Mercaptoethanol	Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire.
Triton X-100, purified	"

Trichloroacetic Acid (TCA)	Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire
Acetone	"
Acrylamide	"
3-Methyl-1-butanol (isoamyl alcohol)	"
Heparin, freeze dried	Evans Medical Ltd., Speke, Liverpool.
Sucrose, density gradient grade, ribonuclease free	Schwartz/Mann, Organgeburg, New York
Oligo dT-Cellulose	Collaborative Research Inc., Waltham, Massachusetts.
L-Amino Acids, A grade	Calbiochem Ltd., Bishops Stortford, Hertfordshire
NN' Methylene Bisacrylamide	Eastman Kodak Co., Rochester, New York.
Formamide	Fluka AG, Chemische Fabrik, Buchs, Schweiz.
Adenosine 5'-Triphosphate (ATP)	P-L Biochemicals Inc., Milwaukee, Wisconsin
Guanosine 5'-Triphosphate (GTP)	"
Absolute Alcohol, AR grade	James Burrough Ltd., London.
G-25 Sephadex, coarse grade	Pharmacia (Great Britain) Ltd., London.
PM 10 Diaflo ultrafilters	Amicon Ltd., High Wycombe, Buckinghamshire
Whatman 3MM 2.5 cm filter paper discs	Whatman Ltd., Maidstone, Kent.
Whatman 3MM chromatography paper	"
Creatine Kinase	The Boehringer Corporation (London) Ltd., Lewes, East Sussex.
Calf Thymus tRNA	"
Staphylococcus aureus V8 Protease	Miles Laboratories Ltd., Stoke Poges, Slough

N,N-Diallyltartardiamide (DATD) Serva Feinbiochemica, Heidelberg.

2.2 STANDARD SOLUTIONS

2.2.1 Cell Culture Solutions

2.2.1.1 RPMI 1640 Medium (complete)

The composition of RPMI 1640 medium is shown in Table 1. For cell culture, RPMI 1640 was supplemented with 10% foetal calf serum (inactivated at 56°C for 1 hour), 10^5 units/litre of penicillin, 0.01% (w/v) streptomycin, 50 μM 2-mercaptoethanol and 2 mM glutamine. Stock solutions of the above supplements were stored at -20°C in all cases except for 2-mercaptoethanol which was stored at 4°C. All were added immediately prior to use.

2.2.1.2 Phosphate Buffered Saline

Phosphate buffered saline, solution A (PBS-A) consisted of:-

0.17 M NaCl

3.4 mM KCl

10.0 mM Na_2HPO_4

1.8 mM KH_2PO_4

pH 7.2

I am grateful to the tissue culture staff, especially Mrs. I. Gall, for the preparation of the above cell culture solutions.

2.2.2 Scintillation Spectrometry Solutions

Toluene-PPO scintillation fluid was composed of 0.4% (w/v) PPO in toluene.

Triton-Toluene-PPO scintillation fluid consisted of 0.4% ($^{W}/v$) PPO in Triton X-100: toluene (1:2).

Table 1

Composition of RPMI 1640 Medium

Amino Acids	mg/litre
L-Arginine (free base)	200.0
L-Asparagine	65.0
L-Aspartic Acid	20.0
L-Cystine (2 HCl)	65.0
L-Glutamic Acid	20.0
L-Glutamine	300.0
Glycine	10.0
L-Histidine (free base)	15.0
L-Hydroxyproline	20.0
L-Isoleucine (Allo free)	50.0
L-Leucine (Methionine free)	50.0
L-Lysine HCl	40.0
L-Methionine	15.0
L-Phenylalanine	15.0
L-Proline (Hydroxy-L-Proline free)	20.0
L-Serine	30.0
L-Threonine (Allo free)	20.0
L-Tryptophane	5.0
L-Tyrosine	28.94
L-Valine	20.0

Table 1 (continued)

Inorganic Salts	mg/litre
$Ca(NO_3)_2.4H_2O$	100.0
KCl	400.0
$MgSO_4$	48.84
NaCl	6000.0
Na_2HPO_4 (anhyd.)	800.0

Vitamins	mg/litre
Biotin	0.2
D-Ca pantothenate	0.25
Choline Cl	3.00
Folic Acid	1.00
i-Inositol	35.00
Nicotinamide	1.0
Para-aminobenzoic acid	1.0
Pyrodoxine HCl	1.0
Riboflavin	0.2
Thiamine HCl	1.0
Vitamin B_{12}	0.005

Other Components	mg/litre
Glucose	2000.0
Glutathione (reduced)	1.0
Phenol red	5.0

Table 1 (continued)

The above table shows the constituents of RPMI 1640, powder formula, as supplied by Gibco Bio-Cult Ltd.. This was supplemented with 2 g/litre of $NaHCO_3$ and adjusted to pH 7.0 with HCl. After sterilization, medium was stored at $4^{o}C$.

2.3 CELL CULTURE TECHNIQUES

2.3.1 Growing of Cells

Cells were grown in complete RPMI 1640 medium as described in Section 2.2.1.1. For preparative purposes, cultures were grown in continuous suspension in 80 oz roller bottles, each containing up to 500 mls. Stock cultures were maintained in Corning tissue culture flasks (75 cm^2) at a concentration of 1 to 6 x 10^5 cells/ml. All cell cultures were grown in an atmosphere of 5% CO_2:95% air, at $37^{o}C$.

2.3.2 Determination of Concentration and Viability of Cells

A total cell count was obtained by counting in an haemocytometer. The viability was ascertained by addition of Trypan blue dye, in normal saline, to a final concentration of 0.1% ($^{w}/v$), the non-viable cells being stained and counted.

2.4 PREPARATION OF BIOSYNTHETICALLY LABELLED MYELOMA PROTEIN

Generally, 10^6 cells were pelleted from the medium described above at 250 x g for 10 minutes. The cells were resuspended in 200 µl of methionine or leucine-free RPMI 1640 containing 100 µCi of lyophilized ^{35}S-methionine or ^{3}H-leucine, respectively.

2.4.1 Biosynthetic Labelling of Total Intracellular Protein

To label intracellular proteins, the period of incubation was 30 minutes at 37°C. The cells were then pelleted at 250 x g for 10 minutes and the supernatant discarded. The cellular pellet was resuspended and lysed in 500 µl of Lysis Buffer.

Lysis Buffer

25 mM Tris-HCl, pH 8.2

0.88% (W/v) NaCl

1% (V/v) NP-40

For complete cell lysis, the cells were held on ice for 15 minutes. The nuclei were removed by centrifugation for 20 minutes at 2000 x g. The supernatant, when necessary, was stored at -20°C. Prior to electrophoresis, aliquots were removed, centrifuged at 30,000 x g for 20 minutes in an SS-34 Sorvall rotor and precipitated with acetone (Section 2.7.3).

2.4.2 Biosynthetic Labelling of Secreted Proteins

Cells were incubated as described above for 1 hour at 37°C. 1 ml of complete medium was then added and the incubation continued for a further 3 hours. The cells were then pelleted at 250 x g for 10 minutes, the supernatant removed and, where applicable, stored at -20°C.

2.4.3 Immunoprecipitation of Mouse Immunoglobulin

Indirect precipitation of mouse immunoglobulin was carried out, from either cell lysates or media, by the addition of either rabbit anti-mouse Ig (RAMIg), whole antiserum, or affinity-purified RAMIg antibodies. Incubation was for 10 minutes at room temperature. Affinity-purified GARIg antibodies were then added at equivalence with further incubation overnight at 4°C. Generally, either 25 µg of RAMIg antibodies or 5 µl

of whole antiserum were added per 10^6 cells and precipitated at equivalence by 100 µg of GARIg antibodies.

Precipitates were washed thoroughly with Solution B (Section 2.7) or Lysis Buffer (Section 2.4.1). Finally, the immune precipitates were dissolved in 2% ($^{W}/v$) SDS and stored at -20°C.

2.5 PREPARATION OF MESSENGER RNA

2.5.1 Preparation of Polysomes

Polysomes were prepared from mouse myeloma cells in tissue culture by a modification of the method of Fitzmaurice, Bennett and Williamson (unpublished results). Prior to harvesting of the cells in mid-log phase, the medium was made 100 µg/ml with cycloheximide and the cells incubated for a few minutes. All steps from this point were carried out at 4°C. The cells were then pelleted in the MSE 6L centrifuge for 10 minutes at 250 x g. The medium was carefully but thoroughly removed and the cells resuspended in ice-cold PBS-A, containing 100 µg/ml of cycloheximide, transferred to Falcon tubes and washed twice with 50 mls of ice-cold PBS-A, containing 100 µg/ml of cycloheximide.

To each cellular pellet was added approximately 5 volumes of Cytoplasmic Lysis Buffer (A) and mixed thoroughly. The lysed cells were then centrifuged at 48,000 x g for 20 minutes in an SS-34 Sorvall rotor. The supernatant was held on ice and the nuclear pellet re-homogenized in Cytoplasmic Lysis Buffer (B). Centrifugation was again at 48,000 x g for 20 minutes. The supernatants were pooled and layered over a 10 ml cushion of 1.5 M sucrose in Solution C. This was centrifuged at 45,000 rpm in a 60-Ti rotor in the Beckman L5-65 ultracentrifuge at 2°C for 3 hours. For storage of polysomes, the pellet was resus-

pended in 0.25 M sucrose in de-ionised water and stored at -70°C.

Solutions:

Solution C

50 mM Tris-HCl, pH 7.4

25 mM NaCl

5 mM MgOAc

7 mM 2-Mercaptoethanol

Cytoplasmic Lysis Buffer (A)

0.88 M Sucrose

1 mg/ml Heparin

100 µg/ml Cycloheximide

0.08% (v/v) Triton X-100

in Solution C

Cytoplasmic Lysis Buffer (B)

0.88 M Sucrose

1 mg/ml Heparin

100 µg/ml Cycloheximide

0.08% (v/v) Triton X-100

0.2 M KCl

0.06% (w/v) Deoxycholic Acid

(Na salt)

in Solution C

2.5.2 Sucrose Density Gradient Centrifugation of Polysomes

Linear sucrose gradients, 0.5 M to 1.25 M sucrose in Solution C (see Section 2.5.1), were poured into polyallomer tubes for the Beckman SW 41 rotor. Polysomes (5 to 10 A_{260} units) in 0.25 M

sucrose were overlaid on each gradient and centrifuged for 90 minutes at 40,000 rpm in an SW 41 rotor in the Beckman L5-65 ultracentrifuge at 4°C.

A_{260} profiles were obtained by passing the sucrose gradient through a 1 mm path-length flow cell, the reading being displayed on a chart recorder.

2.5.3 Phenol Extraction of Total Polysomal RNA

Extraction Buffer

0.1 M NaCl

0.1 M Tris-HCl, pH 9.0

1 mM EDTA

2% (w/v) SDS

Polysome pellets were resuspended in Extraction Buffer at a concentration of approximately 10 A_{260} units/ml and extracted with an equal volume of buffered phenol (redistilled): chloroform: isoamyl alcohol (50:50:1) (Mendecki et al., 1972). The mixture was shaken at room temperature for 10 minutes. The two layers were separated by centrifugation and the aqueous layer removed. The phenol phase was again extracted with an equal volume of buffer, the two aqueous phases pooled and re-extracted with an equal volume of phenol: chloroform: isoamyl alcohol. The final aqueous phase was removed carefully, one tenth volume of 4 M NaCl added and the RNA precipitated overnight at -20°C by the addition of 2 volumes of ethanol.

2.5.4 Oligo dT-Cellulose Chromatography

Loading Buffer

0.5 M LiCl

1 mM EDTA

0.1% (W/v) SDS

10 mM Tris-HCl, pH 7.5

Intermediate Buffer

0.1 M LiCi

1 mM EDTA

0.1% (W/v) SDS

10 mM Tris-HCl, pH 7.5

Elution Buffer

1 mM EDTA

0.1% (W/v) SDS

10 mM Tris-HCl, pH 7.5

Ethanol precipitated polysomal RNA was pelleted, dried with a stream of nitrogen and dissolved in a small volume of Loading Buffer. The oligo dT-cellulose column was equilibrated with Loading Buffer prior to application of the sample. After applying the sample, the column was washed thoroughly with Loading Buffer and the eluant monitored until the A_{260} reading was less than 0.04. This was repeated with Intermediate Buffer and both eluants discarded. Poly (A) RNA was eluted with a small volume of Elution Buffer, one tenth volume of 4 M NaCl added and precipitated overnight at -20°C, by the addition of 2 volumes of ethanol. The RNA was pelleted at 12,000 x g in a Sorvall SS-34 rotor, dissolved in a small volume of distilled water and re-precipitated as described above. The RNA was finally pelleted at 12,000 x g, dried with a stream of nitrogen and redissolved in a small volume of distilled water. This was stored at -70°C.

2.6 THE WHEAT GERM CELL-FREE SYSTEM

2.6.1. Wheat Germ Extract

Extraction Buffer

20 mM Hepes

100 mM KCl

1 mM MgOAc

2 mM $CaCl_2 6H_2O$

6 mM 2-Mercaptoethanol

adjust to pH 7.6 with KOH.

Column Buffer

20 mM Hepes

120 mM KCl

5 mM MgOAc

6 mM 2-Mercaptoethanol

adjust to pH 7.6 with KOH. The final concentration of potassium is thus altered to 132 mM.

The wheat germ extract was prepared according to the method of Marcu and Dudock, 1974. All steps were performed at 4°C. All glassware was baked at 200°C to inactivate ribonuclease. Untoasted wheat germ (2 g) was ground in a mortar for 60 seconds with an equal weight of powdered glass. Extraction Buffer (4 mls) was then added and the paste removed and centrifuged for 10 minutes in a Sorvall SS-34 rotor at 30,000 x g. After centrifugation, the supernatant was removed and applied to a G-25 (coarse) Sephadex column (25 x 1.4 cm, approximately).

The G-25 Sephadex was prepared by swelling in de-ionized water, overnight at room temperature, containing 0.1% ($^{v}/v$) diethyl pyrocarbonate to inactivate ribonuclease. After pouring the column, the Sephadex was

washed thoroughly with Column Buffer to eliminate the diethyl pyrocarbonate.

Approximately 1 ml of wheat germ supernatant was applied to the column, eluted in Column Buffer and the turbid fractions further centrifuged for 20 minutes at 30,000 x g in an SS-34 Sorvall rotor. The supernatant from these pooled fractions was frozen in small aliquots at -70°C or in liquid nitrogen.

2.6.2 Dialysis of Wheat Germ Extract

Wheat germ extracts were prepared as described in Section 2.6.1 and dialysed prior to freezing as follows. Wheat germ extracts were diluted five-fold with Column Buffer (Section 2.6.1) and concentrated to the original volume by dialysis in an Amicon ultrafiltration unit using a PM10 membrane. The extract was subsequently stored in liquid nitrogen. This procedure took about 1 hour and was carried out at 4°C.

2.6.3 ATP Mix (20 x final assay concentration)

60 mg of ATP was dissolved in 2 mls of de-ionized water and neutralized with KOH. To this was added 0.2 g of creatine phosphate and 1 mg of GTP. The volume of the resultant solution was then adjusted to 5 mls and stored at -20°C in small aliquots.

2.6.4 Amino Acid Mixture

A 400 μM solution of 19 amino acids (leucine or methionine excepted), was prepared and the pH adjusted to 7.4 with KOH. The contribution of potassium to the final concentration was negligible, in this case. The amino acid solution was stored at -20°C in small aliquots.

2.6.5 Dithiothreitol

A solution of 0.12 M DTT was made, deaerated by flushing with nitrogen and the solution stored in 20 µl aliquots at -20°C.

2.6.6 Creatine Kinase

Creatine kinase was freshly prepared in water and added to give the final concentrations as given in the Results Section.

2.6.7 ^{3}H-Leucine

Unless otherwise stated, the final concentration of ^{3}H-leucine was 200 µCi/ml, with a specific activity of 55 to 60 Ci/mmole.

2.6.8 ^{35}S-Methionine

^{35}S-Methionine was added as indicated in the Results Section.

2.6.9 Spermidine

A 160 mM aqueous stock solution of spermidine was stored at -20°C.

2.6.10 Salt Mixtures

Salt Dilution Solution

0.57 M Hepes, pH 7.8

24 mM MgOAc

320 mM K^{+} (contributed by KOH used to adjust the pH)

Salt Solution

0.56 M Hepes, pH 7.8

24 mM MgOAc

1.7 M KCl

250 mM K^{+} (contributed by KOH used to adjust the pH)

Concentrated Salt Solution

0.6 M Hepes, pH 7.8

25 mM MgOAc

2.6 M KCl

280 mM K^+ (contributed by KOH used to adjust the pH)

Mg^{2+}-Free Salt Solution

0.6 M Hepes, pH 7.8

280 mM K^+ (contributed by KOH used to adjust the pH)

These solutions were stored at -20°C.

2.6.11 Salt Supplements

Salt supplements consisted of 0.4, 0.8, 1.2, 1.6 and 2.0 M KCl in water and 50, 30, 20, and 10 mM MgOAc, also in water. These were used in conjunction with the Salt Mixtures to vary the Mg^{2+} and K^+ final assay concentrations. These solutions were stored at -20°C.

2.6.12 Energy Mix

The energy mix was made freshly before use and consisted of:-

	Volume µl
ATP Mix	25
Amino Acid Mixture or H_2O	25
DTT	10
Creatine Kinase	5
Salt Mixture	10
Salt Supplement or H_2O	25
Total Volume	100

2.6.13 Assay Mix

	Volume µl
Energy Mix	10
Wheat Germ Extract	20
or	
Wheat Germ Extract	15
and	
Mg^{2+}-Free Salt Solution	5
RNA, Labelled Amino Acid, Spermidine and/or H_2O	30
Total Volume	50

2.7 PRECIPITATION OF TOTAL PROTEIN FROM A WHEAT GERM CELL-FREE SYSTEM

After the incubation period, 50 µl assays were diluted to 250 µl with either Solution A or Solution B and centrifuged for 4 minutes at 8,000 x g in an Eppendorf centrifuge.

Solution A

10 mM Tris-HCl, pH 8.0

0.15 M NaCl

Solution B (Dr. T. Mosmann, personal communication)

0.1 M Tris-HCl, pH 8.0

0.1 M KCl

0.005 M $MgCl_2$

1% ($^{w}/_{v}$) Deoxycholic Acid (Na salt)

1% ($^{v}/_{v}$) Triton X-100

0.5% ($^{w}/_{v}$) SDS

Aliquots were then removed for precipitation.

2.7.1. TCA Precipitation

To estimate the amount of labelled amino acid incorporated, 10% (25 µl) of each diluted assay was removed and an equal volume of 0.2 N NaOH added. Incubation was for 15 minutes at 37° C to hydrolyse charged tRNA molecules. Bovine serum albumin or normal rabbit immunoglobulin (10 to 20 µg) was added as carrier and precipitation carried out by addition of 50 µl of 25% (^{W}/v) TCA and 1 ml of 10% (^{W}/v) TCA. The assays were held on ice for 30 minutes before pelleting the precipitates and washing three times with 10% (^{W}/v) TCA. The final precipitates were dissolved in 200 µl of 0.2 N NaOH. This was buffered by the addition of 50 µl of 5% (^{V}/v) acetic acid. Ten volumes of Triton-Toluene-PPO scintillation fluid were added to each sample and the radioactivity measured by liquid scintillation spectrometry.

2.7.2 TCA Precipitation on Filter Paper Discs

Aliquots (5 µl) were removed from undiluted wheat germ assays and spotted on Whatman 3 MM filter paper discs. The discs were immediately submersed in ice-cold 10% (^{W}/v) TCA for 30 minutes and then washed for 10 minute periods in 5% (^{W}/v)TCA at room temperature, 5% (^{W}/v) TCA at 90°C, ethanol: ether (1:1) and ether only. The discs were then dried and the radio-activity determined by liquid scintillation spectrometry in Toluene-PPO scintillation fluid.

2.7.3 Acetone Precipitation

Protein was precipitated from diluted wheat germ cell-free assays by the addition of 5 volumes of acetone. Precipitation was complete in 30 minutes, the protein pelleted and the acetone removed.

2.8. IMMUNOPRECIPITATION FROM THE WHEAT GERM SYSTEM

For the purpose of immunoprecipitation, wheat germ assays were diluted with Solution B only, as described in Section 2.7. Indirect precipitation of 5563 Ig heavy and light chains was carried out by the addition of anti-5563 antibodies and incubation for 10 minutes at room temperature followed by the addition of GARIg antibodies and further incubation overnight at 4°C. Generally, 25 µg of anti-5563 antibodies and 100 µg of GARIg antibodies were added per 250 µl of diluted wheat germ assay. The precipitates formed were pelleted, washed three times with Solution B and dissolved in 2% (W/v) SDS. Aliquots were either removed for determination of radioactivity by liquid scintillation spectrometry in Triton-Toluene-PPO scintillation fluid or re-precipitated with 5 volumes of acetone (Section 2.7.3), prior to electrophoresis.

2.9 POLYACRYLAMIDE GEL ELECTROPHORESIS

2.9.1 SDS-Polyacrylamide Gel Electrophoresis (Tris-Glycine Buffer System)

SDS-polyacrylamide gels were prepared by a slight modification of the method of Laemmli, 1970. The gels were prepared as slabs of dimensions 20 x 25 x 0.15 cm, containing both a separating and a stacking gel, the compositions of which are shown below. The gels were electrophoresed overnight at 4°C at 15 to 20 mA/gel.

Separating Gel

0.375 M Tris-HCl, pH 8.8

0.1% (W/v) SDS

12.5% (W/v) Acrylamide

0.337% (W/v) Bis-acrylamide

The separating gel was polymerised by the addition of ammonium persulphate to a concentration of 0.1% ($^{W}/v$) and Temed to a concentration of 0.0325% ($^{V}/v$).

Stacking Gel

0.065 M Tris-HCl, pH 6.8

0.1% ($^{W}/v$) SDS

5% ($^{W}/v$) Acrylamide

0.135% ($^{W}/v$) Bis-acrylamide

The stacking gel was polymerised by the addition of ammonium persulphate to a concentration of 0.1% ($^{W}/v$) and Temed to a concentration of 0.1% ($^{V}/v$).

Reservoir Buffer

0.025 M Tris

0.192 M Glycine

0.1% ($^{W}/v$) SDS

at pH 8.8

2.9.1.1 Preparation of Samples

Protein precipitates to be electrophoresed on SDS-polyacrylamide gels, as described above, were dissolved in Sample Buffer and boiled for 2 minutes prior to loading on the gel.

Sample Buffer

0.065 M Tris-HCl, pH 6.8

10% ($^{V}/v$) Glycerol

100 mM DTT

2% ($^{W}/v$) SDS

Bromophenol blue

2.9.1.2 Processing of SDS-Polyacrylamide Gels

2.9.1.2 (a) Fluorography

Gels were processed for fluorography according to the method of Bonner and Laskey, 1974. After electrophoresis, the gels were immersed in three successive baths of DMSO for a total period of about 2 hours. The gel was impregnated with PPO by immersion in 200 mls of 20% ($^{W}/v$) PPO in DMSO, with gentle shaking for one hour. The excess solution was then decanted and the PPO precipitated in the gel by the addition of water. The gel was washed with several changes of water to remove any remaining DMSO. The gel was dried under vacuum onto Whatman 3MM chromatography paper.

A fluorograph was obtained by placing a sheet of Kodak X-Omat-R film in contact with the gel, held in position between two glass plates. This was kept at -70^{o}C for the necessary time of exposure.

2.9.1.2 (b) Autoradiography

After electrophoresis, the gels were immersed in two successive baths of 10% ethanol for a period of one hour. The gels were then dried and placed in contact with film as described in Section 2.9.1.2 (a), with the exception that exposure was carried out at room temperature.

2.9.2 SDS-Polyacrylamide Gel Electrophoresis (Phosphate Buffer System)

Separating Gel

0.1 M Na phosphate, pH 7.2

0.1% ($^{W}/v$) SDS

6 M Urea

10% ($^{W}/v$) Acrylamide

0.27% ($^{W}/v$) DATD

Reservoir Buffer

0.1 M Na phosphate, pH 7.2

0.1% (^{W}/v) SDS

SDS-polyacrylamide gels were prepared according to the method of Summers et al., 1965. Cylindrical gels were of composition as shown above and of dimensions 6 cm x 0.5 cm. Polymerisation of the gels was carried out by the addition of ammonium persulphate to a concentration of 0.2% (^{W}/v) and Temed to a concentration of 0.05% (^{V}/v). Electrophoresis was for 4 hours, using constant current at 10 mA/gel.

2.9.2.1 Preparation of Samples

Samples to be electrophoresed were dissolved in a one tenth volume of 6 M urea containing 2% (^{W}/v) SDS. To this, one tenth volume of Reservoir Buffer was added. Reduction and alkylation of the samples followed. The samples were made 50 mM in DTT and incubated for 30 minutes at 37^{O}C. Iodoacetamide was then added to give a final concentration of 125 mM and further incubated for 30 minutes at 37^{O}C. Finally, a small volume of bromophenol blue in 50% (^{W}/v) sucrose was added and the samples boiled for 2 minutes prior to loading on the gel.

2.9.2.2 Analysis of Electrophoresed Gels

After electrophoresis, the cylindrical gels were frozen in dry ice and cut into 1 mm slices. The slices were digested for 1 hour at 37^{O}C in 0.3 ml of 2% (^{W}/v) periodic acid. Ten volumes of Triton-Toluene-PPO scintillation fluid was then added and the radioactivity measured by liquid scintillation spectrometry.

2.9.3 Formamide-Polyacrylamide Gel Electrophoresis

Formamide-polyacrylamide gels were prepared according to the method of Duesberg and Vogt, 1973. Formamide was buffered (pH 7.0) by the addition of 1 mmole of Na_2HPO_4 and 1 mmole of NaH_2PO_4 to 100 mls of pure formamide.

Formamide-Polyacrylamide Gel

3% (^{W}/v) Acrylamide

0.525% (^{W}/v) Bis-acrylamide

in Buffered Formamide, pH 7.0

The gels were polymerised by the addition of ammonium persulphate to a concentration of 0.075% (^{W}/v) and Temed to a concentration of 0.2% (^{V}/v). RNA samples were applied in 50% (^{V}/v) glycerol in buffered formamide containing bromophenol blue. Gels were electrophoresed in 0.04 M Na phosphate, pH 7.0, for 6 hours at 100 volts.

After electrophoresis, gels were frozen and cut into 1 mm slices. The slices were digested in 500 µl of a 90% (^{V}/v) aqueous solution of Protosol for one hour at 60°C. Toluene-PPO scintillation fluid (5 mls) was added prior to measurement of radioactivity by liquid scintillation spectrometry.

2.10 PEPTIDE MAPPING BY LIMITED PROTEOLYSIS IN SDS-POLYACRYLAMIDE GELS

Mouse myeloma IgG heavy chains were digested with Staphylococcus aureus V8 protease, according to the method of Cleveland et al., 1977. Heavy chains, labelled with ^{35}S-methionine, were identified in 12.5 or 15% SDS-polyacrylamide gels by autoradiography as described in Section 2.9.1.2(b) and the appropriate part of the gel cut out.

Gel slices were allowed to swell in 0.125 M Tris-HCl, pH 6.8, 0.1% ($^{W}/v$) SDS and 1 mm EDTA. Slab gels were prepared as described in Section 2.9.1, with a few modifications.

Separating Gel

0.375 M Tris-HCl, pH 8.8

0.1% ($^{W}/v$) SDS

1 mM EDTA

15% ($^{W}/v$) Acrylamide

0.405% ($^{W}/v$) Bis-acrylamide

The gel was polymerised by the addition of ammonium persulphate to a concentration of 0.1% ($^{W}/v$) and Temed to a concentration of 0.1% ($^{V}/v$).

Stacking Gel

0.125 M Tris-HCl, pH 6.8

0.1% ($^{W}/v$) SDS

1 mM EDTA

3% ($^{W}/v$) Acrylamide

0.081% ($^{W}/v$) Bis acrylamide

The gel was polymerised by the addition of ammonium persulphate to a concentration of 0.1% ($^{W}/v$) and Temed to a concentration of 0.1% ($^{V}/v$).

Larger than usual stacking gels were cast (3.5 cm) with larger than usual wells to accommodate the gel slice. The wells were filled with 0.125 M Tris-HCl, pH 6.8, 0.1% ($^{W}/v$) SDS and 1 mM EDTA. Each gel slice was pushed to the bottom of a well and covered with 0.125 M Tris-HCl, pH 6.8, 0.1% ($^{W}/v$) SDS, 1 mM EDTA containing 20% ($^{V}/v$) glycerol and bromophenol blue. Staphylococcus aureus V8 protease was then added, at the amounts indicated in the Results Section, in the same buffer containing 10% glycerol

Electrophoresis was carried out at 15 mA at room temperature until the bromophenol blue was about 0.5 cm from the separating gel. The current was then switched off for 30 minutes. Electrophoresis was resumed at $4^{o}C$ at 15 to 20 mA.

After electrophoresis, the gel was processed for fluorography as described in Section 2.9.1.2 (a).

3. RESULTS

3.1 CHARACTERIZATION OF THE WHEAT GERM CELL-FREE TRANSLATION SYSTEM

For the purpose of studying primary translation products of mouse myeloma messenger RNA, a wheat germ cell-free protein synthesizing system was chosen. This system has certain advantages over other cell-free systems in that it contains low levels of endogenous mRNA which thus facilitates the identification of proteins synthesized by added mRNA. Furthermore, the wheat germ extract itself is inexpensive and easy to prepare and renders a cell-free system capable of efficient mRNA translation.

Several mRNAs have been faithfully translated including globin (Roberts and Paterson, 1973), TMV RNA (Roberts et al., 1973), collagen (Harwood et al., 1975; Benveniste et al., 1976) and actin mRNA (Paterson et al., 1974; Gozes et al., 1975). Myeloma mRNA has also been translated in the wheat germ cell-free translation system (Schmeckpeper et al., 1974; Sonenshein and Brawerman, 1976; Bedard and Huang, 1977; Ono et al., 1977) although immunoglobulin heavy chain has not been as efficiently synthesized as light chain (Green et al., 1976).

It has been reported that the wheat germ system, like the Krebs II ascites system, prematurely terminates partially synthesized proteins (Schechter and Burstein, 1976a). No evidence of this phenomenon has been seen in the following work although the system does have a tendency to synthesize small proteins (<30,000 molecular weight) more efficiently than larger proteins. These small proteins appear to be complete polypeptide chains since proteins of equivalent migration in SDS-polyacrylamide gels can be found in a corresponding cell lysate.

The following experiments were therefore designed to enhance the synthesis of immunoglobulin heavy chain to enable characterization of the cell-free synthesized product.

3.1.1 Assay of Ribonuclease Activity

It is important to establish the amount of nuclease activity in any system used to assay messenger RNA. To ascertain the endogenous nuclease activity in the wheat germ system, all the reagents used in the cell-free translation system were incubated under normal assay conditions in the presence of ribosomal RNA, labelled with ^{3}H-uridine. (This preparation was a gift from Mr. P.A. Singer). After the incubation, the ribosomal RNA was analysed by formamide-polyacrylamide gel electrophoresis to determine the amount of RNA degradation (see Figures 1A to 1E). This provides a very sensitive method for the detection of RNA degradation owing to the denaturing property of the gels.

All the ribonuclease activity was shown to be associated with the creatine kinase and the wheat germ extract. All the other reagents contained no detectable ribonuclease as they gave profiles identical to the control profile in Figure 1C (results not shown). The nuclease associated with the creatine kinase seemed to be a general contaminant since it was present in creatine kinase from two separate sources. As shown in Figures 1A and 1B, ribonuclease activity was present in wheat germ extracts from different sources. Such nuclease activity has also been found by other workers in a complete wheat germ system but cannot be clearly assigned to any particular ingredient from the data given (Pelham and Jackson, 1976; Hunter et al., 1977a). Messenger RNA degradation by nuclease may account for the synthesis of incomplete proteins reported by Schechter and Burstein, 1976a.

Figures 1A to 1E

Assay of Nuclease Activity by Polyacrylamide-Formamide Gel Analyses

All assays contained 1.3 µg of ribosomal RNA labelled with ^{3}H-uridine, incubated in the presence of the following:-

A) 2.4 A_{260} units of Texan wheat germ extract.

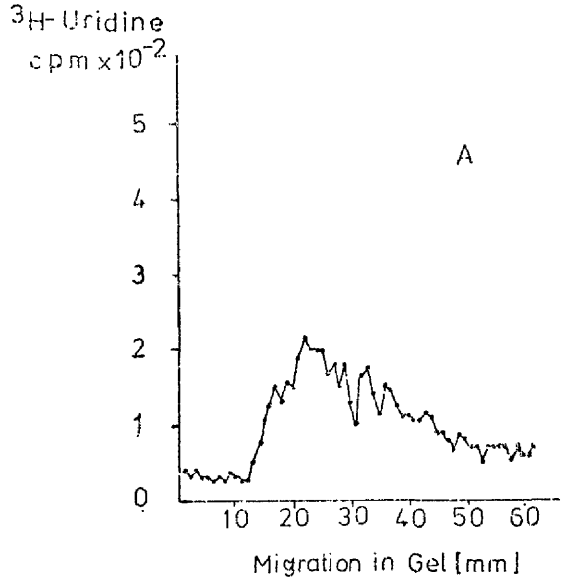
B) 1.2 A_{260} units of Israeli wheat germ extract

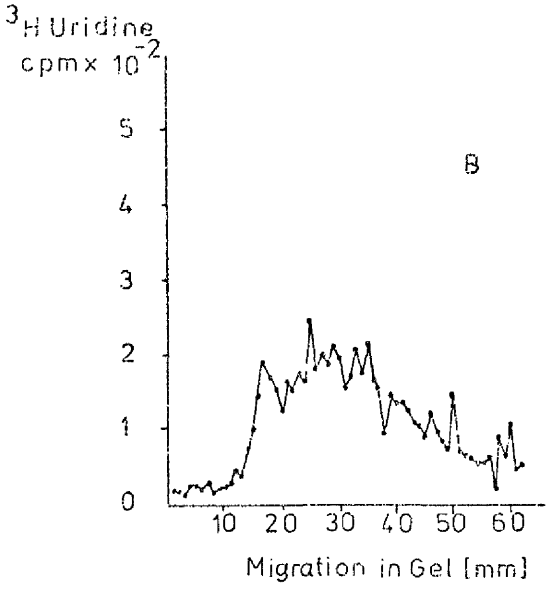
C) H_2O (control)

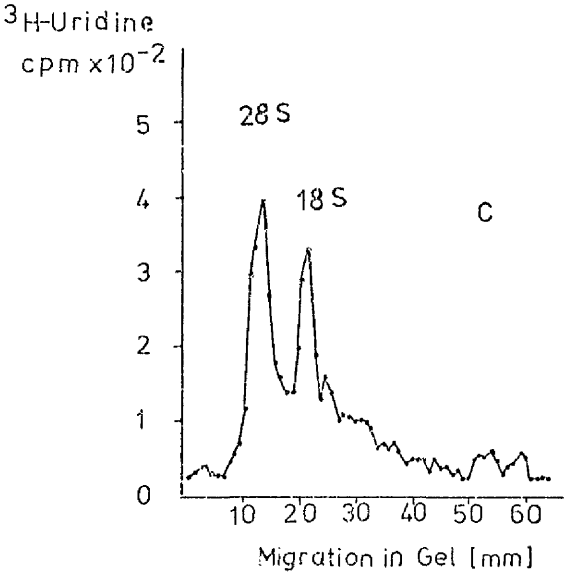
D) 5 µg of creatine kinase, 7.85 mM creatine phosphate, 1.1 mM ATP, 175 µM GTP, 2.4 mM DTT, 2.5 mM Mg^{2+}, 74 mM K^{+}, 1.2 A_{260} units of Israeli wheat germ extract, in 20 mM Hepes, pH 7.6.

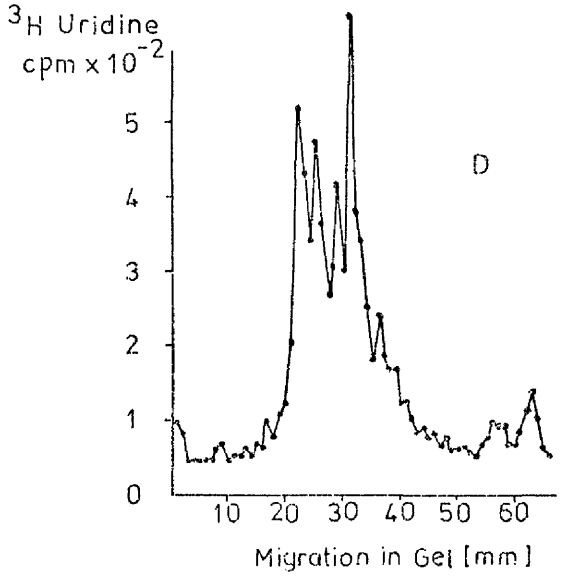
E) 5 µg of creatine kinase in 20 mM Hepes, pH 7.6

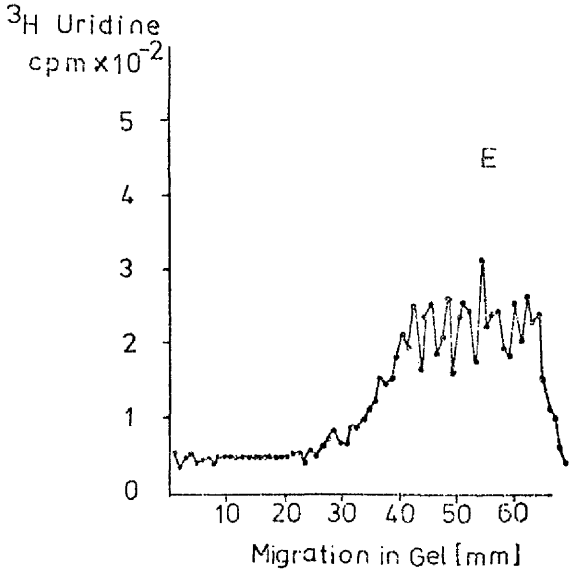
All assays (50 µl) were incubated for 90 minutes at 25°C, except in D, where incubation was for 30 minutes only. D was then held at -20°C for one hour. The samples were phenol extracted as described in Section 2.5.3, and precipitated with 2 volumes of ethanol in the presence of 10 µg of tRNA and 0.4 M NaCl. Precipitation was carried out overnight at -20°C and the RNA pelleted and dried as described in Section 2.5.4. Samples were applied to 3% polyacrylamide-formamide gels which were cut into 1 mm slices, after electrophoresis, and the radioactivity determined for each slice (Section 2.9.3.).


^{3}H-Uridine
cpm x10^{-2}
5
4
3
2
1
0
A
10 20 30 40 50 60
Migration in Gel [mm]


^{3}H Uridine
cpm x 10^{-2}
5
4
3
2
1
0
B
10 20 30 40 50 60
Migration in Gel [mm]


^{3}H-Uridine
cpm x10^{-2}
28 S
18 S
C
5
4
3
2
1
0
10 20 30 40 50 60
Migration in Gel [mm]


^{3}H Uridine
cpm x 10^{-2}
D
5
4
3
2
1
0
10 20 30 40 50 60
Migration in Gel [mm]


^{3}H Uridine
cpm x10^{-2}
E
5
4
3
2
1
0
10 20 30 40 50 60
Migration in Gel [mm]

Since much of the ribonuclease activity was associated with the creatine kinase, an experiment was carried out to determine the minimum amount of creatine kinase required for maximum efficiency of protein synthesis. From the results shown in Table 2, it was decided that 0.5 µg of creatine kinase per 50 µl wheat germ assay would decrease the nuclease contamination while still allowing adequate translational efficiency.

3.1.2 Assay of Protease Activity

To ascertain the amount of proteolytic activity in the wheat germ system, a lysate of P1.17 cells was incubated in the absence and presence of all ingredients of a wheat germ cell-free translation assay. The immunoglobulin heavy and light chains were then precipitated indirectly by the addition of anti-5563 and GARIg, whole antisera, and analysed by SDS-polyacrylamide gel electrophoresis. The gel profiles are shown in Figure 2. The amount of protease activity evident by this method of analysis is negligible.

3.1.3 Effect of Potassium Concentration on the Efficiency of Cell-Free Translation

Although the wheat germ cell-free system readily synthesizes peptides of low molecular weight e.g. globin (Roberts and Paterson, 1973), more difficulty has been experienced in the synthesis of high molecular weight proteins e.g. collagen (Harwood et al., 1975; Benveniste et al., 1976) and immunoglobulin heavy chain (Schmeckpeper et al., 1974). It has been shown that the optimum potassium concentration for total protein synthesis, in the wheat germ system, is 70 to 80 mM. However, at elevated potassium concentrations (150 to 180 mM), although the amount of total protein synthesis is reduced, Harwood et al., 1975 and Benveniste et al., 1976,

Creatine Kinase (µg)	5.0	4.0	3.0	2.0	1.0	0.5	0.0
(A) ^{3}H-Leucine (cpm x 10^{-5})	6.46	6.50	7.62	8.77	8.12	8.04	3.03
(B) ^{3}H-Leucine (cpm x 10^{-5})	4.55	4.27	5.33	5.28	4.53	4.86	1.01

TABLE 2: Effect of Creatine Kinase on Translational Efficiency of the Wheat Germ System

Wheat germ assays (50 µl) were prepared as described in Section 2.6 and contained 10 µCi of ^{3}H-leucine (60 Ci/mmol), 74 mM potassium, 2.5 mM magnesium and 0.5 µg of 5563 poly(a) RNA. The amount of creatine kinase added to each 50 µl assay is shown in the table above. Column (A) represents the amount of ^{3}H-leucine per 50 µl assay incorporated into protein in the presence of 2.4 A_{260} units of Texan wheat germ extract. Column (B) represents the amount of ^{3}H-leucine per 50 µl assay incorporated into protein in the presence of 1.2 A_{260} units of Israeli wheat germ extract.
Assays were incubated for 90 minutes at 26°C and aliquots removed for the determination of ^{3}H-leucine incorporated as described in Section 2.7.1.

Figure 2

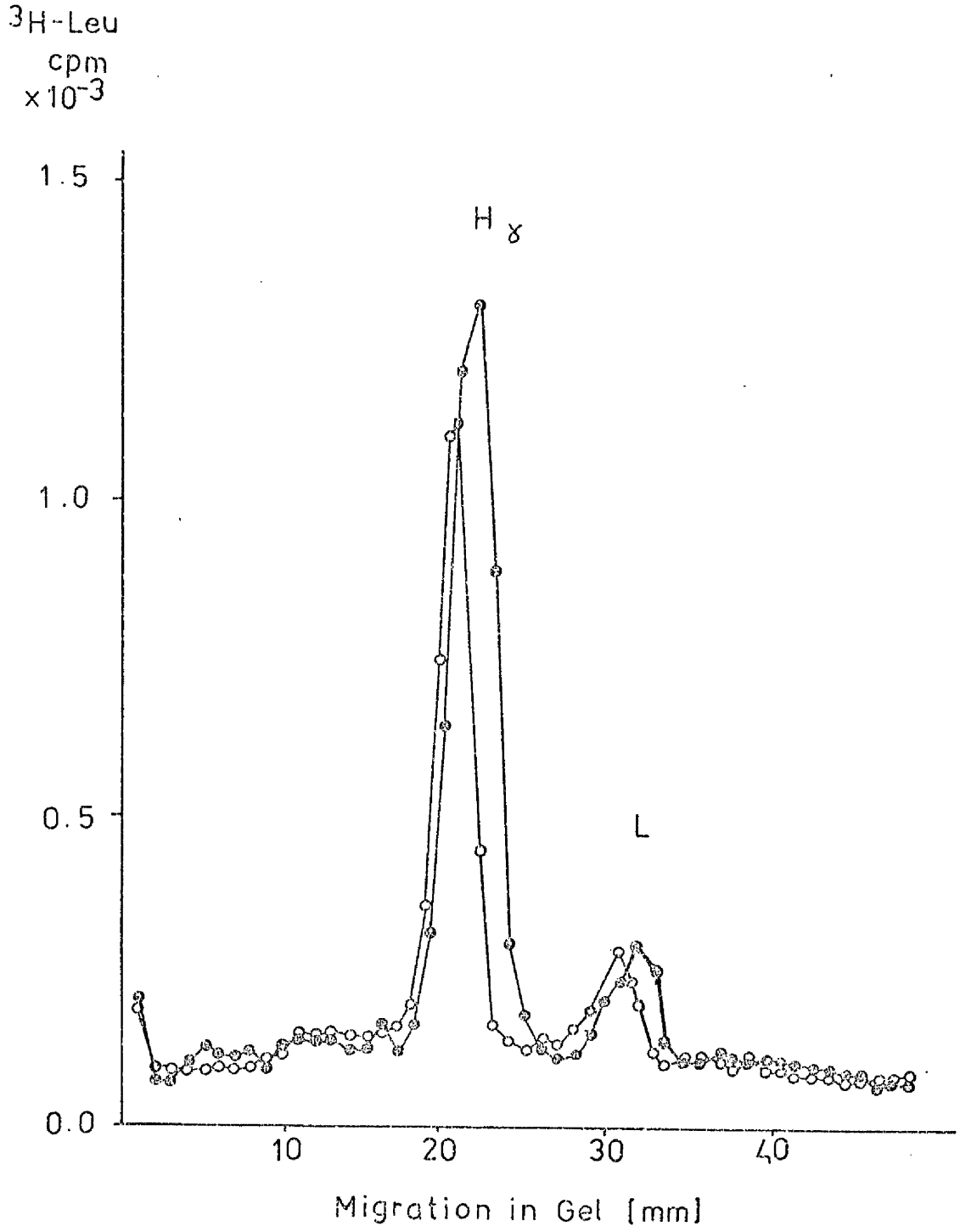
Assay of Protease Activity

Pl.17 cells were labelled with ^{3}H-leucine and the cells lysed according to Section 2.4.1. An aliquot of the total cell lysate (100 µl) was incubated in the presence of 50 µl of energy mix (Section 2.6.12) and 100 µl of St. Vincent St. wheat germ extract. Pl.17 cell lysate (100 µl) only was incubated as a control.

After the incubation, 1 hour at 30°C, the 100 µl control was diluted to 250 µl with PBS-A and the IgG indirectly precipitated from both samples by the addition of 1 µl of anti-5563 and 25 µl of GARIg, whole antisera (Section 2.4.3). The immune precipitates were prepared for analysis on 10% polyacrylamide cylindrical gels linked with DATD (Section 2.9.2). The gels were sliced, dissolved in periodic acid and the amount of radioactivity measured in Triton-Toluene-PPO scintillation fluid (Section 2.9.2.2).

(o—— o) represents ^{3}H-labelled Pl.17 IgG, precipitated after incubation in the presence of wheat germ assay ingredients.

(●——●) represents ^{3}H-labelled Pl.17 IgG, precipitated after incubation in the absence of wheat germ assay ingredients.


^{3}H-Leu
cpm
x10^{-3}
1.5
1.0
0.5
0.0
H γ
L
10
20
30
40
Migration in Gel [mm]

have shown more efficient synthesis of collagen. This has also been suggested for the synthesis of mouse IgA heavy chain (Schmeckpeper et al., 1974) where the optimum potassium concentration was found to be 104 mM, although greater total protein synthesis was achieved at 74 mM. In fact, at this lower concentration, no detectable IgA heavy chain was synthesized. This has also been shown in the Krebs II ascites cell-free system where elevated potassium concentrations were required for complete translation of encephalomyocarditis viral RNA (Mathews and Osborn, 1974).

The effect of potassium concentration on the translation of mouse myeloma poly(A) RNA was ascertained over a range of potassium concentrations from 56 to 211 mM. As can be seen from Figure 3, cell-free translation is almost completely inhibited by concentrations of potassium exceeding 110 mM. This is very different from the results obtained by Harwood et al., 1975, for collagen mRNA. A similar result, however, has been obtained by Marcu and Dudock, 1974, for the translation of TMV RNA.

Although the optimum potassium concentration for total protein synthesis was 74 mM, the products of all the assays containing 56 to 111 mM potassium were analysed by SDS-polyacrylamide gel electrophoresis to test the hypothesis that larger polypeptides are synthesized more efficiently at higher than optimum potassium concentrations. However, as shown in Figure 4, the proteins synthesized at potassium concentrations greater than 74 mM appear to be similar in molecular weight distribution to those synthesized at concentrations optimal for total protein synthesis. In this system, there is therefore no advantage in translating mouse myeloma poly(A) RNA at potassium concentrations higher than the optimum for total protein synthesis, since the inhibitory effect of high concentrations of potassium overrides any specific enhancement in the synthesis of high molecular weight proteins.

Figure 3

Effect of Potassium Concentration on the Translation of Mouse Myeloma Poly(A) RNA

Wheat germ cell-free assays (50 µl) were prepared as described in Section 2.6, and contained 1.5 A_{260} units of Israeli wheat germ extract, 0.5 µg of Pl.17 poly(A) RNA, 10 µCi of ^{3}H-leucine (specific activity, 55 Ci/mmole), 5 µg of creatine kinase, 2.5 m<u>M</u> Mg^{2+} and a range of K^{+} concentrations from 56 to 211 m<u>M</u>. Incubation was for 90 minutes at 30°C.

Aliquots were removed to determine the amount of labelled amino acid incorporated (Section 2.7.1). The figure opposite represents the ^{3}H-leucine incorporated per 50 µl assay at the K^{+} concentrations indicated.

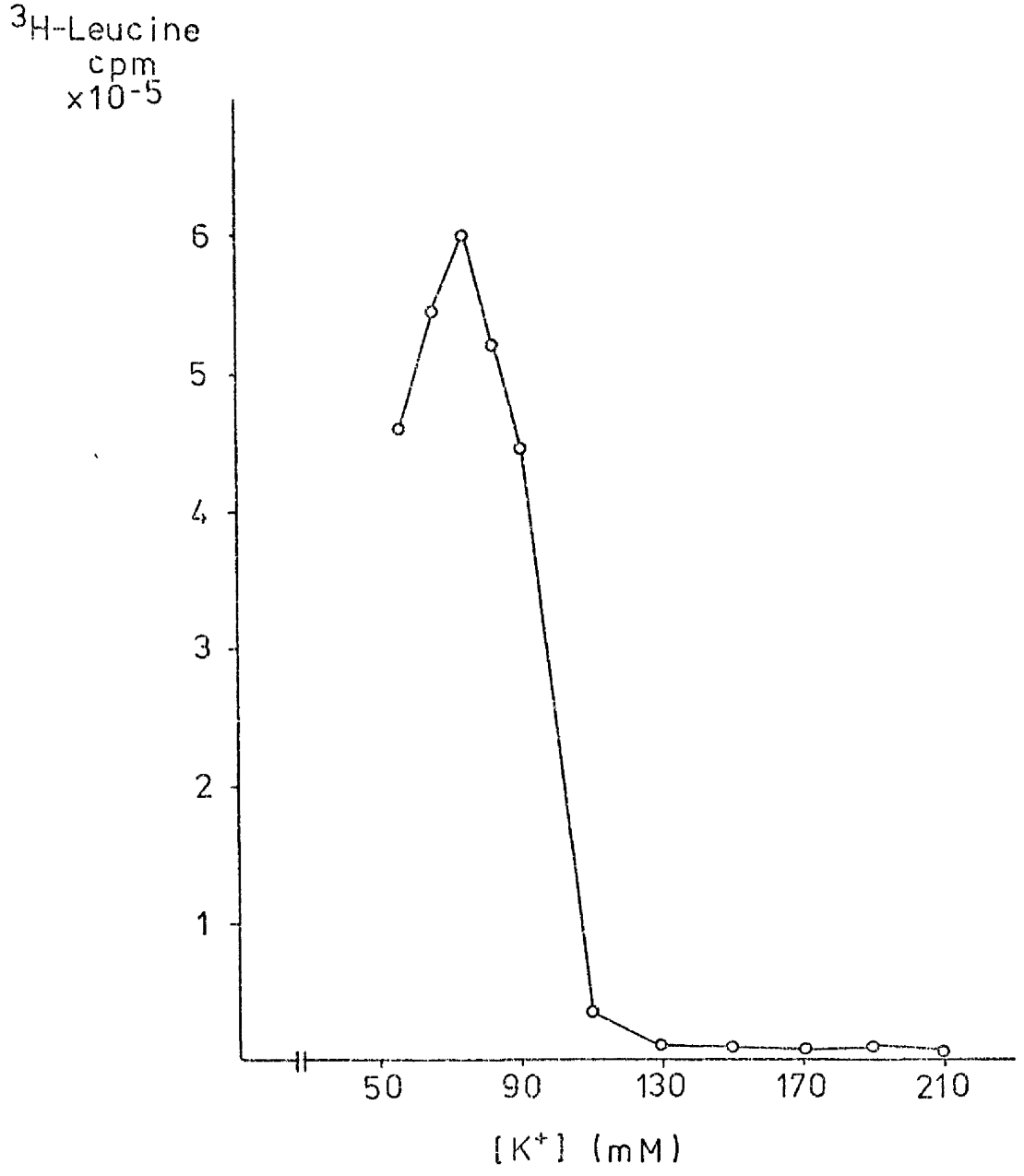
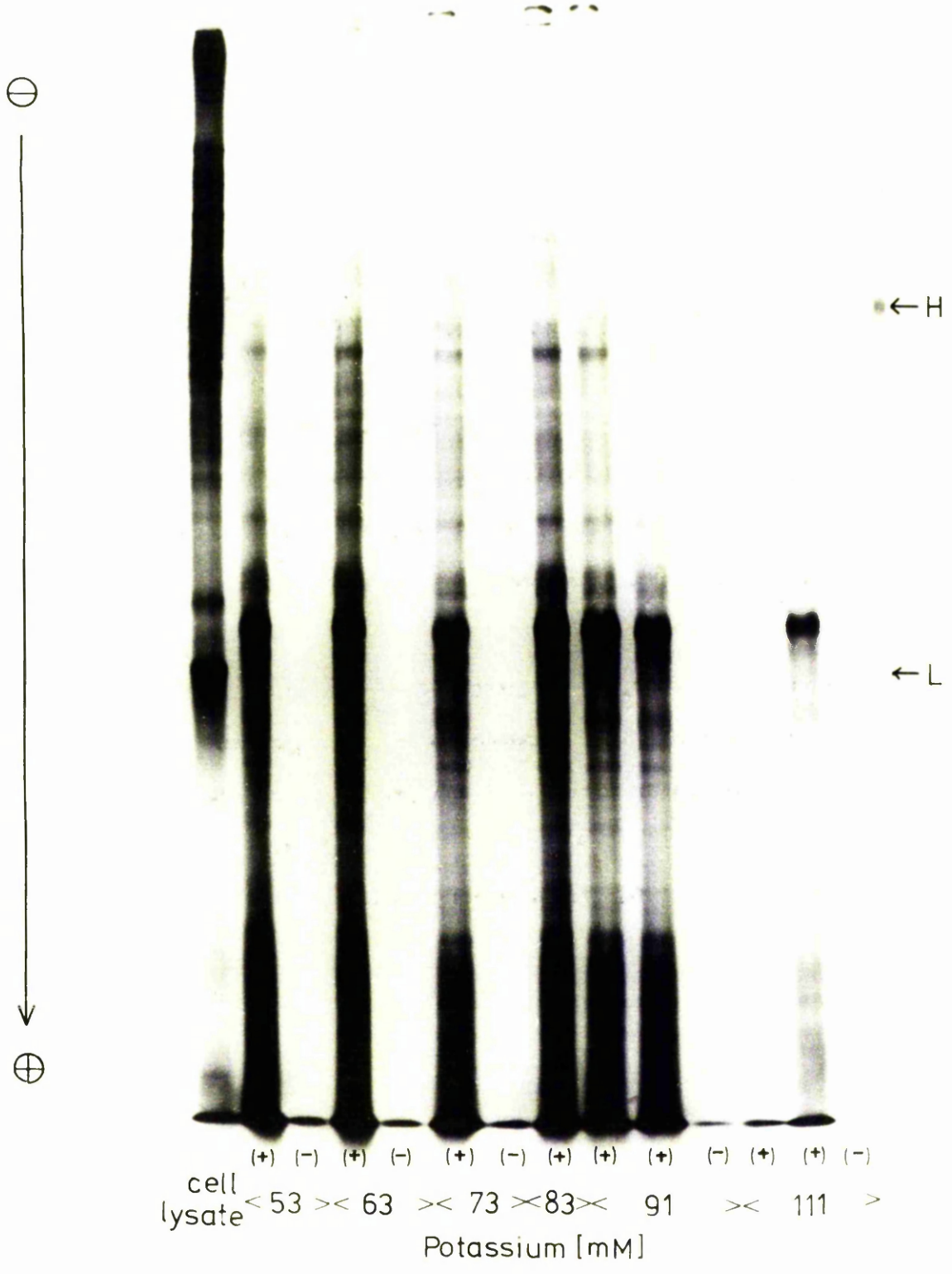

^{3}H-Leucine
cpm
x10^{-5}
6
5
4
3
2
1
50
90
130
170
210
[K$^+$] (mM)

Figure 4

Effect of Potassium Concentration on the Synthesis of Myeloma Cell Proteins

The fluorograph of an SDS-polyacrylamide gel shows an analysis of the cell-free synthesized proteins in the absence (-) and presence (+) of 0.5 µg of P1.17 poly(A) RNA at the potassium concentrations indicated.

Wheat germ assays were prepared as described for Figure 3. Aliquots (40%) were removed for acetone precipitation as described in Section 2.7.3 and prepared for electrophoresis on an SDS-polyacrylamide gel (Section 2.9.1). The gel was processed for fluorography (Section 2.9.1.2a).


⊖
⊕
←H
←L
(+) (−) (+) (−) (+) (−) (+) (+) (+) (−) (+) (+) (−)
cell lysate
53
63
73
83
91
111
Potassium [mM]

3.1.4 Optimum Magnesium Concentration for the Translation of Mouse Myeloma Poly(A) RNA

To determine the optimum concentration of magnesium for the translation of mouse myeloma poly(A) RNA, 5563 poly(A) RNA was translated in the presence of 2.5 to 5 mM magnesium at the optimum potassium concentration of 74 mM. From Figure 5, the optimum magnesium concentration is 3.0 mM at the above potassium concentration. This is slightly lower than the optimum magnesium concentration described by Schmeckpeper et al., 1974, for the translation of mouse myeloma poly(A) RNA. This higher magnesium optimum may be due to the presence of EDTA in the latter wheat germ system.

3.1.5 Effect of Polyamines on Cell-Free Translation

The role played by polyamines is unclear but it has been shown that spermine enhances translation of natural messenger RNA in cell-free extracts from wheat germ (Marcu and Dudock, 1974), with a particularly large enhancement of the synthesis of larger polypeptides (Atkins et al., 1975). While polyamines may play a part in at least one of the reactions of peptide chain initiation (Konecki et al., 1973), Hunter et al., 1977a, have shown that optimal concentrations of spermidine increase the rate of chain elongation approximately two-fold in a wheat germ cell-free translation system.

To ascertain the effect of spermidine on the translation of mouse myeloma poly(A) RNA, 5563 poly(A) RNA was translated in the presence of 0 to 0.8 mM spermidine. Since the requirement for magnesium is lowered, although not eliminated, by the addition of polyamines (Atkins et al.,1975), a magnesium concentration of 1.5 mM was used in assays containing spermidine compared to 2.5 mM in assays containing no spermidine. The addition of spermidine to a wheat germ assay containing mouse myeloma poly(A) RNA increases protein synthesis by approximately 50% at an optimum spermidine concentration of 0.4 mM (see Figure 6). Analysis of the cell-free synthesized proteins

Figure 5

Determination of Optimum Magnesium Concentration for the Translation of Mouse Myeloma Poly(A) RNA

Wheat germ assays (50 µl) were prepared as described in Section 2.6. These contained 1.5 A_{260} units of Israeli wheat germ extract, 10 µCi of ^{3}H-leucine (55 Ci/mmole), 5 µg of creatine kinase, 74 mM potassium and magnesium as shown. Incubation was for 90 minutes at 30°C. Aliquots were removed and the radioactivity measured by the method described in Section 2.7.1. The ^{3}H-leucine incorporated per 50 µl assay, at different magnesium concentrations, is represented in the figure opposite.

(o—— o) represents assays containing 0.55 µg of 5563 poly(A) RNA.

(●—— ●) represents assays containing endogenous mRNA only.

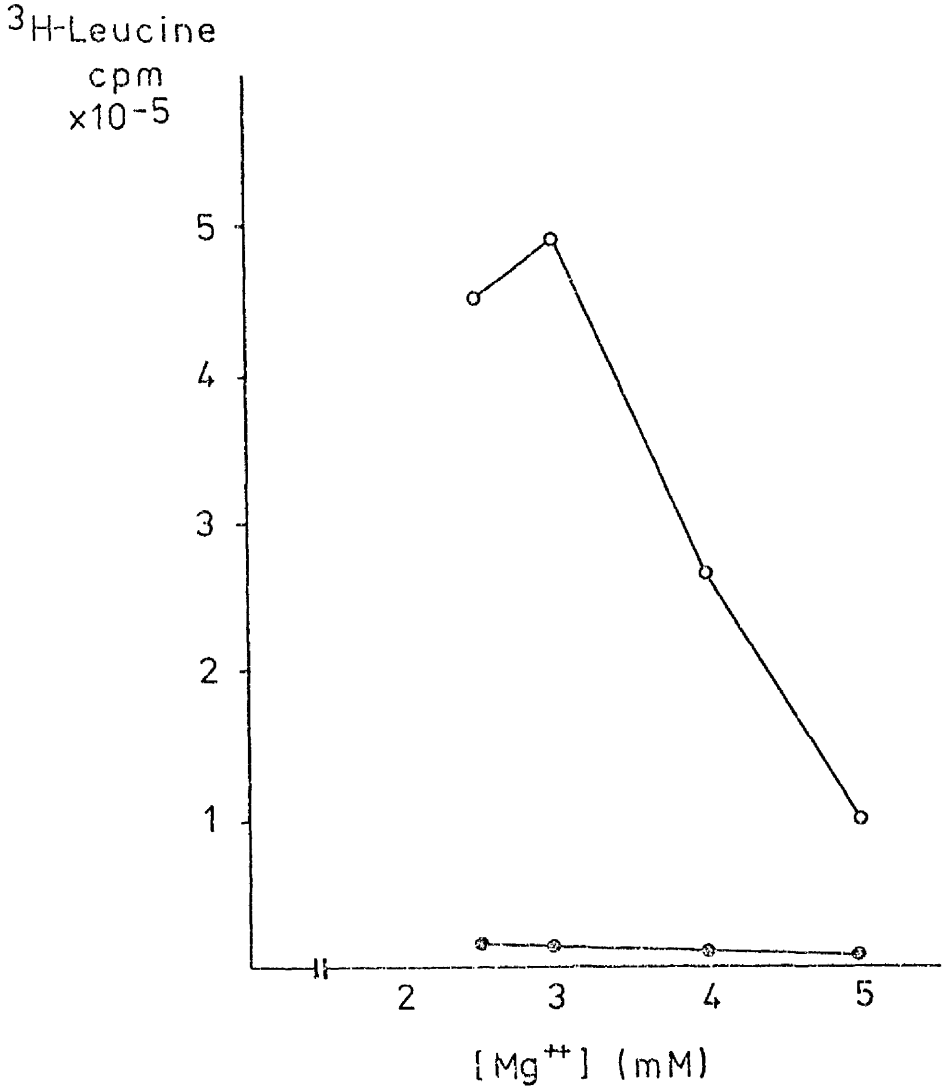
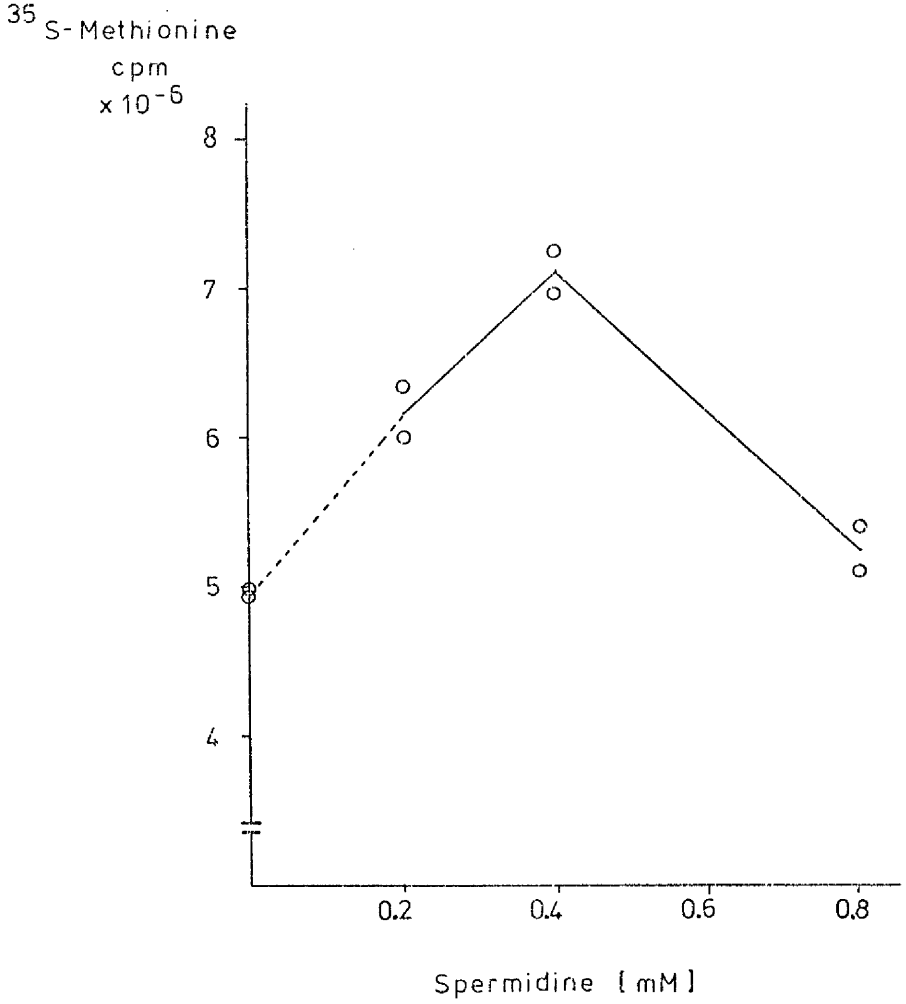

^{3}H-Leucine
cpm
x10-5
5
4
3
2
1
2
3
4
5
[Mg^{++}] (mM)

Figure 6

Effect of Spermidine on the Translation of Mouse Myeloma Poly(A) RNA

Cell-free assays (50 µl) contained 1.8 A_{260} units of Texan wheat germ extract, o.6 µg of 5563 poly(A) RNA, 74 m$\underline{M}$ K^+, 0.5 µg of creatine kinase, 17 µCi of ^{35}S-methionine (specific activity, 1027 Ci/mmole). The Mg^{2+} concentration was 1.5 m$\underline{M}$ in assays containing spermidine and 2.5 m$\underline{M}$ in the absence of added spermidine. All other reagents were as described in Section 2.6. Incubation was for 90 minutes at 25°C. Aliquots were removed to determine the amount of labelled amino acid incorporated (Section 2.7.1).

The figure opposite shows the incorporation of ^{35}S-methionine per 50 µl assay. Assays were carried out in duplicate.


35 S-Methionine
cpm
x 10-6
8
7
6
5
4
0.2
0.4
0.6
0.8
Spermidine [mM]

by polyacrylamide gel electrophoresis shows an increase in amount of both large and small proteins (see Figure 7), at optimum spermidine concentrations.

3.1.6 Endogenous Levels of Amino Acids in Wheat Germ Extract

In the preparation of the wheat germ extract, the extract is passed over a column of G25 coarse grade Sephadex. The combination of coarse grade bed material and a high flow rate tends to give large zone broadening. Wheat germ extracts prepared in this manner may well contain endogenous amino acids, either free or attached to tRNA. In order to calculate the pmoles of amino acids that the cell-free system can incorporate into protein, it is essential to know the endogenous levels of amino acids.

A preliminary experiment was designed to assess the dependence of the cell-free translation system on added amino acids. As shown in Table 3, protein synthesis, as measured by the incorporation of ^{3}H-leucine, remains unaffected by changes in the amount of added exogenous amino acids. The endogenous amino acid pool is therefore sufficiently large to support cell-free protein synthesis directed by 0.6 µg of 5563 poly(A) RNA in a standard 50 µl assay.

To obtain an estimate of the amount of endogenous leucine and methionine in the wheat germ extract, different amounts of exogenous ^{3}H-leucine and ^{35}S-methionine, respectively, were added. Since the total amount of protein synthesis i.e. pmoles of total amino acid incorporated, remains constant (see Table 3), with increasing amounts of exogenous amino acid, the percentage of total amino acid incorporated i.e. endogenous plus exogenous, decreases. The percentage of total amino acid incorporated

Figure 7

Enhanced Synthesis of Proteins by Addition of Spermidine

The fluorograph shows an analysis by SDS-polyacrylamide gel electrophoresis of proteins synthesized in the presence of various amounts of spermidine, as indicated.

Cell-free assays (50 µl) contained 1.8 A_{260} units of Texan wheat germ extract, 0.6 µg of 5563 poly(A) RNA, 74 mM K^+, 0.5 µg of creatine kinase, 17 µCi of ^{35}S-methionine (specific activity, 1027 Ci/mmole). The Mg^{2+} concentration was 1.5 mM in assays containing spermidine and 2.5 mM in the absence of added spermidine. All other reagents were as described in Section 2.6. Incubation was for 90 minutes at 25°C. The incorporation of ^{35}S-methionine is shown in Figure 6.

Aliquots (5%) from each assay were precipitated with acetone and prepared for polyacrylamide gel electrophoresis as described in Sections 2.7.3 and 2.9.1, respectively. After electrophoresis, the gel was processed for fluorography (Section 2.9.1.2a).

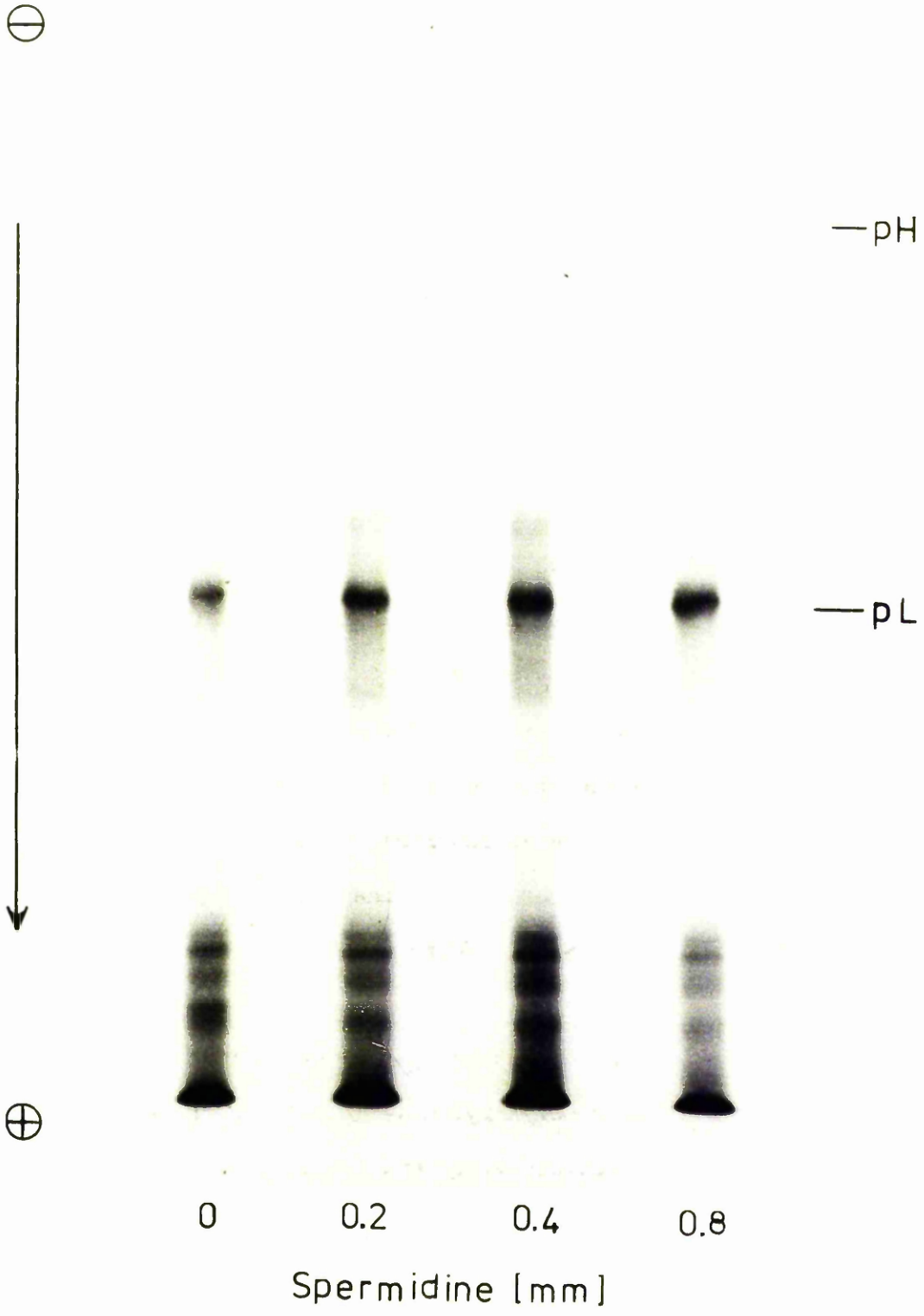

⊖
—pH
—pL
⊕
0
0.2
0.4
0.8
Spermidine [mm]

TABLE 3: Dependence of the Wheat Germ Cell-Free Translation System on Added Amino Acids

Added Amino Acids (nmoles)	^{3}H-Leucine Incorporated (cpm x 10^{-5})
1.0	9.60
0.4	9.31
0.1	10.28
0.05	9.89
0.025	9.67
0.01	9.19
0.00	9.56

Assays (50 µl) were prepared as described in Section 2.6 and contained 0.6 µg of 5563 poly(A) RNA, 10 µCi of ^{3}H-leucine (specific activity, 55 Ci/mmole), 2.3 A_{260} units of Texan wheat germ extract, 5 µg of creatine kinase, 74 m$\underline{M}$ K^{+} and 2.5 m$\underline{M}$ Mg^{2+}. Exogenous amino acids (19 excluding leucine) were added as shown above. Incubation was for 90 minutes at 26°C. Aliquots were removed for determination of radioactivity as described in Section 2.7.1. The above table shows the incorporation of ^{3}H-leucine per 50 µl assay in the presence of exogenous amino acids as indicated.

is maximal with no added exogenous amino acid. At the point where this maximum percentage is reduced to 50% by added exogenous amino acid, the amount of endogenous amino acid is equal to the amount of exogenous amino acid. This data is shown in Figures 8A and B. In both cases, the 50% inhibition point for leucine and the 40% inhibition point for methionine is based on the percentage of total amino acid incorporated with small amounts of added exogenous amino acid, 8.75 and 6.3 pmoles of leucine and methionine, respectively. This amount, in each case, must be subtracted from the calculated endogenous amino acid pool. The amount of endogenous leucine and methionine in 2.3 A_{260} units of Texan wheat germ extract is therefore 160 and 85 pmoles, respectively.

These calculations have been based on the following assumptions. Firstly, the amount of exogenous amino acid added was calculated from the specific activity as supplied by Amersham. It has been assumed, therefore, that this given figure is accurate. Secondly, in the case of methionine, it has been assumed that the total protein synthesis remained constant regardless of added exogenous amino acids. In the experiment calculating the amount of endogenous leucine, however, a small constant amount of ^{35}S-methionine was added to each assay to ensure that the total protein synthesis was constant. The incorporation of leucine was corrected for the incorporation of methionine in every case.

3.1.6.1 Dialysis of Wheat Germ Extract

The incorporation of added labelled amino acids could be increased if the wheat germ extract was dialysed prior to use (Section 2.6.2). A comparison of dialysed and non-dialysed extracts is shown in Table 4. The increase in the percentage of exogenous amino acids incorporated is probably due to a decrease in the endogenous amino acid level.

Figure 8A

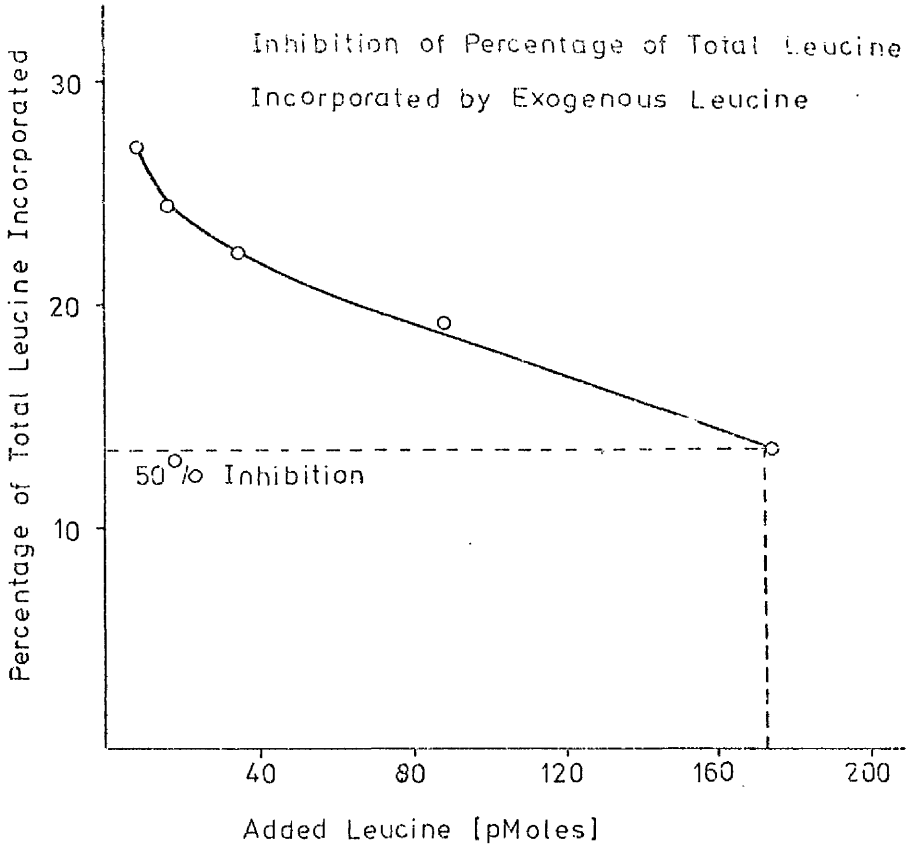
Inhibition of Percentage of Total Leucine Incorporated by Exogenous Leucine

Assays (50 µl) contained 0.6 µg of 5563 poly(A) RNA, 2.3 A_{260} units of Texan wheat germ extract, 5 µg of creatine kinase, 74 mM K^+, and 2.5 mM Mg^{2+}. A trace amount of ^{35}S-methionine was added to each assay to determine the total amount of protein synthesis in each case. This was a constant value and the values of ^{3}H-leucine have thus been corrected. The specific activity of ^{3}H-leucine was 55 Ci/mmole. Various amounts of ^{3}H-leucine, 10, 5, 2, 1, and 0.5 µCi, were added to 50 µl assays. Incubation was for 90 minutes at 26°C. Aliquots were removed from each assay and the amount of labelled amino acid incorporated determined as described in Section 2.7.1. The ^{3}H-leucine incorporated (cpm) was calculated as a percentage of the total ^{3}H-leucine added (cpm) and plotted as a function of the added leucine (pmoles).

Figure 8B

Inhibition of Percentage of Total Methionine Incorporated by Exogenous Methionine

Assays (50 µl) contained 0.6 µg of 5563 poly(A) RNA, 2.3 A_{260} units of Texan wheat germ extract, 0.02 µg of creatine kinase 74 mM K^+ and 2.5 mM Mg^{2+}. ^{35}S-methionine, 15.5, 7.75, 3.1, and 1.55 µCi, was added to individual assays. The specific activity of the ^{35}S-methionine was 247 Ci/mmole. Incubation was for 90 minutes at 26°C. Aliquots were removed from each assay and the amount of labelled amino acid incorporated determined as described in Section 2.7.1. The ^{35}S-methionine incorporated (cpm) was calculated as a percentage of the total ^{35}S-methionine added (cpm) and plotted as a function of the added methionine (pmoles).


Inhibition of Percentage of Total Leucine
Incorporated by Exogenous Leucine
Percentage of Total Leucine Incorporated
30
20
10
50% Inhibition
40
80
120
160
200
Added Leucine [pMoles]

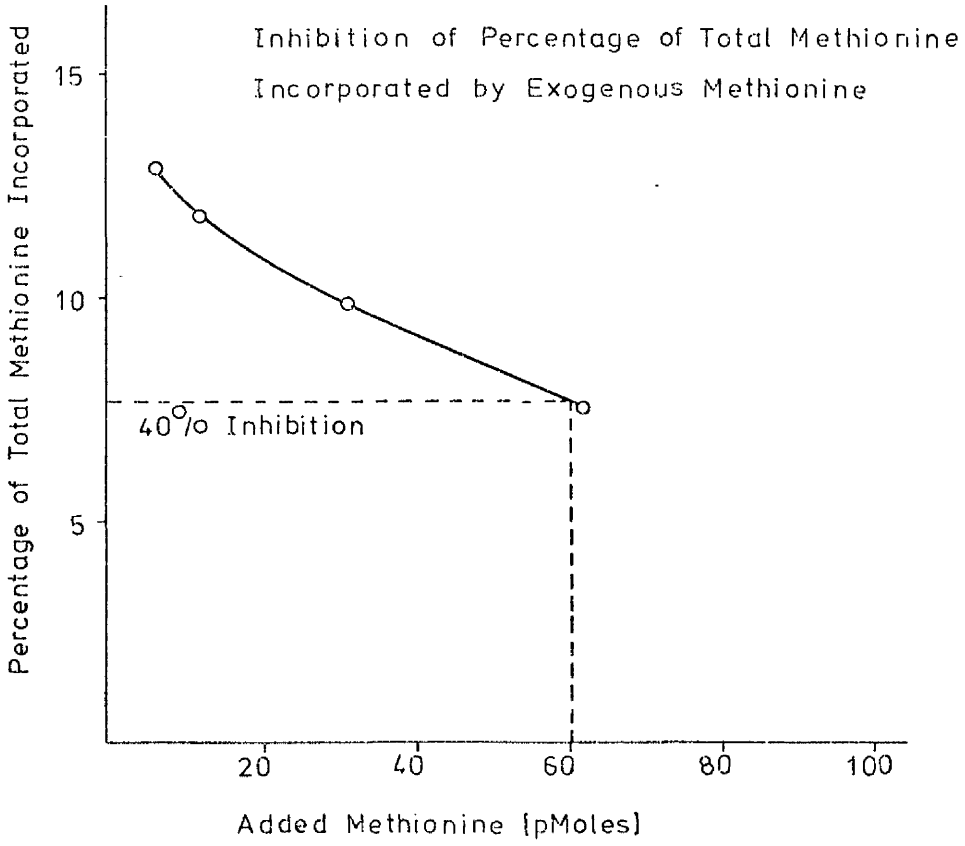

Inhibition of Percentage of Total Methionine
Incorporated by Exogenous Methionine
Percentage of Total Methionine Incorporated
15
10
5
40% Inhibition
20
40
60
80
100
Added Methionine [pMoles]

TABLE 4: Effect of Dialysis on the Incorporation of Exogenous Amino Acids by Wheat Germ Extract

Wheat Germ Extract	Dialysis	A_{260}/ml	^{3}H-Leucine Incorporation (cpm x 10^{-5}
A	-	76	4.17
A	+	65	6.42
B	-	45	3.95
B	+	33	7.74

Wheat germ extracts A and B were both prepared from General Mills wheat germ and the final absorbance recorded at 260 nm before and after dialysis, as shown above. An equal volume (20 µl) was added to each assay. Assays (50 µl) also contained 0.5 µg of 5563 poly(A) RNA, 10 µCi of ^{3}H-leucine (specific activity, 55 Ci/mmole), 0.5 µg of creatine kinase, 2.5 m$\underline{M}$ Mg^{2+} and 74 m$\underline{M}$ K^{+}. All other ingredients were as described in Section 2.6. Incubation was for 90 minutes at 25°C.

Aliquots were removed to determine the incorporation of ^{3}H-leucine as described in Section 2.7.1. The table above shows the incorporation of ^{3}H-leucine per 50 µl assay before (-) and after (+) dialysis of the wheat germ extract.

3.1.7 In Vitro Synthesis of 5563 Immunoglobulin Heavy and Light Chains as a Function of Time

The rate of synthesis of total protein directed by 1.1 µg of 5563 poly(A) RNA is shown in Figure 9. The rate of synthesis of individual complete polypeptides is shown in Figures 10 and 11. To determine whether one round of heavy or light chain synthesis could be detected by autoradiography as described in Section 2.9.1.2b, the following calculation was made.

Assuming a ratio of 1:10, protein molecular weight: mRNA molecular weight, 1.0 µg of mRNA will synthesize 100 ng of protein per round of translation. If it is further assumed that light chain mRNA represents 1% of the total cellular mRNA (probably a low estimate since 8 to 10% of the protein synthesized by 5563 cells is immunoglobulin), it follows that 1.0 ng, or 4.0×10^{-11} mmoles of light chain is synthesized:

$$\text{mmoles of light chain} = \frac{1 \times 10^{-9} \text{ gm}}{25 \times 10^{3} \text{ gm/mole}}$$

$$= 4 \times 10^{-11} \text{ mmoles}$$

5563 light chain contains 3 methionine residues (A.R. Williamson, unpublished results). Therefore, 4×10^{-11} mmoles of light chain contains 1.2×10^{-10} mmoles of methionine. From the specific activity of the ^{35}S-methionine in the assay, the final estimation of disintegrations per minute per round of light chain synthesis can be calculated. The specific activity of the ^{35}S-methionine (1096 Ci/mmole) is altered by the endogenous pool of amino acids in the wheat germ extract (estimated from Section 3.1.6) and is thus taken to be 174 Ci/mmole.

Figure 9

Time Course for Protein Synthesis

Duplicate wheat germ cell-free translation assays (500 µl) contained 12 A_{260} units of General Mills wheat germ extract, 5 µg of creatine kinase, 74 mM K^+, 2.5 mM Mg^{2+}, 11 µg of 5563 poly(A) RNA and 180 µCi of ^{35}S-methionine (specific activity, 1096 Ci/mmole). All other ingredients were as described in Section 2.6.

Incubation was for a total period of 3 hours at 26°C. Aliquots (5 µl) were removed at the times shown in Figure 9 and the amount of labelled amino acid incorporated determined as described in Section 2.7.2. Figure 9 shows the ^{35}S-methionine (cpm) incorporated per 50 µl assay at the time points indicated.

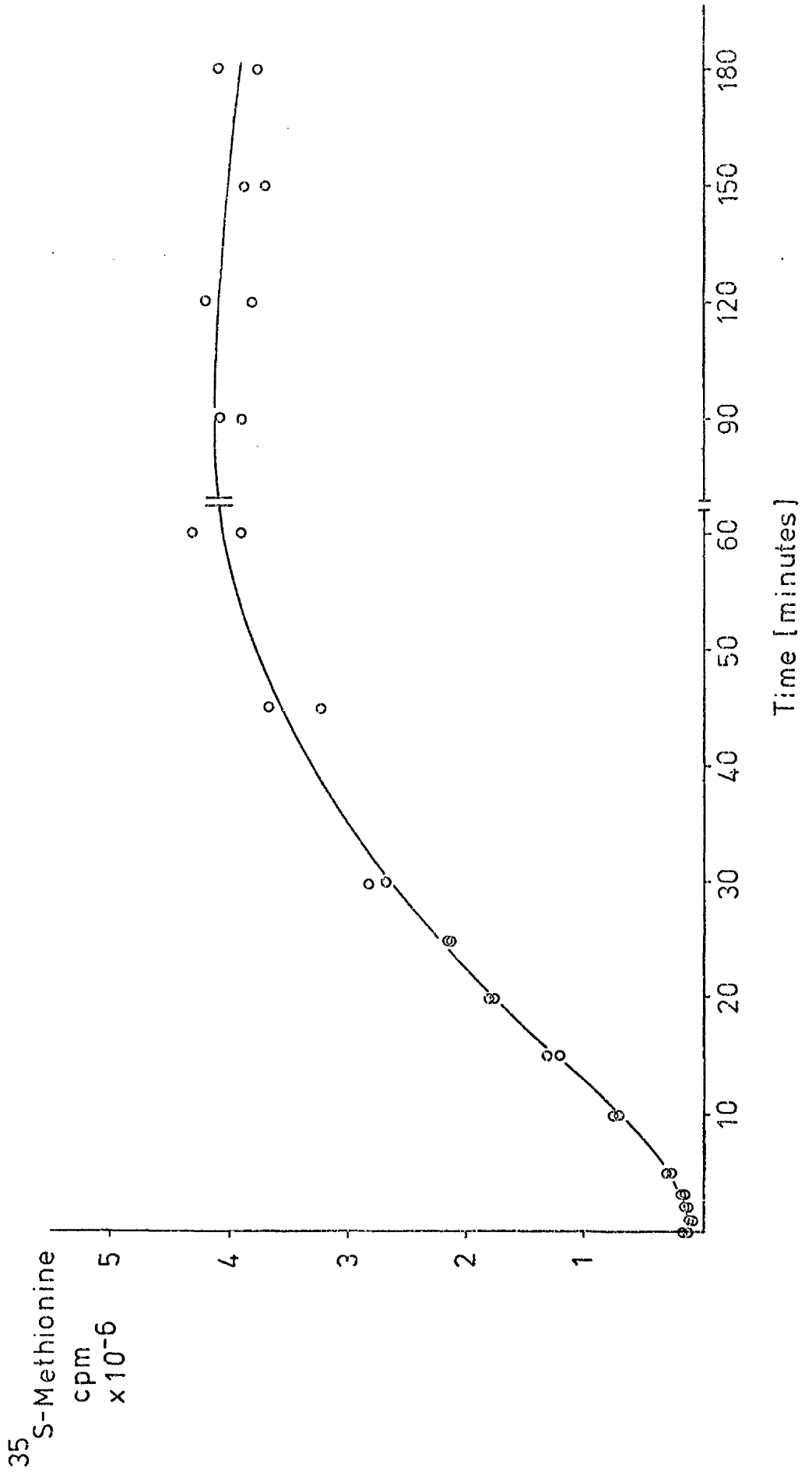

35S-Methionine
cpm
x10-6
1
2
3
4
5
10
20
30
40
50
60
90
120
150
180
Time [minutes]

Figure 10

Elongation of Polypeptide Chains as a Function of Time

Wheat germ assays were prepared as described in Figure 9. Aliquots (10 µl) were removed at time intervals shown in Figure 10, diluted five-fold with Buffer A (Section 2.7) and precipitated with acetone (Section 2.7.3). The precipitates were prepared for SDS-polyacrylamide gel electrophoresis as described in Section 2.9.1. After electrophoresis, the gel was processed for autoradiography (Section 2.9.1.2b). The time of exposure of the film was 18 hours.

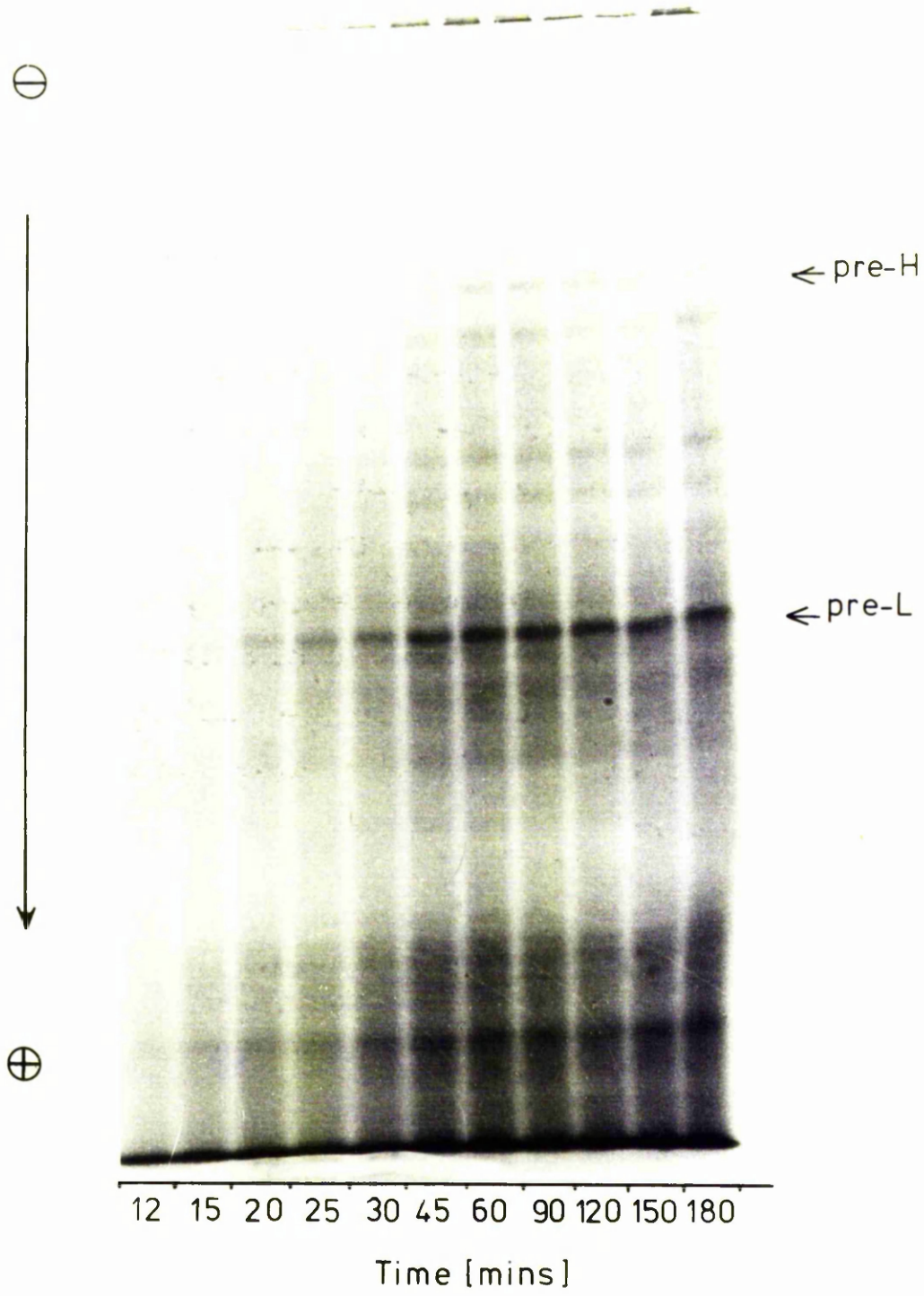
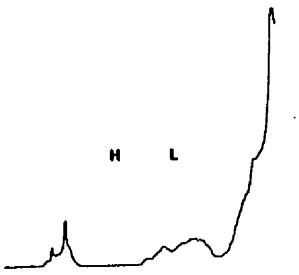

⊖
⊕
pre-H
pre-L
12 15 20 25 30 45 60 90 120 150 180
Time [mins]

Figure 11

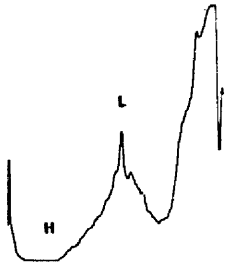
Protein Synthesis as a Function of Time

Figure 11 represents a number of densitometric traces obtained by scanning the autoradiograph described in Figure 10. The autoradiograph was analysed by a Joyce-Loebel densitometer to obtain a more quantitative analysis of the proteins synthesized during the various incubation periods as indicated.

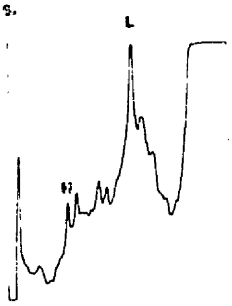
12 mins.



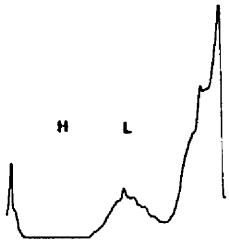
25 mins.



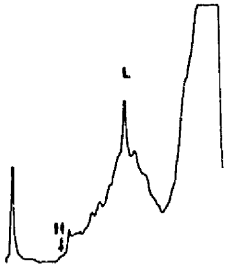
60 mins.



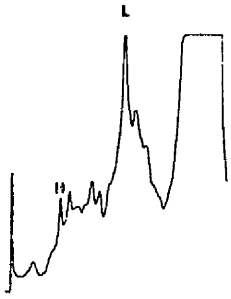
15



30



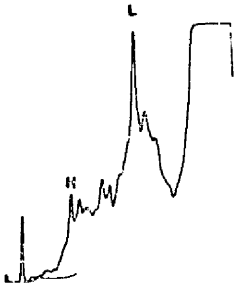
90



20

45

120



Exogenous methionine = 16 pmoles

Endogenous methionine = 85 pmoles

$\therefore$ Final Specific Activity = 1096 Ci/mole x $\frac{16 \text{ pmoles}}{101 \text{ pmoles}}$

= 174 Ci/mmole

Therefore, one round of light chain synthesis has a methionine activity of 4.6×10^4 dpm.

1.2×10^{-10} mmoles x 174 Ci/mmole = 2.08×10^{-8} Ci

$(2.08 \times 10^{-8}$ Ci) $(2.22 \times 10^{-12}$ dpm/ci) = 4.6×10^4 dpm

A similar calculation can be made for heavy chain, assuming a molecular weight of 55,000 and a methionine content of 10 residues (A.R. Williamson, unpublished results). The methionine activity of one round of heavy chain synthesis is thus estimated to be 7×10^4 cpm.

According to Bonner and Laskey, 1974, 1.5×10^3 dpm of ^{35}S-methionine can be detected in a single protein band in 24 hours by autoradiography. Therefore, the heavy and light chains synthesized by 1.1 µg of 5563 poly(A) RNA, under the conditions described in Figure 9, should be detected by autoradiography in less than one hour.

Figure 10 represents 20% of the proteins synthesized by 1.1 µg of 5563 poly(A) RNA as a function of time. From the above calculation, it can be assumed that the first time point at which a protein is detected by autoradiography represents the time required for one round of translation of the corresponding mRNA. Immunoglobulin light chain is first detected by autoradiography after an incubation period of 15 minutes. This period represents the time necessary for initiation, elongation and termination. From Figure 9, the time required for initiation is less than 3 minutes,

since the lag period prior to linear amino acid incorporation represents the time necessary for equilibration of ^{35}S-methionine and tRNA, initiation and polymerisation of sufficient amino acids to enable their precipitation with TCA. The actual time attributable to initiation is probably small compared to the time necessary for elongation. The time required for termination of the polypeptide chain is also probably negligible with respect to the elongation rate since Lodish and Jacobsen, 1972, have shown that the rate of release of complete globin chains from polyribosomes in cell-free extracts of rabbit reticulocytes is about 15 seconds per chain.

The κ light chain of 5563 contains 214 amino acid residues (Fougereau et al., 1976). Light chains synthesized in vitro, however, generally contain an extra N-terminal amino acid sequence of approximately 20 residues (Burstein and Schechter, 1976). Therefore, 5563 light chain synthesized in vitro probably contains about 234 amino acids. If it is assumed that 15 minutes is a close approximation to the elongation time for light chain, the rate of polymerisation is 0.26 amino acids per second at 26°C.

Similarly, the first detectable heavy chain is synthesized in 30 minutes. (A small amount of heavy chain can be detected by the eye in the original autoradiograph although heavy chain can only be detected after 45 minutes by the Joyce-Lobel densitometer). Since γ_{2A} heavy chain contains 447 amino acid residues (Fougereau et al., 1976) and is also likely to be synthesized in vitro as a precursor molecule (Schechter et al., 1977), the rate of polymerisation is calculated to be 0.26 amino acids per second at 26°C.

3.1.8 Efficiency of Translation of Myeloma Poly(A) RNA

As described in the previous sections, several factors affecting translation of mouse myeloma poly(A) RNA have been optimised. Table 5 illustrates the capacity of such an optimised wheat germ cell-free system to translate several different preparations of both mouse and human poly(A) RNA. The results reflect not only the efficiency of the system but also its reproducibility.

The amount of protein synthesis, in each case, has been measured as the number of pmoles of amino acid (exogenous plus endogenous) incorporated, calculated as follows:

$$\frac{\text{Exogenous Amino Acid Incorporated (cpm)}}{\text{Exogenous Amino Acid Added (cpm)}} \quad \% \quad X \quad \text{Endogenous + Exogenous amino acid (pmoles)}$$

(The exogenous amino acid (pmoles) is calculated from the known specific activity; the endogenous amino acid (pmoles) is estimated from the data in Section 3.1.6)

From the amino acid incorporation, the actual number of rounds of synthesis can be estimated with the following assumptions:-

1. The ratio of protein molecular weight to the molecular weight of its equivalent mRNA is 1:10.
2. The average molecular weight of synthesized protein is 30,000.
3. A 30,000 molecular weight protein contains, on average, 15 leucine and 3 methionine residues.

Poly(A) RNA (µg)	Spermidine (0.4 mM)	Incorporation of Leucine (pmoles)	Incorporation of Methionine (pmoles)
0.5 µg 5563	-	49	-
0.5 µg 5563	-	46	-
0.6 µg 5563	-	51	-
0.6 µg 5563	-	-	12.7
0.6 µg 5563	+	-	18.3
1.2 µg 5563	+	-	23.0
0.6 µg Daudi	+	-	15.2
0.8 µg 1788	+	-	16.6

TABLE 5: Incorporation of Amino Acids into Protein by a Wheat Germ Cell-Free System

All assays were carried out using Texan wheat germ extract. Assays with ^{3}H-leucine contained 2.3 A_{260} units of extract while assays with ^{35}S-methionine contained 1.8 A_{260} units. The magnesium concentration was 1.5 mM in the presence of spermidine and 2.5 mM in its absence. All assays contained 74 mM potassium, 0.5 µg of creatine kinase and all other reagents as described in Section 2.6. Incubation was for 90 minutes at 26°C.

Daudi and 1788 are human lymphoid cell lines. The poly(A) RNA from these lines was a gift from Mr. P.A. Singer.

From the data in Table 5, the average number of pmoles of leucine incorporated by 0.5 μg of poly(A) RNA is approximately 48.

$$48 \text{ pmoles of leucine} = \frac{48}{15} \text{ pmoles of protein}$$

$$= 3.2 \text{ pmoles of protein}$$

From the first assumption, 0.5 μg of poly(A) RNA would be expected to synthesize 50 ng of protein per round of translation. From the second assumption, 3.2 pmoles of protein is equivalent to 96 ng of protein. Therefore, the number of rounds of synthesis is approximately 2, in the absence of spermidine.

A similar calculation based on an average incorporation of methionine of 13 pmoles in the absence of spermidine gives the rounds of synthesis as 2, whereas an average incorporation of methionine in the presence of spermidine is equivalent to approximately 3 rounds of synthesis.

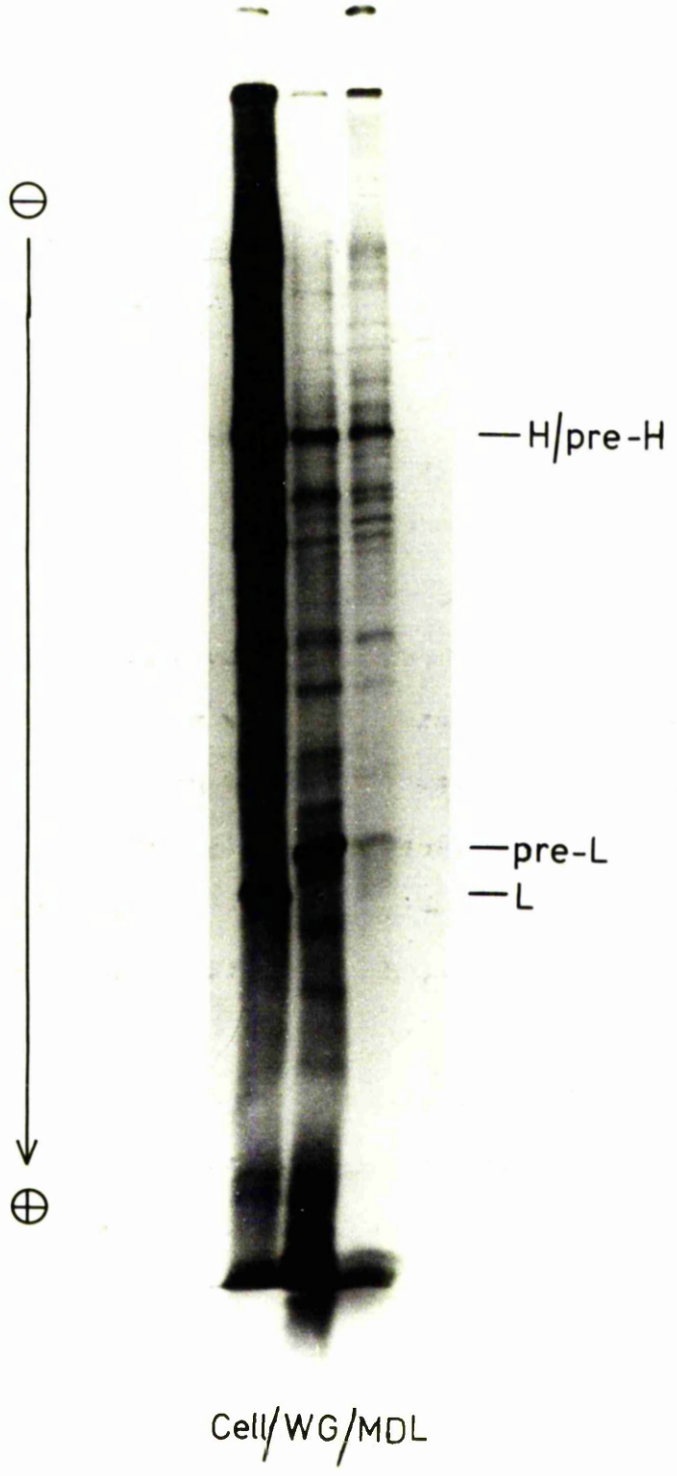
However, it appears that although the above calculations give an average of the rounds of translation of all the mRNA species, the actual number of rounds of translation of particular mRNAs may differ greatly and not necessarily reflect the amount of each mRNA species present. Figure 12 shows a comparison of the cell-free products directed by the same preparation of poly(A) RNA in a messenger-dependent reticulocyte lysate (MDL) (Pelham and Jackson, 1976) and the wheat germ system. (I am grateful to Mr. S. A. Laidlaw for translating this poly(A) RNA sample in the MDL). Further discussion of these results is carried out in the Discussion Section.

Figure 12

A Comparison of Protein Synthesis Directed by Mouse Myeloma Messenger RNA in Two Different Cell-Free Systems

The conditions for the translation of 5563 poly(A) RNA in the wheat germ system were as described for Figure 15. The translation of 5563 poly(A) RNA in a messenger dependent reticulocyte lysate cell-free system was carried out by Mr. S.A. Laidlaw. In both cases, the synthesized proteins were labelled with ^{35}S-methionine. The cell line, 5563, was labelled biosynthetically with ^{35}S-methionine and a lysate prepared as described in Section 2.4.1.

All samples were prepared for SDS-polyacrylamide gel electrophoresis as described in Section 2.9.1 and the gel processed for fluorography (Section 2.9.1.2a).


⊖
⊕
—H/pre-H
—pre-L
—L
Cell/WG/MDL

3.2 IDENTIFICATION OF MOUSE IgG HEAVY CHAIN SYNTHESIZED IN VITRO

Two methods of identification of the cell-free synthesized IgG heavy chain were used. These were the identification of IgG by comparison with the cell-free products synthesized by mouse myeloma poly(A) RNA isolated from a non-IgG-producing cell line and identification by immunoprecipitation.

3.2.1 Identification of IgG Heavy Chain by Comparison with the Cell-Free Translation Products of a Non-IgG-Producing Cell Line

For the purpose of this comparison, the three cell lines used were MOPC 315.40 which synthesizes and secretes IgA heavy chain and λ_2 light chain, MOPC 315.26, a variant clone selected by Dr. T. Mosmann, which synthesizes and secretes λ_2 light chain only and 5563 which synthesizes and secretes IgG_{2A} heavy chain and κ light chain. Lysates of these cell lines and immune precipitates of their respective intracellular and extracellular proteins are shown in Figure 13.

Poly(A) RNA was isolated from the above mentioned cell lines as described in Section 2.5. Profiles of the intermediate polysomes are shown in Figure 14, showing no variation in quality and thus no bias in any one preparation for the elimination of specific mRNAs. The poly(A) RNAs were subsequently translated in a wheat germ cell-free system and the synthesized proteins analysed by SDS-polyacrylamide gel electrophoresis (Figure 15). The γ_{2A} and α heavy chains and the κ and λ_2 light chains can easily be identified as the only major proteins which are not common to the three mouse cell lines.

Figure 13

Synthesis of Immunoglobulin by Several Myeloma Cell Lines

The fluorograph shows an analysis by SDS-polyacrylamide gel electrophoresis (Section 2.9.1) of the total intracellular proteins (I), the intracellular immunoglobulin (IIg) and secreted immunoglobulin (SIg) for each cell line.

Cell lines, 5563, P1.17, MOPC 315.40 and MOPC 315.26 were incubated for one hour in medium containing ^{35}S-methionine, as described in Section 2.4. Cell lysates were prepared as described in Section 2.4.1. Immunoglobulin was precipitated from both the cell lysates and the medium by addition of specific antisera as described in Section 2.1.3 and the soluble complex formed precipitated by addition of GARIg antibodies, at equivalence, as described in Section 2.4.3.

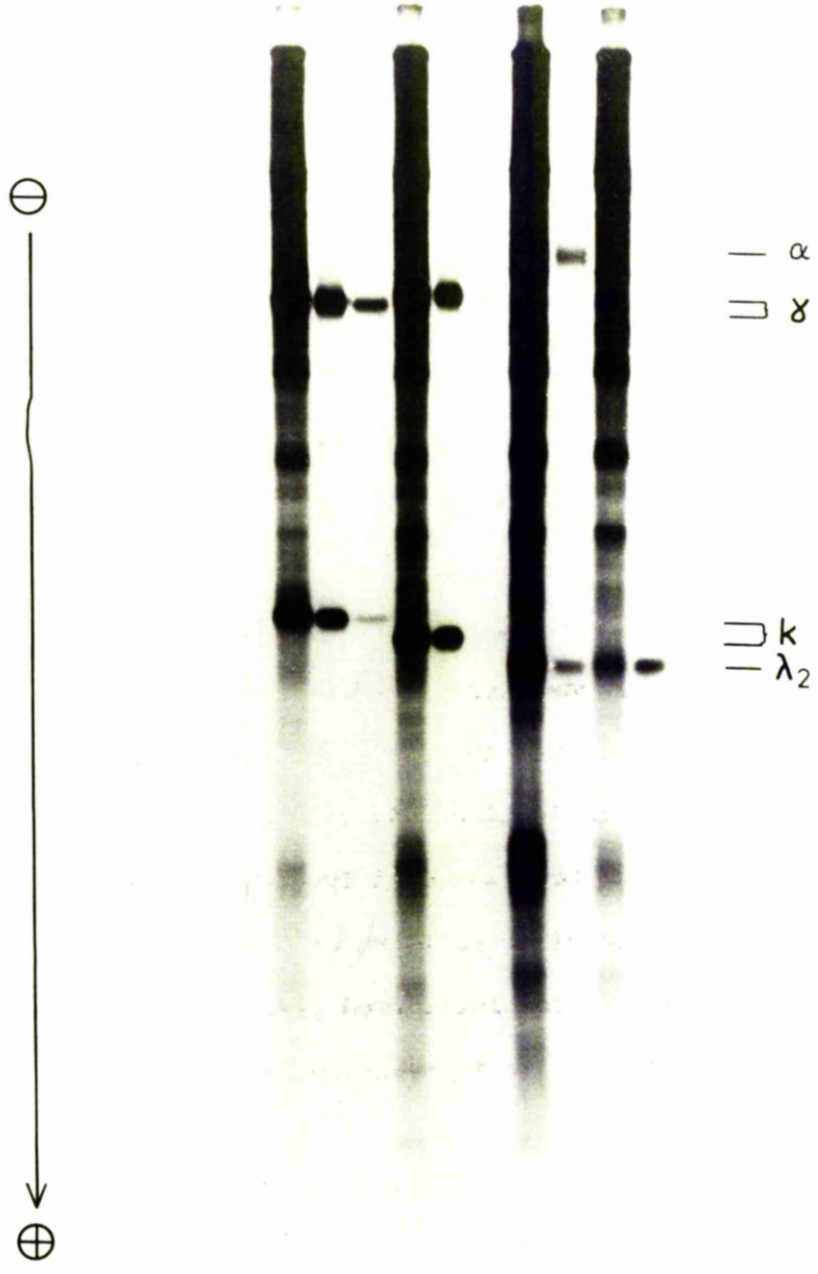

⊖
⊕
α
γ
k
λ2
I IIg SIg I IIg SIg I IIg I IIg
P1.17
5563
315.40
315.26

Figure 14

Sucrose Density Gradient Analyses of Polysomes
Prepared from Different Myeloma Cell Lines

Polysomes were prepared from 5563, MOPC 315.40 and MOPC 315.26 cell lines as described in Section 2.5.1. The polysomes were analysed by sucrose density gradient centrifugation as described in Section 2.5.2.

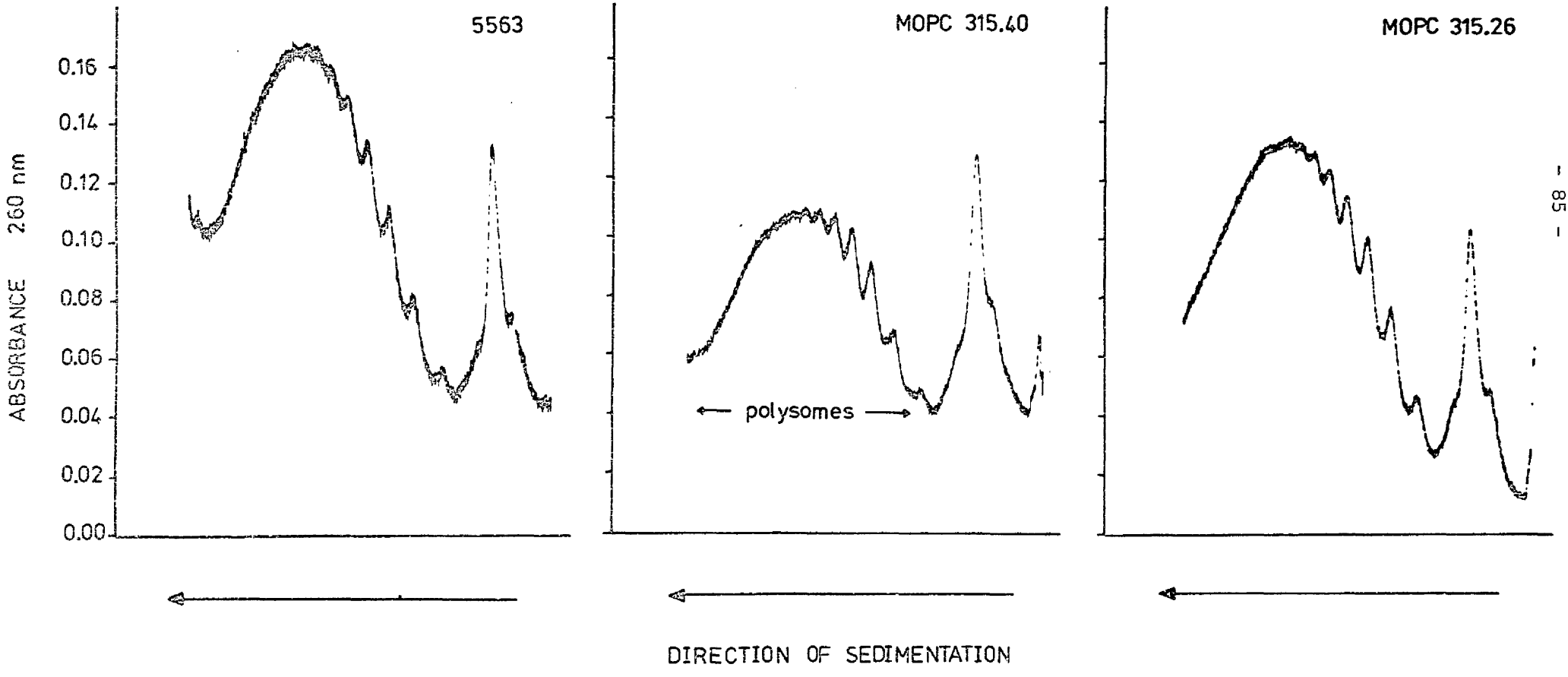
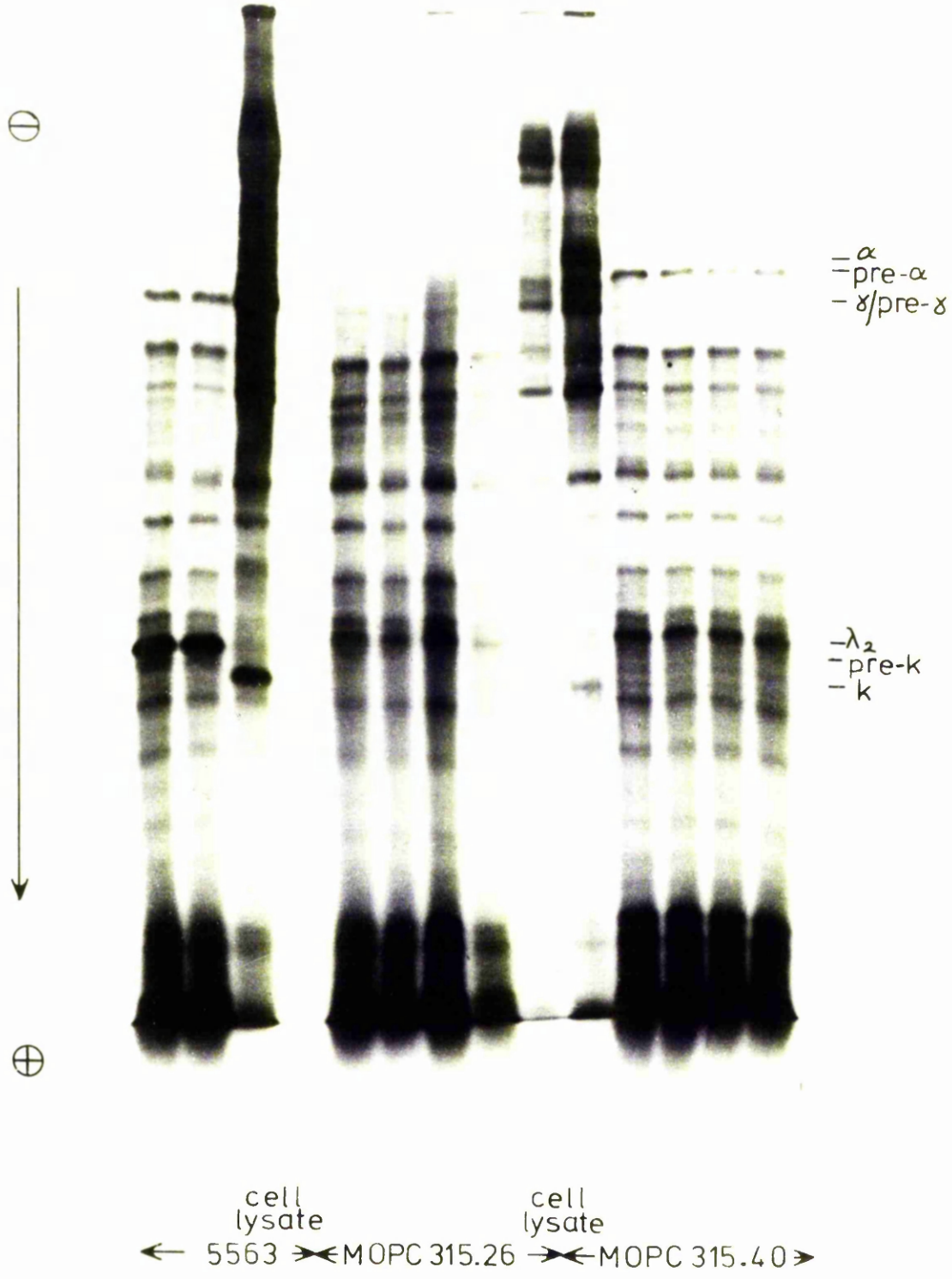

5563
MOPC 315.40
MOPC 315.26
ABSORBANCE 260 nm
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
polysomes
DIRECTION OF SEDIMENTATION

Figure 15

Comparison of the Cell-Free Translation Products of Poly(A) RNA from Different Myeloma Cell Lines

Wheat germ assays (50 µl) contained 2.4 A_{260} units of Texan wheat germ extract, 0.02 µg of creatine kinase, 74 m$\underline{M}$ K^+, 2.5 m$\underline{M}$ Mg^{2+}, 15.5 µCi of ^{35}S-methionine (specific activity, 247 ci/mmol) and approximately 1.0 µg of poly(A) RNA as indicated in the figure opposite. All other reagents were as described in Section 2.6. Incubation was for 90 minutes at 26°C.

Samples were prepared for SDS-polyacrylamide gel electrophoresis as described in Section 2.9.1.


⊖
⊕
α
pre-α
γ/pre-γ
λ2
pre-k
k
cell lysate
cell lysate
← 5563 ⋊ MOPC 315.26 → ⋊ MOPC 315.40 →

It is interesting to compare the mobilities of the heavy and light chains synthesized *in vitro* and *in vivo*. Both κ and λ_2 light chains synthesized *in vitro* migrate more slowly in SDS-polyacrylamide gels suggesting a size difference. Several cell-free synthesized mouse light chains have been sequenced by Burstein and Schechter, 1976, showing an extra N-terminal piece of polypeptide chain. It seems likely that a similar explanation would be applicable to the κ-chain of 5563.

No conclusive evidence has yet been published showing a similar extension of the variable region of cell-free synthesized heavy chains. Comparing the mobilities of IgA cell-free and cellular heavy chains in SDS-polyacrylamide gels, a marked difference can be seen with the cellular product migrating more slowly. This difference can be attributed to the presence of carbohydrate (Maizel, 1971) on the cellular product while cell-free synthesized proteins are believed to lack carbohydrate. However, when a similar comparison is made with 5563 IgG heavy chain, no difference in mobility can be detected. IgG_{2A} heavy chain has one point of carbohydrate attachment (Fougereau *et al.*, 1976) which would be likely to retard its migration in SDS-polyacrylamide gels. It is possible, therefore, that the cell-free synthesized heavy chain contains some extra feature to thus enable its co-migration with the cellular glycoprotein. These possibilities are further discussed later.

3.2.2 Identification of IgG by Immunoprecipitation

Further evidence that the wheat germ system is capable of synthesizing immunoglobulin heavy chain was obtained by specific immune precipitation of the heavy and light chains as shown in Figure 16. This result confirms the synthesis of IgG heavy chain and its migration in SDS-polyacrylamide gels as described in Section 3.2.1. However, since γ_{2A} heavy chain appears

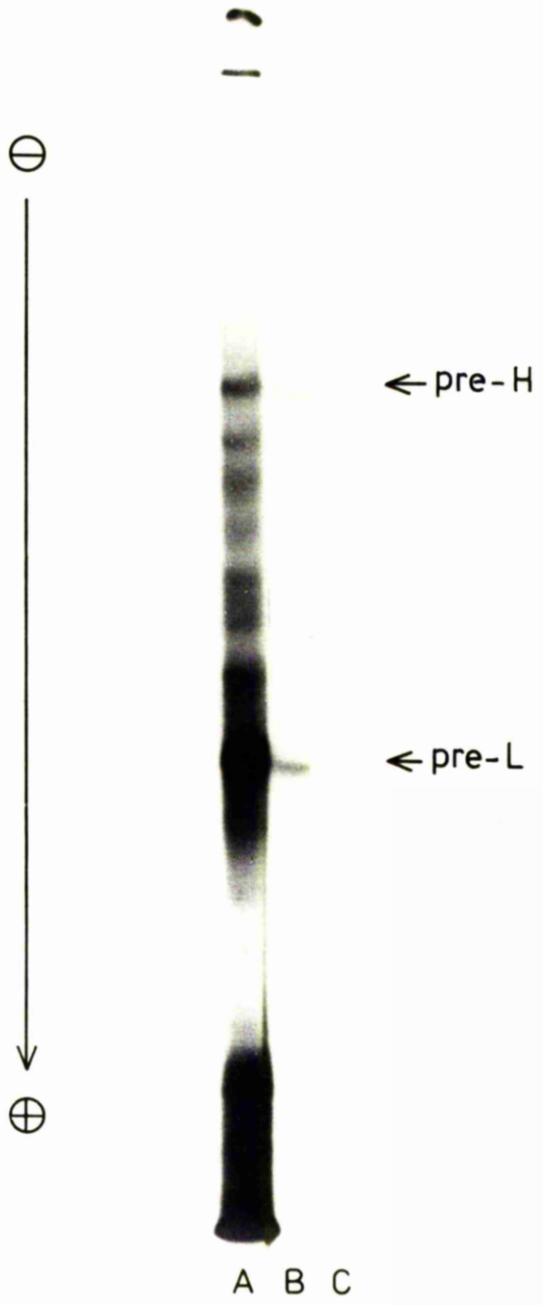
Figure 16

Immunoprecipitation of Immunoglobulin Heavy and Light Chains Synthesized in Vitro

The fluorograph shows an analysis by SDS- polyacrylamide gel electrophoresis (Section 2.9.1) of total protein and immunoglobulin precipitated from 5% of a wheat germ assay by addition of acetone (A), anti-5563 antibodies (B) and NorIg (C). The necessary time of exposure of the film by fluorography for samples (B) and (C) was five times that of (A).

The wheat germ assay (50 µl) contained 1.8 A_{260} units of Texan wheat germ extract, 0.6 µg of 5563 poly (A) RNA, 74 m$\underline{M}$ K^+, 1.5 m$\underline{M}$ Mg^{2+}, 0.4 m$\underline{M}$ spermidine, 0.5 µg of creatine kinase and 17 µCi of ^{35}S-methionine (specific activity, 1027 Ci/mmole). All other reagents were as described in Section 2.6. Incubation was for 90 minutes at 25°C.

The assay was diluted with Solution B and centrifuged (Section 2.7). An aliquot was removed for precipitation of total protein with acetone (Section 2.7.3) and 40% of the assay was used for immune precipitation of 5563 heavy and light chains by addition of 12.5 µg and anti-5563 purified antibodies and 50 µg of GARIg antibodies (see Sections 2.1.3 and 2.8). A non-specific precipitation was also carried out on 40% of the wheat germ assay by addition of NorIg (12.5 µg) and GARIg antibodies (50 µg).


⊖
⊕
← pre-H
← pre-L
A B C

to be well separated from other major proteins (Figure 15), it appears that immune precipitation of the cell-free synthesized chain is not quantitative (Figure 16). The immune precipitable γ_{2A} heavy chain requires a much longer exposure to cause blackening of the film by fluorography than does the corresponding γ_{2A} heavy chain band in the total proteins.

3.3. CHARACTERIZATION OF IMMUNOGLOBULIN HEAVY CHAIN SYNTHESIZED IN VITRO

Two methods of characterization of heavy chain synthesized *in vitro* have been employed, namely comparison of the protease digestion products of heavy chains synthesized *in vitro* and *in vivo* and amino acid sequence analysis.

3.3.1 Peptide Mapping by Limited Proteolysis

Analysis of proteins purified by SDS-polyacrylamide gel electrophoresis is often hampered by technical difficulties such as elution from the gel and the sensitivity of several analytical systems to SDS. Cleveland *et al.*, 1977, have described a method of peptide mapping which eliminates these problems. This method has been described in detail in Section 2.10 and involves identifying heavy chains, synthesized *in vitro* or *in vivo*, by SDS-polyacrylamide gel electrophoresis, digestion of the protein in the gel by proteolysis and subsequent analysis of the digestion products by further SDS-polyacrylamide gel electrophoresis.

To determine the reproducibility of such a peptide map of γ_{2A} heavy chain, IgG labelled with ^{35}S-methionine was isolated from the media of 5563 cells by immune precipitation and analysed by SDS-polyacrylamide gel electrophoresis (see Sections 2.4.2 and 2.9.1). Immunoglobulin heavy chain bands were identified by autoradiography (Section 2.9.1.2 (b)), isolated

and digested with Staphylococcus aureus V8 protease as described in Section 2.10. This protease cleaves polypeptide chains specifically at glutamic acid residues. The peptide maps of γ_{2A} heavy chain obtained at different enzyme concentrations are shown in Figure 17. The digestion products were reproducible for a given enzyme concentration and the peptide maps remained similar over a wide range of concentrations which suggested that such a system could provide a valuable method for the comparison of heavy chains synthesized *in vitro* and *in vivo*.

A comparison of heavy chains synthesized *in vitro* and *in vivo* is shown in Figure 18. 5563 γ_{2A} heavy chain synthesized *in vivo* was prepared as described above. 5563 γ_{2A} heavy chain was synthesized *in vitro* in the presence of ^{35}S-methionine and identified according to mobility in an SDS-polyacrylamide gel as described in Section 3.2.1, with the exception that the gel was processed for autoradiography (Section 2.9.1.2(b)). A comparison of the peptide maps of heavy chain synthesized *in vitro* and *in vivo* is complicated by the fact that heavy chain synthesized *in vivo* is synthesized as a glycoprotein while heavy chain synthesized in the wheat germ cell-free system lacks the carbohydrate portion. Although γ_{2A} heavy chain has only one point of carbohydrate attachment (Fougereau *et al.*, 1976), the mobility in SDS-polyacrylamide gels of several of the partial digestion products shown in Figure 18 is further complicated by inadequate resolution of the peptide chain fragments, especially from the cell-free product. However, comparing peptides A and B from the cellular heavy chain to C and D from the heavy chain synthesized *in vitro* suggests the possibilities that (1) these peptides are derived from the same part of the polypeptide chain since in both cases they contain a major portion of the ^{35}S-methionine and (2) the mobility differences cannot be attributable to

Figure 17

Limited Protease Digestion of IgG Heavy Chains

IgG, labelled with ^{35}S-methionine, was isolated from the media of 5563 cells by immune precipitation as described in Section 2.4. The immune precipitates were analysed by SDS-polyacrylamide gel electrophoresis, the heavy chain bands removed and digested with Staphylococcus aureus V8 protease as described in Section 2.9.1 and 2.10, respectively. The amount of protease added to each sample is shown in the figure opposite.

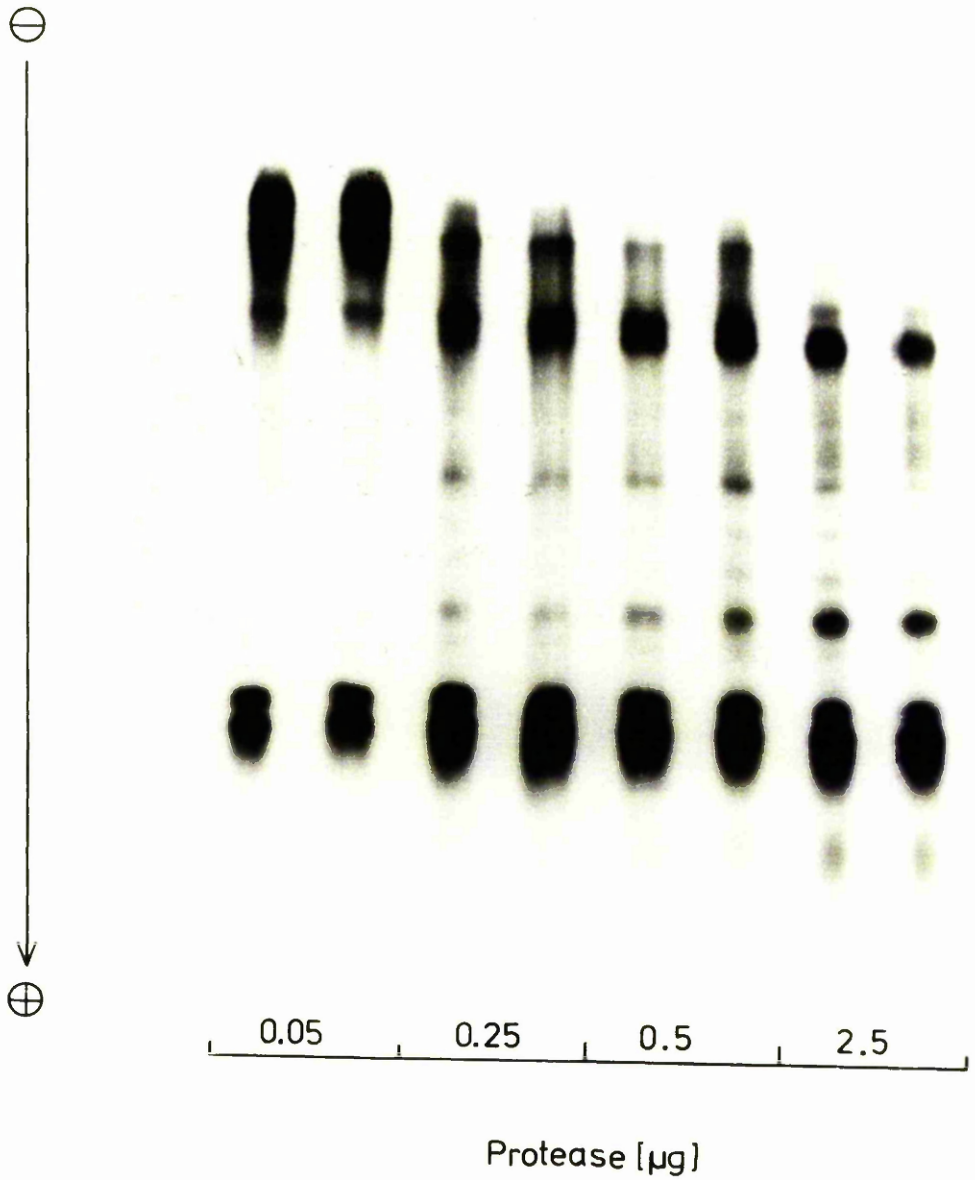
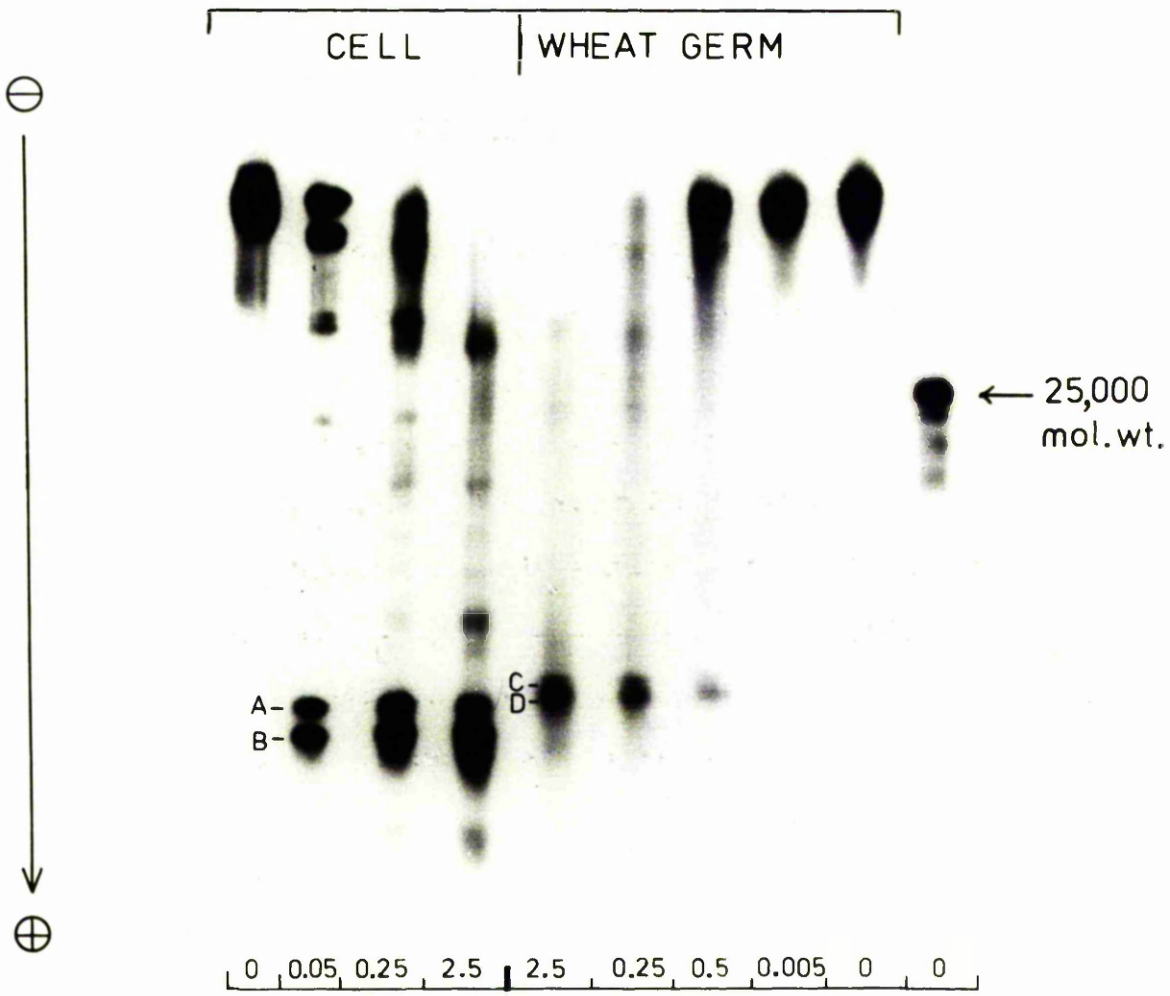

⊖
⊕
0.05
0.25
0.5
2.5
Protease [µg]

Figure 18

Comparison of Protease Digestion Products of Heavy Chain Synthesized in Vivo and In Vitro

IgG heavy chain was prepared as described for Figure 17. 5563 heavy chain was synthesized *in vitro* in the wheat germ system as described for Figure 9, with an assay incubation period of 3 hours. The heavy chain was identified by its mobility in an SDS-polyacrylamide gel (see Section 3.2). Heavy chains synthesized *in vitro* and *in vivo* were digested with Staphylococcus aureus V8 protease as described in Section 2.10, with amounts of protease as shown in the figure opposite.


CELL
WHEAT GERM
⊖
⊕
← 25,000
mol.wt.
A-
B-
C
D
0 0.05 0.25 2.5 2.5 0.25 0.5 0.005 0 0
PROTEASE [µg]

carbohydrate content since the cell-free product migrates more slowly in SDS-polyacrylamide gels which leads to the possibility that the mobility is affected by extra C or N-terminal amino acid residues.

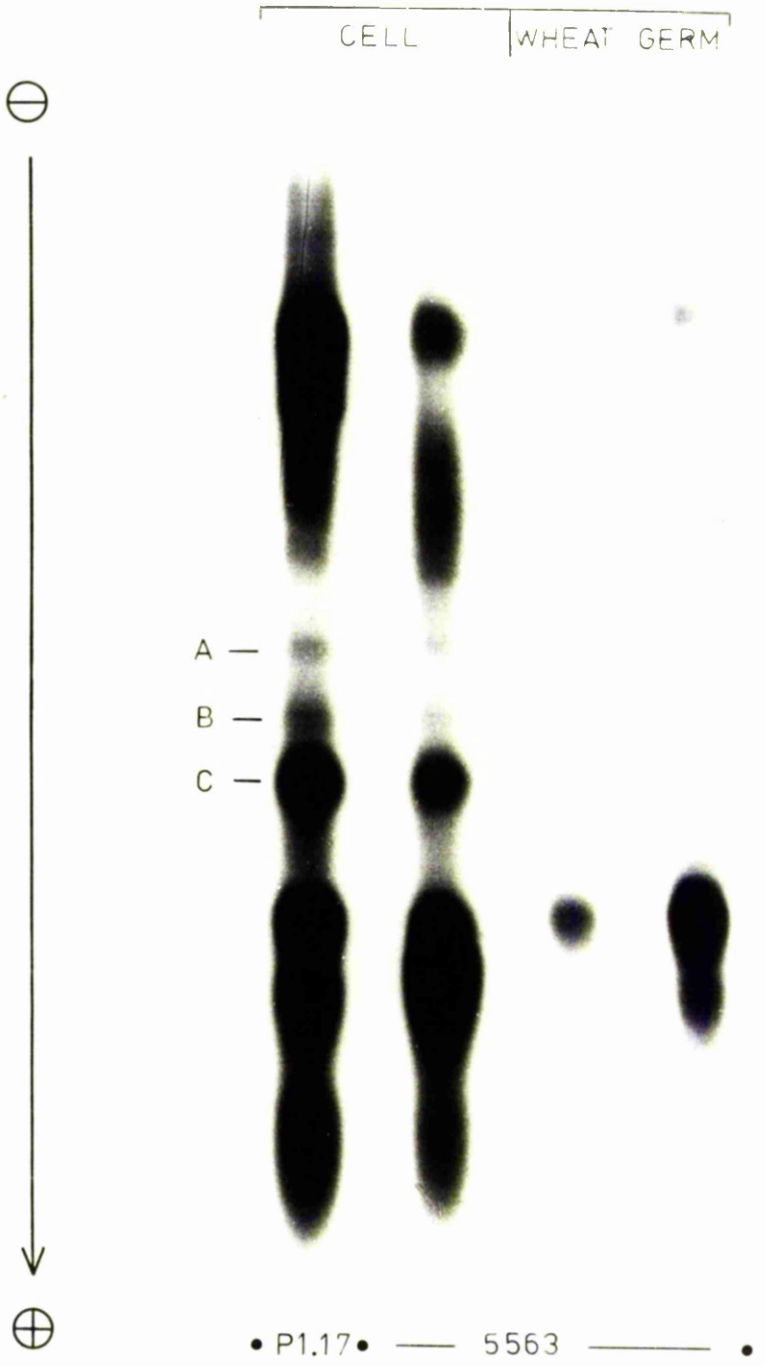
In an attempt to simplify the interpretation of the peptide maps obtained by proteolytic digestion, the digestion products of P1.17 and 5563 heavy chains synthesized *in vivo* were compared since the immunoglobulin produced by both these cell lines belongs to the same subclass. These heavy chains therefore share identical constant region amino acid sequences which should yield identical protease digestion products. This should enable the identification of those peptides which differ between 5563 heavy chains synthesized *in vitro* and *in vivo* because of carbohydrate content and identification of the variable region digestion products. It was intended that this would lead to the assignment of peptides A and B, and C and D, to either the V- or C-region of the heavy chain.

A comparison of the digestion products of P1.17 and 5563 heavy chains synthesized *in vivo* and 5563 heavy chain synthesized *in vitro* is shown in Figure 19. Peptides A, B, and C appear to represent peptides from the constant region of the molecule since they are present in the digestion products of both the cellular P1.17 and 5563 heavy chains. The identification of A, B and C constant region peptides in 5563 heavy chain synthesized *in vitro* is hindered by the lower activity of ^{35}S-methionine and also the absence of carbohydrate moieties which complicates the comparison with the cellular heavy chain. It is difficult to assign the other peptides to the V- or C-region of the molecule since most of the

Figure 19

Comparison of Protease Digestion Products of P1.17 and 5563 Heavy Chains

IgG was isolated from the media of P1.17 and 5563 cells as described for Figure 17. 5563 γ_{2A} heavy chain was synthesized *in vitro* and isolated by immune precipitation and SDS-polyacrylamide gel electrophoresis as described for Figure 16. Digestion of heavy chains synthesized *in vitro* and *in vivo* was carried out as described in Section 2.10 at protease concentrations as shown in the figure opposite. Each immune precipitate contained 6 to 12 µg of protein.


CELL
WHEAT GERM
⊖
A
B
C
⊕
• P1.17 • —— 5563 ——— •

peptides shown in Figure 19 lack resolution. Hence each band may contain combinations of both V- and C-region peptides. It is, therefore, difficult to determine the existence of an N-terminal precursor in the γ_{2A} heavy chain synthesized in vitro, unless a gel system is applied which gives better resolution of the digestion products.

3.3.2 Amino Acid Sequence Analysis of 5563 Heavy Chain Synthesized in Vitro

The proteins synthesized in the wheat germ cell-free system by total poly(a) RNA isolated from 5563 mouse myeloma cells were labelled with ^{35}S-methionine, ^{3}H-leucine, ^{3}H-lysine, ^{3}H-phenylalanine, ^{3}H-proline and ^{3}H-serine. The immunoglobulin heavy chain was identified according to its mobility in SDS-polyacrylamide gels (see Section 3.2) and eluted from the appropriate part of the gel by incubation for several hours at 37^{o}C in 0.25 M Tris, pH 8.0, 2% SDS, 10 mM DDT. Since the heavy chain was eluted in a volume of approximately 25 mls, the solution was concentrated to 1 or 2 mls in an Amicon ultrafiltration unit using a PM10 membrane. At this stage, a small aliquot was removed and re-run on an SDS-polyacrylamide gel as shown in Figure 20. Sperm whale apomyoglobin was then added as carried protein. This was a generous gift from Dr. J. Coggins. The protein was alkylated by addition of iodoacetic acid to a concentration of 25 mM prior to precipitation of the protein by the addition of five volumes of acetone. The precipitate was washed thoroughly with acetone to remove any SDS or Tris buffer, dissolved in 30% acetic acid, diluted to 3% with water and lyophilized.

Amino acid sequence analysis was carried out by Dr. T.J. Kindt and Mr. D. Gates. The sample at the time of analysis contained 400,000 cpm of tritium and 25,000 cpm of ^{35}S, approximately, of which 50% was used for the analysis. Figure 21 shows the radioactivity recovered at each sequencer cycle; each amino acid was identified by high pressure liquid chromatography. From the data shown, the following assignments

Figure 20

Preparation and Identification of 5563 IgG Heavy and Light Chains for Sequence Analysis

A, B and C represent the eluted light and heavy chains and a sample of the total proteins synthesized in the wheat germ system, respectively.

Wheat germ assays (50 µl) contained 1.2 A_{260} units of General Mills wheat germ extract, 0.5 µg of 5563 poly(A) RNA, 74 m$\underline{M}$ K^+, 2.5 m$\underline{M}$ Mg^{2+} and 0.5 µg of creatine kinase. Amino acids, leucine and phenylalanine, lysine and serine, valine and proline (10 µCi of each) were added to twenty wheat germ assays at specific activities as shown in Section 2.1.4. Two assays contained 15 µCi of ^{35}S-methionine (specific activity, 1026 Ci/mmol). All assays were incubated for 90 minutes at 26°C and prepared for SDS-polyacrylamide gel electrophoresis as described in Section 2.9.1. Heavy and light chain bands were identified according to mobility and eluted from the gel as described in Section 3.3.2. An aliquot of each eluant was removed, prior to alkylation, precipitated in the presence of 0.3 A_{260} units of wheat germ extract as carrier protein, and analysed by SDS-polyacrylamide gel electrophoresis as described in Section 2.9.1. The gel was processed for fluorography (Section 2.9.1.2a).

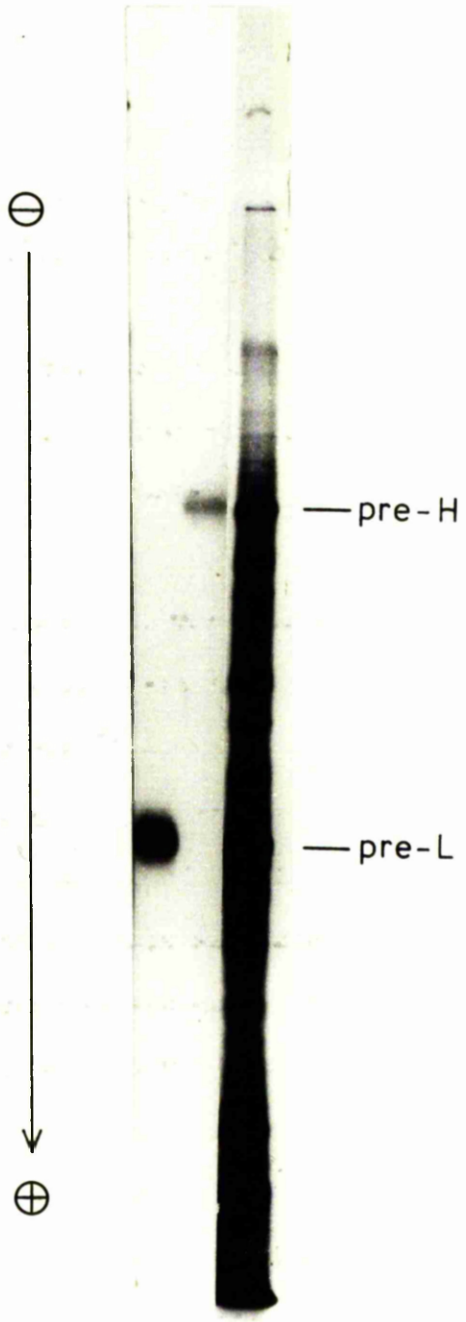
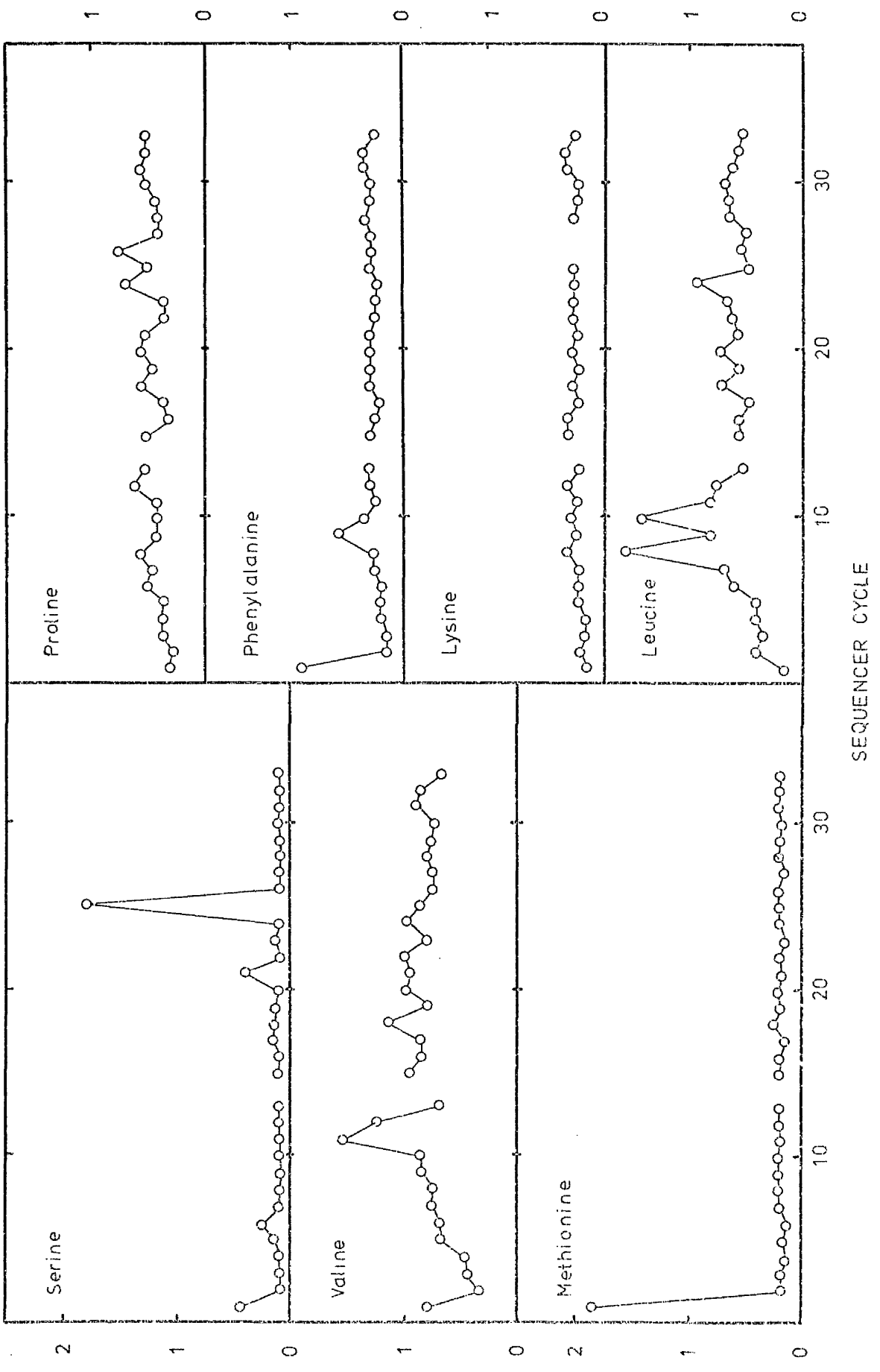

⊖
⊕
pre-H
pre-L
A B C

Figure 21

Sequence Analysis of 5563 IgG Heavy Chain Synthesized in Vitro

IgG_{2A} heavy chain was labelled in vitro with six radioactive amino acids and isolated from an SDS-polyacrylamide gel, as described in Figure 20. The heavy chain sample was analysed in an automatic sequencer and each radioactive amino acid identified by high pressure liquid chromatography. The radioactivity recovered at each sequencer cycle is shown in the figure opposite.


Serine
Valine
Methionine
Proline
Phenylalanine
Lysine
Leucine
SEQUENCER CYCLE
10⁻² x RADIOACTIVITY RECOVERED [cpm]

can be made: position 1, phe or met, position 8, leu, 9 phe, 10 leu, 11 val, 12 also val, 18 val, 24 leu or pro, 25 ser and 26 pro. Assignments at positions 18 and 24 to 26 are only tentative.

The interpretation of the heavy chain sequence data leads to the conclusion that this sequence lacks any of the invariant residues that one would expect to find at the beginning of a mouse heavy chain.

4. DISCUSSION

4.1 PREFERENTIAL SYNTHESIS OF SMALL PROTEINS IN THE WHEAT GERM CELL-FREE SYSTEM

Like the Krebs II ascites system, the wheat germ system has become notorious for 'premature termination' of polypeptide chains (Benveniste et al., 1976; Schechter and Burstein, 1976a). This phenomenon, although well documented, has not been assigned to any particular deficiency in the protein synthetic machinery. In the course of the work reported in this thesis, experimental evidence was gathered which suggested that ribonuclease activity was responsible for the preferential synthesis of small proteins. RNA degradation could also explain the 'premature termination' of polypeptide chains observed by other workers. Recently, Hunter et al., 1977a, reported their observations of nuclease activity in the wheat germ system, also suggesting that mRNA degradation could account for the low efficiency of synthesis of high molecular weight proteins.

Several experiments described in this thesis substantiate this hypothesis:

(1) The generation of small proteins in the wheat germ system is unlikely to be due to an imbalance in the mRNA population since mouse myeloma poly(A) RNA translated in a messenger-dependent reticulocyte lysate cell-free system closely reflects, both quantitatively and qualitatively, the balance of protein synthesis in the intact cell while protein synthesis in the wheat germ system, although accurate qualitatively, is not quantitative (Figure 12). An alternative explanation is that protein synthesis in the cell and the messenger-dependent reticulocyte lysate is subject to control by similar mammalian regulatory factors while the wheat germ system, lacking such factors, more closely reflects the distribution of the mRNA population.

This latter interpretation seems less probable since the regulatory factors would have to distinguish mRNA species on the basis of size, discrimination being approximately proportional to molecular weight.

(2) No protease activity was found in the wheat germ system (Figure 2). Therefore, the generation of small proteins by proteolytic cleavage is unlikely.

(3) The preferential synthesis of small proteins cannot be attributed to factors influencing either initiation or elongation. Assuming that the time required for initiation of protein synthesis is negligible with respect to the time required for elongation (Section 3.1.7), the elongation rates of heavy and light chains are equal. Differences in the rates of initiation of individual mRNA species do not readily explain the above phenomenon since initiation would have to be inversely proportional to molecular weight. Such distinction between mRNA species on the basis of size, as already mentioned in (1), is improbable. Degradation of mRNA by nuclease could readily explain the imbalance in protein synthesis since there is an increased probability of RNA degradation proportional to mRNA size i.e. the longer the mRNA, the longer the time required for polypeptide synthesis and hence the lower the probability of completion of one round of synthesis prior to degradation of the mRNA by nuclease.

(4) Analysis of the nuclease activity in the wheat germ system is consistent with the hypothesis that RNA degradation is the main factor affecting protein synthesis (Figures 1A and 1E). The wheat germ extract and creatine kinase both cause considerable degradation of ribosomal RNA as shown when, after the incubation under normal assay conditions, the ribosomal RNA is analysed

under denaturing conditions (Figure 1). Although this data refers to the degradation of ribosomal RNA rather than to poly(A) RNA, there is undoubtedly sufficient nuclease in the system to affect messenger RNA which is normally more susceptible to degradation by nuclease. Since creatine kinase appears to contribute a large proportion of the nuclease contamination, it would be advantageous to obtain an enzyme preparation free of nuclease activity. However, even a reduction in the amount of creatine kinase, from 5 µg to 0.5 µg per 50 µl assay, not only lowers the amount of nuclease but also increases total protein synthesis (Table 2). The nuclease contamination in the wheat germ extract was found in extracts prepared from two different sources of wheat germ (Figure 1). Two different preparations of creatine kinase also exhibited nuclease activity (Figure 1 and Table 2). It is therefore likely that nuclease contamination of wheat germ extracts and creatine kinase is a general phenomenon. The elimination or reduction of nuclease activity in the wheat germ extract poses a more difficult problem and has not been dealt with in this thesis.

4.2 FACTORS AFFECTING PROTEIN SYNTHESIS

Polyamine concentration and potassium concentration are both reputed to affect protein synthesis *in vitro*. Spermidine is the predominant polyamine in wheat germ (Hunter *et al.*, 1977a). Wheat germ extracts prepared by gel filtration, however, may contain suboptimal concentrations of polyamines causing a decrease in protein synthetic efficiency. Addition of spermidine to cell-free wheat germ assays increases protein synthesis, as shown in Figures 6 and 7. Hunter *et al.*, 1977a, have shown that the increase in overall protein synthesis in the wheat germ system, on addition of spermidine, is due to an increase in the efficiency of elongation with no apparent effect on initiation.

There is also some evidence to suggest that potassium concentration has an effect on the rate of elongation of polypeptide chains in cell-free systems. Unlike spermidine, however, potassium not only increases elongation but also reduces the amount of total protein synthesis, probably by inhibiting initiation. The most convincing data have been reported by Mathews and Osborn, 1974, showing the effect of increased potassium concentration on protein synthesis in the Krebs II ascites system. The rate of chain elongation increased with increasing potassium concentration although total protein synthesis was reduced at high levels of potassium. Increased elongation rates enhanced the synthesis of high molecular weight proteins. Elevated potassium concentrations in the wheat germ system have also been reported to enhance the synthesis of larger polypeptide chains (Schmeckpeper et al., 1974; Harwood et al., 1975; Benveniste et al., 1976; Hunter et al., 1977a). These results apply to the translation of TMV RNA, collagen and mouse myeloma mRNAs. However, the results presented in this thesis show no obvious relative enhancement of the synthesis of immunoglobulin heavy chain at concentrations of potassium greater than 74 mM. Furthermore, at potassium concentrations greater than 110 mM, protein synthesis was almost completely inhibited. Although in most cases mentioned above the inhibitory effect of potassium appears to be less striking than that shown in Figure 3, a similar effect of potassium concentration on the translation of TMV RNA has been observed by Marcu and Dudock, 1974. The different degrees of effect produced by elevated potassium concentrations, therefore, cannot be dependent only on the species of mRNA since Hunter et al., 1977a, report an enhancement in the synthesis of large TMV proteins at 125 mM potassium while Marcu and Dudock, 1974, indicate that synthesis of TMV proteins at such potassium concentrations is almost completely inhibited. Moreover, the synthesis of both IgG and IgA heavy chains at a potassium concentration of 74 mM has been clearly demonstrated in this thesis (Figures 15 and 16) while Schmeckpeper et al., 1974, reported the

dependence of IgA heavy chain synthesis on potassium concentrations above 74 mM.

One explanation of this apparent contradiction is that there are fewer fragments of protein released in the wheat germ system described in this thesis thereby decreasing the enhancement of chain completion achieved in other cell-free systems at elevated potassium concentrations. It would therefore be less beneficial to use potassium concentrations deviating from the optimum concentration required for initiation. This would not, however, apply to spermidine, since it is not known to inhibit initiation.

In the wheat germ system, an increase in efficiency of elongation or initiation will not only increase the total amount of protein synthesis but will also lessen the effect of mRNA degradation by nuclease. An increase in elongation rate increases the probability of one round of protein synthesis being completed prior to degradation of the mRNA by nuclease. This greatly enhances the synthesis of heavy chain and other high molecular weight proteins since an increase in elongation rate decreases the time that the part of the mRNA between the ribosome and the 3' end of the mRNA is exposed to nuclease, prior to completion of the first round of protein synthesis. This also applies to the transit of subsequent ribosomes i.e. the faster the ribosome travels along the mRNA, the more times a message will be read during its life span. Therefore, in the presence of a factor enhancing the efficiency of elongation, a larger percentage of the mRNA molecules will be translated by the first and subsequent ribosomes.

An increase in initiation rate increases the loading of ribosomes on the mRNA, which increases the protection of the mRNA against degradation by nuclease. This protection will allow the mRNA to have a longer life span and thus participate in a greater number of rounds of protein synthesis.

Addition of poly(A) as an inhibitor of initiation may increase the synthesis of certain proteins whose mRNA molecules are susceptible to nuclease degradation since the poly(A) may compete for binding sites on the nuclease molecules. Similarly, addition of high levels of potassium as a means of studying its effect on initiation of certain proteins may also have an effect on elongation, resulting in an increase in the number of complete proteins synthesized. The effect of nuclease degradation may thus complicate the interpretation of experiments designed to study translational control (Sonenshein and Brawerman, 1976). Nuclease activity also renders the wheat germ system less useful for the determination of purity of mRNA preparations since the system does not accurately reflect the distribution of a mRNA population.

4.3 ENDOGENOUS AMINO ACID CONTENT OF THE WHEAT GERM SYSTEM

In order to accurately calculate the incorporation of amino acids into protein by the wheat germ system, it is essential to determine the endogenous amino acid pool in the wheat germ extract. From the results illustrated in Table 3, it is apparent that the wheat germ system contains sufficient endogenous amino acids to support protein synthesis although it has been generally assumed until now that the addition of exogenous amino acids is necessary (Roberts and Paterson, 1973). Slight differences in the methods of preparation of the wheat germ extract, however, may cause variations in endogenous amino acid concentration. The endogenous amino acid pool can be slightly lowered by dialysis to give an increase in the amount of labelled amino acid incorporated (Table 4 and Ms. A. Alexander, personal communication).

The size of the endogenous amino acid pool is particularly important for the synthesis of labelled proteins for sequence analysis. In this respect, the wheat germ system is preferable to other cell-free systems which are

not prepared by gel filtration, such as the reticulocyte lysate cell-free system, since the wheat germ system has a lower endogenous amino acid content.

4.4. DETECTION OF A PRECURSOR TO MOUSE IgG HEAVY CHAIN

Precursor amino acid sequences have been identified at the N-terminus of several secreted proteins (Section 1.4) including immunoglobulin light chain (Burstein and Schechter, 1976). The evidence for the existence of a precursor to immunoglobulin heavy chain is less conclusive, however. Comparisons of the trypsin or cyanogen bromide digestion products of heavy chains synthesized *in vitro* and *in vivo* have provided contradictory results (Cowan and Milstein, 1973; Bedard and Huang, 1977) and although it has been reported that preliminary sequence analysis of heavy chain synthesized *in vitro* shows the presence of a precursor segment no data has yet been published (Schechter *et al.*, 1977). To determine whether immunoglobulin heavy chain is synthesized as a precursor molecule, it is necessary to study the primary translation products of myeloma mRNA in a cell-free system since, in the cell, the precursor peptide is expected to be cleaved rapidly, by analogy with immunoglobulin synthesis in the murine myeloma, MOPC 41 (Blobel and Dobberstein, 1975). If the heavy chain synthesized *in vitro* contains a precursor segment, the sequence of amino acids at the N-terminus should be both hydrophobic and hypervariable as has been found for several light chain precursors (Schechter and Burstein, 1976; Burstein and Schechter, 1976).

Three methods of analysis of 5563 heavy chain synthesized *in vitro* have been employed. An initial comparison of the mobilities in SDS-polyacrylamide gels of the cellular and cell-free products provided

some preliminary information on the relative sizes of the two heavy chains (Figure 15). However, the presence of carbohydrate moieties on the cellular heavy chain hinders such an analysis. Moreover, SDS-polyacrylamide gels, prepared by the method of Laemmli, 1970, separate proteins on the basis of charge as well as size and thus limit the validity of such a comparison. Both the 5563 heavy chains synthesized *in vivo* and *in vitro* have the same mobility although the former heavy chain is synthesized as a glycoprotein (Fougereau *et al*, 1976). This tentatively suggests the presence of extra amino acids or some other slight modification of the heavy chain synthesized *in vitro* to enable it to co-migrate with the cellular heavy chain whose mobility is hindered by the presence of carbohydrate.

A more meaningful comparison of the heavy chains synthesized *in vitro* and *in vivo* can be achieved by a comparison of the fragments generated by chemical or enzymic digestion. Digestion of 5563 heavy chain, as described in this thesis, was carried out using the enzyme, Staphylococcus aureus V8 protease, which specifically cleaves proteins at glutamic acid residues. This methodology (Cleveland *et al.*, 1977), has the technical advantages of ability to carry out the digestion in a polyacrylamide gel in the presence of SDS and therefore eliminates the need for elution of the protein from the first gel phase. However, since the digestion achieved by this method is not carried to completion, the pattern of peptide fragments generated is more difficult to interpret than fragments generated by cyanogen bromide cleavage where the peptides produced can be predicted from a known protein sequence.

The digestion patterns of P1.17 cellular heavy chain and 5563 heavy chains synthesized *in vivo* and *in vitro* are shown in Figure 19. Interpretation of the data is further complicated by inadequate resolution of the peptide

fragments. This method of peptide mapping may be improved by analysing the digestion products on higher resolution gels e.g. gradient gels, or by applying a two dimensional gel system.

Sequence analysis is an invaluable technique for the detection of precursors to secreted proteins. Several immunoglobulin light chains synthesized *in vitro* have been identified as precursor molecules by this method of analysis (Burstein and Schechter, 1976). The preparation of 5563 heavy and light chains has been described in Section 3.3.2 and the results of the sequence analysis of heavy chain, carried out by Dr. T.Kindt and Mr. D. Gates, are shown in Figure 21. The amino acid sequence of 5563 heavy chain synthesized *in vitro* is compared in Table 6 to the known sequences of the different subgroups of mouse heavy chains. Director comparison cannot be made with 5563 cellular heavy chain for which the sequenc is unknown owing to the presence of pyroglutamic acid at the N-terminus (Awdeh *et al.*, 1970). No alignment of the sequence of 5563 heavy chain is possible with the known sequences of the other heavy chains suggesting that (1) 5563 heavy chain is synthesized *in vitro* as a precursor molecule and/or (2) 5563 heavy chain belongs to an immunoglobulin subgroup not yet identified. This latter possibility seems unlikely since any heavy chain subgroup would be expected to contain the invariant amino acid residues represented in all known subgroups, as outlined in Table 6. (The number of heavy chain sequences analysed in each subgroup is indicated in brackets Moreover, the hydrophobic nature of this preliminary sequence is indicative of a precursor by analogy with other known precursor sequences (Devillers-Thiery *et al.*, 1975; Schechter and Burstein, 1976).

The detection of two amino acid residues at position 1, methionine and phenylalanine, suggests that the sequence shown in Figure 21 may represent the amino acid sequence analysis of more than one protein. There is, however, an alternative explanation. Unblocked methionine is the initiato:

TABLE 6: Variable Regions of Mouse Immunoglobulin Heavy Chains

(Kabat, Wu and Bilogsky (1976) in 'Tabulations and Analyses of Amino Acid Sequences)

	Invariant Residues of Different Subgroups					Sequence of
	I(3)	II(3)	III(26)	IV(2)	V(11)	5563 H-Chain
0						
1				Glu	Glu	Phe/Met
2	Val	Val	Val	Val	Val	
3	Gln	Gln		Gln	Gln	
4	Leu	Leu		Leu	Leu	
5	Gln			Gln	Gln	
6	Glu	Glu				
7	Ser	Ser	Ser	Ser	Ser	
8	Gly	Gly	Gly	Gly	Gly	Leu
9	Pro		Gly	Thr	Ala	Phe
10			Gly	Val	Glu	Leu
11	Leu	Leu	Leu	Leu	Leu	Val
12	Val	Val	Val	Ala	Val	Val
13	Lys		Gln	Arg	Lys	
14	Pro	Pro		Pro	Ala	
15	Ser	Gly	Gly	Gly	Gly	
16	Gln		Gly	Ser	Ser	
17		Ser		Ser	Ser	
18	Leu		Leu	Leu	Val	Val
19	Ser	Lys		Lys	Lys	
20	Leu		Leu	Met	Met	
21						
22						
23						
24						Leu/Pro
25						Ser
26						Pro

residue for protein synthesis in both plant and animal cells (reviewed by Schechter and Burstein, 1976a). Most proteins, synthesized as precursors in the wheat germ cell-free translation system have been shown, by sequence analysis, to retain this methionine residue (Kemper et al., 1976; Strauss et al., 1977). However, the product of satellite tobacco necrosis virus lacks methionine at the N-terminus, when synthesized in a wheat embryo cell-free system (Lundquist et al., 1972). It has therefore been suggested that the removal of the initiator methionine residue depends on the structure of the 5' end of each protein molecule. Although methionine has been found at the N-terminus of most immunoglobulin light chains which have been synthesized in the wheat germ system, sequence analysis of MOPC 104E light chain precursor indicated that only 10% of the initiator methionine residues were retained (Burstein et al., 1976). Sequence analysis of MOPC 41 light chain precursor, synthesized in the wheat germ system, indicated the presence of two precursor moieties, one species two N-terminal residues shorter than the other (Burstein and Schechter, 1976). These results show that the peptidase responsible for the removal of the N-terminal methionine is present in the wheat germ cell-free system and may account for the presence of both methionine and phenylalanine at the N-terminus of 5563 heavy chain precursor. If only a small percentage of the synthesized protein retains the methionine residue, the specific activities of all the other amino acids besides methionine will be too low to enable the detection of more than one protein sequence.

Sequence analysis of 5563 light chain synthesized in the wheat germ system failed to generate a single amino acid sequence owing to the impurity of the preparation. Future preparations of both 5563 heavy and light chains will be isolated from the wheat germ system by immunoprecipitation, as shown in Figure 16, to ensure that the sequence analyses represent a single protein.

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