POST-TRANSCRIPTIONAL CONTROL OF IMMUNOGLOBULIN SYNTHESIS

by

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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

ATA	Aurintricarboxylic acid
BSS	Balanced salt solution
DATD	Diallyltartardiamide
Dansyl chloride	1-dimethylaminonapthalene-5-sulphonyl chloride
DNS	Dansyl (when referring to amino acids)
DMSO	Dimethyl sulphoxide
DTT	Dithiothreitol
EGTA	Ethyleneglycol-bis-(β aminoethyl ether) NN' tetraacetic acid
FCS	Foetal calf serum
MDL	Messenger-dependent lysate
NaDOC	Sodium deoxycholate
NP-40	Nonidet P-40 (Non-ionic detergent)
NRS	Normal rabbit serum
PAGE	Polyacrylamide gel electrophoresis
PAG	Polyacrylamide gel
PBS	Phosphate buffered saline
PBSA	Phosphate buffered saline (no Ca^{++} or Mg^{++})
PCI	Phenol/Chloroform/Isoamyl alcohol
PEB	Phenol extraction buffer
poly(A) RNA	RNA species containing poly(A) sequences at their 3' termini - functionally equivalent to mRNA in this study
PPO	2,5 diphenyloxazole

a) General (Contd):-

SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid
TEMED	NN N'N' tetramethylethylenediamine
TMV	Tobacco mosaic virus
TPCK	L-1-Tosylamide-2-phenylethyl -chloromethyl ketone

b) Immunological

Ig	Immunoglobulin
H	Ig heavy chain
L	Ig light chain
γ_{2a}, γ_{2b}	classes of Ig H chains
α	
μ	
κ	classes of Ig L chains
λ_1, λ_2	
pre L	precursor to Ig L chain

α- - anti-

CONTENTS

SUMMARY

Studies on the translation of mRNA of several secreted proteins and polypeptides in heterologous cell-free protein synthesizing systems have indicated that such secreted proteins are synthesized *in vitro* as precursor molecules. These precursors contain additional amino acid residues at their N-termini which appear to be rapidly removed *in vivo*. In cell-free systems, mRNA from Ig-secreting myeloma cells, which generally produce and secrete Ig H and L chains, has been shown to produce a higher MW precursor to L chain. Much less evidence exists for the presence of a precursor to H chains *in vitro*. Production of precursors to H and L chains of Ig was investigated in this project, using a recently developed cell-free system.

The presence of two major polypeptide products of myeloma mRNA also allowed the relative effect of various inhibitors of protein synthesis on different mRNA's to be investigated.

Cell-free translation of both polysomes and poly(A) RNA from several myeloma cell lines was investigated in this project.

Ig H and L chains were identified as products of cell-free translation of Pl.17 polysomes in rabbit reticulocyte lysate by specific immunoprecipitation.

More rigorous identification, involving both direct and indirect immunoprecipitation and comparison of producing and non-producing cell lines was carried out in a messenger-dependent lysate system.

From/

From these studies the following conclusions were drawn:-

1. In all cell lines investigated, Ig L chain was synthesized as a higher MW precursor, when produced in the cell-free system. In one cell line (5563) further characterisation by tryptic mapping showed the cell-free product to be related to the cellular product. This precursor also did not appear to have methionine as its N-terminal amino acid.

2. In all cell lines investigated, H chains, whether of type α, γ_{2a} or γ_{2b}, appeared to be synthesized in cell-free systems with a lower apparent MW than the appropriate cellularly synthesized H chain. Inhibition of carbohydrate addition intracellularly mimicked this effect in one cell line (5563).

The work in this project left unresolved the question of the existence of a precursor to H chains.

Studies on the effect of inhibitors of initiation of protein synthesis on cell-free protein synthesis were carried out with Pl.17 mRNA. It was concluded that:-

1. Ig H and L chain mRNA's were much more resistant to inhibition of initiation than other myeloma mRNA's.

2. Ig H chain mRNA was more resistant to inhibition of initiation than Ig L chain mRNA.

Finally, an improved cell-free protein synthesizing system for the translation of polysomes was developed from the existing M.D.L. system by removal of endogenous amino acids.

1. Murine myelomas.

1.1. Introduction.

Much of the work discussed in this section has appeared in Potter's reviews (1972, 1975).

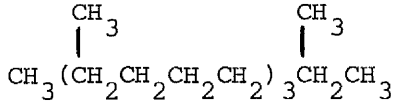
Myelomas of plasma cells, (plasmacytomas), producing myeloma proteins, are derived from mature differentiated lymphocytes which normally synthesize and secrete immunoglobulin (Ig) molecules. Plasmacytomas generally synthesize, and may secrete, one species of Ig molecule (although myelomas derived from lymphocytes at earlier stages of lymphocyte differentiation may synthesize and secrete more than one Ig species e.g. SAMM 368, Results, Section 9.) The Ig produced is generally assumed to be a functional antibody but since myelomas apparently arise from previously stimulated lymphocytes, the specificity of the antibody is generally unknown. (In some cases, e.g. MOPC 315, screening studies against a wide range of antigenic substances have revealed antibody-like affinities for some antigens).

The use of myeloma cell-lines producing and secreting a single Ig species has elucidated many features of Ig structure and function, most notably on the assembly of Ig molecules, and the amino acid sequence of Ig molecules. More recently, in view of their high levels of mRNA coding for Ig H and L chains, they have proved popular in studies aimed at producing purified mRNA's and studying their properties. (Mach, Faust and Vassali, 1972; Swan, Aviv and Leder, 1972). They may also prove useful as model systems for the *in vivo* response to antigen stimulation, i.e. the formation of antibody-producing clones, although caution must be used in extrapolating the features/

features of a pathological condition to that of the normal in vivo situation. Finally, the use of cDNA probes derived from purified Ig mRNA may offer insights into the genetic mechanisms involved in the origin of antibody diversity (e.g. numbers of Ig genes, point of linking of V and C genes in the genome, etc.). These possibilities have been comprehensively reviewed in recent years (Rabbitts and Milstein, 1977).

1.2. Origins of plasmacytomas.

Plasmacytomas may occur either spontaneously or by chemical induction. Chemical induction, whose effect is confined mainly to inbred strains of mice such as BALB/c or NZB, involves intraperitoneal injection or implantation of some foreign substance, most commonly mineral oils or plastic discs. Mineral oil injection at regular intervals produces plasmacytoma tumours within 6 months of the initial injection in over 60% of BALB/c mice (Potter, 1972). The inducing factors in the oil are believed to be long chain and branched aliphatic hydrocarbons, such as pristane.



Pristane:2,6,10,14 tetramethylpentadecane

The high incidence of such mineral oil induced plasmacytomas in BALB/c and NZB mice (and the low incidence in other strains of mice) may be due to several reasons:-

1) Genetic: BALB/c and NZB mice are much more susceptible to the change/

change in control within plasma cells resulting in their conversion to rapidly proliferating species. This suggests a genetic susceptibility in these particular inbred strains. F_1 crosses of BALB/c mice, with other mice which do not produce plasmacytomas in response to normal induction methods (pristane, etc), yield hybrid mice which are inducible (Potter, 1967).

2. Hormonal factors such as testosterone may also influence plasma cell tumour development either positively or negatively.

3. Virus involvement: Plasmacytoma cells from both spontaneous and chemically induced tumours have been shown to contain large numbers of virus-like particles. Although no direct link between such particles and tumour formation has been made, it remains possible that the pathological transformation of plasma cells to plasmacytoma cells may be virus-linked. Moreover, it has been demonstrated (Potter et al., 1973) that Abelson virus (MLV-A) can dramatically increase the occurrence of lymphosarcomas and plasmacytomas in response to pristane injection. The tumours also develop in a much shorter time.

1.3. Maintenance of plasmacytomas.

Plasma tumours are normally passaged in mice by insertion of small pieces of tumour tissue into previously tumour-free mice. Subcutaneous insertion results in the formation of solid tumours. Intraperitoneal insertion yields ascites fluid rich in plasmacytoma cells. The tumour is normally maintained as a solid tumour; ascites fluid is used for preparation of large amounts of secreted Ig. The solid tumour generally maintains its synthetic capacity over many passages.

Plasma/

Plasma tumour cells may also be adapted to in vitro culture, growing in specially prepared medium, usually supplemented with serum. Once established, the cell-lines are generally grown in suspension. Under these conditions, the cell-line is more prone to lose some or all of its Ig synthetic activity, always losing synthesis of Ig heavy chain first, than when maintained in solid tumour form.

1.4. Differences between plasma cells and plasmacytoma cells.

Differences exist between the normal and tumour cells which must be borne in mind when extrapolating data from tumour cells to the in vivo situation.

1) Mature plasma cells are non-proliferative whilst tumour cells have generation times of as little as 12 hours.

2) Plasma cells are diploid; tumour cells are oligoploid, varying between hyperdiploid and hypertetraploid, with a tendency to tetraploidy.

3) Tumour cells are well-characterised as regards production levels, balance (or imbalance) of H and L chain synthesis; plasma cells are not extensively characterised in this manner. It may not be valid to assume that what holds for tumour cells will hold for plasma cells.

In this study, two tissue culture cell lines were extensively utilised. One, (X5563) arose spontaneously in a C3H mouse. It has been maintained as a tumour and as a cell line continuously for almost twenty years (see Materials and Methods for details) without change of immunoglobulin synthesized. The other, AdjPC5 (P1.17) was mineral oil-induced in a BALB/c mouse. This line has recently

been/

been re-cloned (T.R. Mossman, unpublished work) and clones producing either high levels of Ig or L chains only were isolated and adapted to large-scale culture (Pl.17.1 and Pl.17.4) (see Materials and Methods).

2. Messenger RNA (mRNA).

2.1. The messenger RNA hypothesis.

The formulation of the messenger RNA hypothesis (Jacob and Monod, 1961) was swiftly followed by demonstrations of the existence of messenger RNA (mRNA) species in prokaryotic organisms (Nathans et al., 1962 and others). Debate as to the nature and even existence of mRNA in eukaryotic organisms, however, continued throughout the 1960's (see Singer and Leder, 1966). Most of the controversy arose from the lack of clearly defined criteria for the isolation and identification of mRNA species. This, coupled with their occurrence in relatively low amounts in cells, compared to other RNA species, made their isolation, even in impure form, difficult. (In HeLa cells the relative proportions of mRNA, rRNA, tRNA and nuclear RNA have been measured as 3:79:12:5 approximately by Darnell, 1968).

2.2. Early criteria for identification of mRNA.

Most early attempts to distinguish mRNA from other RNA species were hampered by the fact that the criteria used to identify mRNA were not exclusive properties of mRNA. Base composition studies (Darnell, 1968), using the supposed "DNA-like" base composition of

mRNA, and hybridisation data studies were both prone to difficulties in interpretation due to the problems in removing contaminating RNA species.

Use of the inhibitor of rRNA synthesis, Actinomycin D, in pulse labelling experiments proved more fruitful. Using low levels (0.04µg/ml) to block synthesis of rRNA, Penman et al., 1968 demonstrated the appearance in the cytoplasm of HeLa cells of low levels of radioactively labelled RNA species. These species were found to associate with polysomes.

Association of RNA species with polysomes was another criterion used for identifying mRNA species. Electron microscopic investigations (most convincingly, those of Rich et al., 1963 and of Mathias et al., 1963) had revealed the existence of thin threads of material joining linear arrays of ribosomes from both eukaryotic and prokaryotic sources. The dimensions of these threads were consistent with those of RNA. Studies by Gierer (1963) had implicated mRNA in the formation of ribosome aggregates which could incorporate amino acids.

Disruption of polysomes by the addition of metal-ion chelating agents, e.g. EDTA (Chantrenne et al., 1967; Darnell, 1968; Labrie, 1969; Penman et al., 1968; Lebleu et al., 1971) or by run-off due to addition of puromycin (Darnell, 1968; Penman et al., 1968; Blobel, 1972) yielded RNA species which could be purified of ribosomal contamination. Using polysomes isolated from cells producing no predominant protein species, the range of size of released RNA was wide. In HeLa cells it was found to be 8S - 30S (Penman et al., 1968). Polysomes from reticulocytes, which produced mainly globin, however, were found to release a fairly discretely sedimenting 9S species (Chantrenne et al., 1967/

1967 and others).

2.3. Recent developments in mRNA isolation.

As has been noted in two recent reviews (Mathews, 1974; Brawerman, 1974)[a,b] two major breakthroughs allowed the isolation and evaluation of pure mRNA on a relatively straightforward basis.[a,b]

2.3.1. Eukaryotic cell-free protein synthesizing systems.

The first of these advances was the demonstration of the translation of 9S RNA, isolated from mouse reticulocytes by EDTA release, in a heterologous eukaryotic cell-free protein synthesizing system derived from rabbit reticulocytes (Lockard and Lingrel, 1969; Lingrel et al., 1971). Previously, only synthetic polynucleotides e.g. poly(U) had been shown to be translated in eukaryotic cell-free systems (Arnstein, Cox and Hunt, 1962; Arlinghaus and Schweet, 1962; Williamson et al., 1967). Earlier work had attempted to demonstrate mRNA activity in cell extracts by translation in cell-free systems (Arnstein et al., 1964; Knip et al., 1964). Since the RNA species and cell-free system used had both been derived from rabbit reticulocytes, however, stimulation could not be unambiguously attributed to the translation of exogenous mRNA.

Prokaryotic cell-free systems, such as that described by Nirenberg et al., 1963, had been stimulated by the addition of eukaryotic mRNA-like species but had not yielded identifiable polypeptide products (Hardesty et al., 1963; Schaeffer et al., 1964).

Such/

Such lack of success may have been a combination of imperfect mRNA isolation techniques and of incompatibility between eukaryotic mRNA and prokaryotic protein synthetic machinery. Several eukaryotic cell-free systems have been subsequently developed (see Introduction, Section 3). The translation of "mRNA-like" species into identifiable polypeptides provided the one unambiguous criterion for the identification of mRNA.

2.3.2. Poly(A) as 3' terminus of mRNA.

Secondly, following the initial reports of Kates, 1970, and Lim and Canellakis, 1970, it was established by several groups that most eukaryotic mRNA's (as well as several viral RNA's) can be identified by the presence of regions of nucleotide sequence consisting predominantly, although not necessarily exclusively (Burr and Lingrel, 1971) of adenine nucleotides (Mendecki et al., 1972; Adesnik et al., 1972; Molloy et al., 1972, also Kates, 1974 for review). Later work established that the adenine-rich or poly(A) sequence was to be found at the 3' end of the mRNA molecule (Mendecki et al., 1972) and that its length was between 150 and 200 nucleotides (Mendecki et al., 1972; Nakazato et al., 1973). This sequence is generally thought to be added post-transcriptionally (Darnell et al., 1971).

The possible roles of the poly(A) sequence in control of mRNA translation will be discussed in Section 4.3.3.2 of the Introduction.

2.3.3. Purification of mRNA using the poly(A) sequence.

The poly(A) sequence on mRNA has served as a basis for purification of mRNA both in phenol extraction techniques (Brawerman et al., 1972) and in methods utilising its affinity to immobilised polynucleotides such as Poly(U) (Adesnik et al., 1972) or oligo(dT) (Swan et al., 1972). These extraction methods have been recently reviewed by Brawerman, 1974 and Mathews, 1974. By these methods cellular mRNA can be recovered in a purified form, essentially free from rRNA or tRNA contamination.

2.4 Purification of individual mRNA species.

2.4.1. Purification prior to poly(A)-based enrichment.

Several properties of polysomes have been used to enrich preparations for particular mRNA's. Preliminary purification of mRNA has involved fractionation of cellular polysomes on the basis of size (Heywood and Nwagwa, 1969). Polysome size is roughly proportional to the size of protein coded for by the mRNA. This rule does not, however, hold true in all cases (Hunt et al., 1968). This technique is useful when attempting to isolate mRNA's which are very large or very small and whose polysomes therefore lie outside the normal size range of polysomes.

Another widely exploited technique has been the isolation of specific mRNA's by antibody precipitation of their polysomes. This makes use of the antigenicity of the nascent polypeptides distributed along the polysomes. It has been studied most thoroughly by Schimke and co-workers (Rhoads et al., 1971; Palmiter et al., 1972; Palacios et al., 1973). In spite of many problems related to non-specific/

specific interaction, trapping of non-specific polysomes and nuclease activity in antibody preparations, it has been used to isolate a number of mRNA species, such as ovalbumin (Rhoads et al., 1971 et seq; Schutz et al., 1977), catalase and serum albumin (Uenoyama and Ono, 1972), Ig L chain (Delovitch et al., 1972; Schechter, 1974), vitellogenin (Wetekam et al.,1975), or histone H5 (Scott et al., 1975). Polysomes detected generally show a wide range of size (Berridge et al., 1976) but poly(A) RNA isolated from them shows a discrete size on PAGE (Palacios et al., 1973; Delovitch et al., 1972). This may reflect heterogeneity in polysome size for individual mRNA's.

2.4.2. Purification subsequent to poly(A)-based enrichment.

Poly(A) RNA can be purified of remaining contaminating species (rRNA, tRNA) and individual mRNA species isolated by standard sucrose gradient centrifugation, and PAGE techniques. Globin mRNA, for example, has been partially separated into α and β globin mRNA's by electrophoresis in fully denaturing conditions using formamide-polyacrylamide gels (Morrison et al., 1974). Such separation is not, however, necessarily complete due to poly(A) size heterogeneity and complete separation of α and β globin mRNA's required removal of the poly(A) sequence (Maniatis et al., 1976). Such procedures may prove necessary for all mRNA's.

3. Eukaryotic cell-free protein synthesizing systems.

3.1. Introduction.

The use of cell-free protein synthesizing systems derived from eukaryotic/

eukaryotic cells has increased rapidly in the last ten years. Such systems provide a means of characterising non-homologous mRNA preparations and allow the polypeptide products coded for by such mRNA's to be assayed both qualitatively and quantitatively. This can be done under controlled conditions in the absence of some of the possible constraints which operate on their translation *in vivo*. They also allow the investigation of the effect of controlled changes in various physical parameters (salt concentration, pH, temperature, inhibitors) whose effect is difficult to interpret *in vivo*. *In vivo*, factors such as membrane permeability, continued transcription of RNA in the nucleus and the operation of regulatory mechanisms within the cell combine to complicate interpretation of simple changes in the cellular environment.

Effective cell-free systems should meet (in part or in whole) the following criteria:-

1. Non-limiting levels of substrates and intact protein synthetic apparatus.
2. Low levels of endogenous activity.
3. Repeated faithful translation of added mRNA.
4. Reproducibility of preparation.

Several cell-free systems have been developed. The most widely used of these are discussed below.

3.2. Reticulocyte lysate.

The reticulocyte lysate system originating from work on whole reticulocytes by Borsook's group (Borsook *et al.*, 1957; Lingrel et al., 1963), was first described by Lamfrom and Knopf (1964). It was not, however, until the work of Adamson *et al.*, (1968) with the introduction of haemin at an early stage in the preparation, that sustained/

sustained protein synthesis was achieved with the system. Using this system, Lockard and Lingrel (1969) first demonstrated unambiguously the translation of eukaryotic mRNA in a heterologous cell-free system. The system, consisting of cytoplasm from lysed reticulocytes (usually collected from rabbits or mice suffering from chemically induced anaemia) is generally supplemented with amino acids, salts and an energy source (e.g. creatine phosphate/creatine kinase). The addition of low levels of haemin to the system allows relatively long incubations without inactivation of protein synthesis (see Introduction, Section 4.2.4.).

The system has been shown to be very efficient in translating added mRNA. Palmiter, for example, calculated that each ovalbumin mRNA was translated between 18 and 47 times during incubation in a rabbit reticulocyte lysate (Palmiter, 1973). This indicated a substantial amount of re-initiation of translation by the endogenous ribosomes.

The major disadvantage of the reticulocyte lysate was, until recently, the presence of large quantities of endogenous mRNA (in particular, globin mRNA). Added mRNA had to compete for substrates, ribosomes, etc., against this mRNA, under conditions which would probably favour the endogenous mRNA. The recent modification of the system described by Pelham and Jackson (1976) has removed this disadvantage. Another drawback in radioactive labelling experiments is the existence of large pools of endogenous unlabelled amino acids. These dilute the specific activity of the added radioactive amino acid and hence reduce the amount of radioactivity incorporated. Whilst this is, to some extent, compensated for by the high efficiency

of/

of translation, it nevertheless poses problems when trying to work with gel systems, where the capacity of the gel is limited by protein weight. Attempts to overcome this problem were made in the course of the present work.

3.3. Wheat germ system.

Two methods of preparing a cell-free system from wheat germ have been developed by Shih and Kaesberg, 1973,(using wheat germ embryos),and by Roberts and Paterson, 1972,(using whole wheat germ), respectively. The method of Shih and Kaesberg involves isolating whole embryos of wheat germ by flotation in organic solvents to remove chaff. As such its reproducibility is variable and its preparation rather tedious. The more widely used system (Roberts and Paterson, 1972) was developed from methods first applied to peanut cotyledons, (Marcus and Feeley, 1964) and subsequently adapted for a number of other cell sources, such as mouse ascites tumour cells (McDowell et al., 1972). The preparation involves the rupture of wheat germ cells in a buffered salt solution by grinding with sand. Centrifugation removes large debris and the cytoplasmic extract is purified further by passing over a G-25 Sephadex column.

The column eluant fraction is normally supplemented with added amino acids, salts, and energy (creatine phosphate / kinase; ATP; GTP). A pre-incubation step is often included after the initial centrifugation step to lower background incorporation.

For many small size mRNA's, the wheat germ system is the system of choice for translation. Low background levels of incorporation are/

are combined with high levels of incorporation of radioactivity due to the absence of large endogenous amino acid pools. Much of the work which revealed the existence of precursor molecules to polypeptide hormones was carried out using the wheat germ system (see Introduction, Section 5).

Unfortunately, the system has a tendency to cause premature termination of translation, especially with non-viral eukaryotic mRNA and particurly with longer mRNA's. Even short mRNA's are often only partially translated yielding a spectrum of complete and partially complete polypeptides (see, for example, Burstein et al., 1976). This drawback has tended to lessen the effectiveness of the system. Moreover, the activity of the system varies with wheat germ from different sources (Marcu and Dudock, 1974). Consequently, the system, whilst very effective at optimum can be unreliable and will not necessarily reflect the translational capacity of an unfractionated mRNA mixture.

3.4. Ascites cell-free systems.

Cell-free systems have been prepared from Krebs II (Mathews and Korner, 1970) and Ehrlich (Green et al., 1976), ascites tumour cells by a method closely related to that used in preparing the wheat germ system. The system often requires supplementation by heterologous tRNA (Aviv et al., 1971: Metafora et al., 1972). It similarly offers lower background incorporation and high levels of incorporation of radioactivity. Its rate of translation, however, is much lower than that found in the reticulocyte lysate system/

system (Osborn and Mathews, 1973). Moreover, it also suffers from premature termination problems (Swan, Aviv and Leder, 1972; Schechter et al., 1974) leading to synthesis of a range of polypeptides from full-size down. These have been shown, in the case of Ig L chain, to contain identical N-terminal sequences and hence presumably have varying lengths of polypeptide depending on the point of termination. Moreover, as has been conclusively demonstrated by Boime et al., 1977, and implied by Swan et al., 1972 and Milstein et al., 1972, Krebs ascites cell-free systems are often contaminated by variable levels of membrane fragments. These tend to cause processing of the primary translation products of added mRNA, removing precursor peptides. Consequently, results in the ascites cell-free system must be interpreted with caution.

3.5. Other cell-free systems.

The most widely used alternative method of producing a cell-free system involves the purification of the various components of protein synthesis (tRNA, ribosomes, cytosol "factors", etc.), often from different cell-types. These components are then mixed to re-constitute an active cell-free system. Such systems have been devised by Crystal et al.,(1972),Shafritz et al.,(1972)using reticulocyte components and by Blobel and Dobberstein (1974)using both rabbit reticulocyte and dog pancreas components. While such systems may reveal interesting phenomena related to protein synthesis, they may suffer to some extent from incompatibility between components and from omission of necessary factors.

4. Regulation of protein production.

4.1. Introduction.

In many biological systems, a balance must be struck. This is true both in a whole animal scale, where food consumption is correlated to energy consumption (except in exceptional circumstances such as starvation) and also at the level of the cell. With cells, the problem of balanced consumption and production are more acute since reserves of food and energy are not always available in the cell as they are in the whole animal. Even in the situation where energy and food supplies are not limiting, there exists a need for balance in the cell to maintain orderly growth. If the cell is growing and dividing, DNA must be replicated at fixed points in the cell cycle, RNA must be transcribed (sometimes also at fixed periods), and protein must be synthesized, both continuously and also at fixed periods. Histones are synthesized predominantly in the S-phase of the cell-cycle (Robbins and Borun, 1967) in close synchrony with DNA replication, while immunoglobulin synthesis is confined to the late G and early S phase of the cell cycle (Finegold et al., 1968) although not in myeloma cells (Cowan and Milstein, 1972). To maintain this "regularity" of growth, a system of checks and balances operates in the cell. DNA synthesis is switched off and on by mechanisms as yet undiscovered, whilst RNA synthesis is dependent on interactions in the cell nucleus between the DNA and its histone and nonhistone protein regulators which bind and are released from the genome in response to chemical signals. Most obvious are those effected by various hormones and their receptors.

Protein/

Protein synthesis is similarly subject to control. Much work on control of protein synthesis has been carried out in the past ten years. This has been mainly due to:-

a) The advent of eukaryotic cell-free protein synthesis systems for use as models.

b) Fractionation of some systems (e.g. reticulocyte) into components and their subsequent reconstitution.

c) Isolation of purified mRNA species from many eukaryotic organisms.

Several points of control of protein synthesis can be envisaged beyond the stage of transcription. These will be considered both as a means of exerting general control and also as a means of closely controlling synthesis of one or more specific proteins.

4.2. General control by substrate availability.

4.2.1. Availability of protein synthetic machinery, by control of rRNA and/or tRNA production could be used to control protein synthesis. Such control could be specific if, for example ribosomes or met $tRNA_f$ species were limiting in the system. In this case, higher initiation rate constant or more plentiful (assuming initiation rates equal) mRNA's would be more likely to be translated (Lodish, 1974 and Section 4.4.1.1.). Similarly, tRNA production, if limiting or unbalanced, could affect the rate of translation of mRNAs. Sharma, Beezley and Roberts, 1976, have demonstrated such an effect using reticulocyte tRNA's in a tRNA-free ascites cell-free system. In this system, globin mRNA is translatable in a normal manner. Non-reticulocyte mRNA species/

species from hen oviduct and encephalomyocarditis (EMC) virus, however, showed much poorer stimulation of, and premature termination of, protein synthesis. Such a system could operate during development.

4.2.2. Low MW Substrates.

Small substrates for protein synthesis, such as ATP, GTP, amino acids, etc., could be limiting for protein synthesis. Live and Kaminskas (1975) have demonstrated that the effect of depletion of energy charge in ascites cells by starvation is to decrease protein synthesis. In myeloma cells, Sonenshein and Brawerman (1976a) have found that whilst protein synthesis decreases during starvation, synthesis of Ig H and L chains is markedly less affected than overall protein synthesis. Rupniak and Quincey, 1975, have shown the importance to protein synthesis of maintaining energy charge in reticulocyte lysate assays. These results show the dependence of protein synthesis on adequate supplies of energy.

It has also been discovered in reticulocyte lysate assays that use of O-methyl threonine, an isoleucine antagonist, (effectively mimicking isoleucine depletion), can lead to preferential inhibition of α over β chain translation due to the differing distribution of isoleucine residues in the two chains. (Temple and Housman, 1972).

4.2.3. Specific protein factors.

Specific protein factors for translation of specific mRNA's have been suggested (Heywood & Nwagwu 1969; Rourke and Heywood, 1972). The/

The widespread translation of mRNA's in heterologous cell-free systems, however, seems to argue against such specific factors except perhaps in isolated incidences (see Introduction Section 4.4. for further discussion).

4.2.4. Non-specific protein factors.

Activation and inactivation of various components of protein synthesis apparatus could be envisaged as a means of controlling protein synthesis. Many workers have demonstrated protein synthesis inhibition due to lack of haemin both in whole reticulocytes (Waxman and Rabinowitz, 1965) and reticulocyte lysates (Gross & Rabinowitz, 1972; Legon et al., 1972). This inhibition is usually reversed upon addition of haemin and is thought to be due to the formation of an inhibitor of translation in reticulocyte lysates, whose formation can be prevented by the presence of haemin. Recent work has tentatively identified this inhibitor as a cyclic AMP independent protein kinase which prevents formation of the 40S - met-$tRNA_f$ complex by phosphorylation of the initiation factor involved (Ranu and London, 1976).

Such a control process would be selective if one were to postulate specific protein factors involved in translation of specific messages (see above). The evidence in the case of the haemin-controlled inhibitor discussed above, however, points to general inhibition of all protein synthesis in reticulocyte lysates including that with exogenous mRNA's (Mathews et al., 1973).

4.3/

4.3. Control exerted on the mRNA itself.

4.3.1. Separation of mRNA from the protein synthetic machinery.

Several possible mechanisms can be envisaged.

4.3.1.1. Prevention of exit of the mRNA from the nucleus.

Although mRNA transfer from the nucleoplasm to the cytoplasm can be blocked, or at least retarded, by the use of cordycepin, an inhibitor of the addition of poly(A) to mRNA 3' terminus (Penman et. al.,1970) no evidence for the prevention of release of specific mRNA's from the nucleus exists. It is interesting, however, in view of the continuing debate over the possible precursor-product relationship of HnRNA and mRNA (for review see Perry, 1976), to speculate that the mRNA phenotype found in cytoplasm is dictated not by regulation of transcription of the genotype but that continuous transcription of the entire genotype is modulated by the selective processing of HnRNA and selective release though the nuclear membrane.

4.3.1.2. Sequestration of the mRNA in inactive messenger ribonucleoprotein (mRNP) particles.

The role, if any, of mRNP's in controlling which mRNA species are translated is still unclear. Several mRNA's have been isolated from post-ribosomal supernatant of cytoplasm in association with proteins, notably α globin mRNA (Jacobs-Lorena and Baglioni, 1972). In this case, the occurrence of α globin mRNA in an mRNP particle may be the result of other control factors (see Introduction 4.4.) rather/

rather than a control itself. Reports of proteins bound to active mRNA released from reticulocyte polysomes by EDTA (Lebleu et al., 1971; Morel et al., 1971) or puromycin (Blobel, 1972) have shown some degree of agreement on the sizes of bound protein. Later work in Brawerman's laboratory (reviewed Brawerman, 1976) suggests that the protein is bound to the poly(A) sequence in such a way as to prevent nuclease digestion.

Several instances of inactive mRNP's releasing active mRNA have nevertheless been documented, notably in sea urchin embryos (reviewed by Kedes (1976). In that case, histone mRNA's manufactured during oogenesis are stored as mRNP's until fertilisation of the ovum.

The possibility of specific control by sequestration of mRNA in inactive particles therefore exists and may play a role in development.

Claims have also been made for a specific interaction between mRNA's and small "translational control" (tc) RNA's (by Heywood and Kennedy, 1976) but as yet the role of such factors is not well defined.

4.3.2. Processing of the mRNA for translation.

The processes leading to the ultimate release of mRNA from the nucleus into the cytoplasm have not yet been clarified. It is possible that mRNA undergoes a similar maturation process to that undergone by rRNA and tRNA, (see Perry, 1976).

4.3.3. Inherent structural features of mRNA.

Several structural features of mRNA have been suggested as possible controlling elements in translation.

4.3.3.1. The 5' terminal "cap".

It has been discovered that an unusual methylated terminal base sequence is found at the 5' termini of many, but not all, virus RNA's and, so far, all eukaryotic mRNA's (reviewed by Shatkin, 1976). The sequence, known as a "cap", is of the form

$$^{7m}GpppX^{m}_{p}Y^{m}_{p}NpNp\cdots \quad \underline{or} \quad ^{7m}GpppX^{m}_{p}NpNp\cdots$$

This led to speculation as to its possible role in translation. As reviewed by Shatkin, 1976, the following evidence for a role in translational control has been found:-

1. In vivo, and in vitro "capped" viral RNA's are found in association with polysomes, whilst "uncapped" RNA's are not.

2. Removal of "cap" from some viral and from eukaryotic mRNA's decreases the ability to direct protein synthesis in cell-free systems, although the stringency of the requirement for "cap" varied between systems. Some evidence suggests that the decrease in synthetic ability may in part be due to the severe conditions used to remove the "cap".

3. Naturally occurring "uncapped" viral mRNA's (e.g. poliovirus) nevertheless direct protein synthesis efficiently in cell-free systems.

4./

4. The 7-methyl guanosine apparently acts in initiation of protein synthesis by binding to the 40S subunit protein(s), after which it is protected from nuclease digestion. After protease digestion of the protected residue, oligonucleotides of chain length 31-65 bases, depending on the mRNA, have been recovered.

5. Evidence from "capped" synthetic polynucleotide studies suggests that binding to ribosomes may also be sequence specific.

Thus a role for the "cap" in regulation of translation is feasible although the preliminary studies cited by Shatkin suggest no significant turnover of "caps" in cell-free systems.

4.3.3.2 3' poly(A) sequences.

The role of poly(A) regions in translational control of protein synthesis has been widely explored. This has generally been done by enzymatic removal of the poly(A) sequence and subsequent translation studies in cell-free systems (Bard et al., 1974, Soreq et al., 1974; Huez et al., 1974; Williamson et al., 1974; Sippel et al., 1974; Doel and Carey, 1976). The effect of poly(A) removal on translational capacity was found to vary depending on the cell-free system used for translation of the deadenylated mRNA (Soreq et al., 1974; Doel and Carey, 1976). The Krebs II ascites and reticulocyte cell-free systems or Xenopus oocytes (Nudel et al., 1976) showed a decrease in incorporation with deadenylated mRNA. This was not found in the wheat germ or wheat embryo cell-free system (Bard et al., 1974; Doel and Carey, 1976) but this may have been due to a low rate of re-initiation in this system. Criticism of such experiments can be made on the grounds of other unspecified damage occurring/

occurring to the mRNA in such deadenylation reactions. The findings with wheat germ cell-free systems, the formation of full-size products in all systems, and the ability of such deadenylated mRNA to be successfully copied into cDNA (Maniatis et al., 1976) argue against this.

Several theories have been proposed (e.g. Sussman, 1970) suggesting a progressive removal of A nucleotides from the poly(A) tail of mRNA's as they are translated, ultimately leading to degradation. Recent work by Lingrel et al., (1974) and by Brawerman (1976), has shown that initial degradation of the poly(A) tail takes place after appearance of the mRNA in the cytoplasm. This rapid degradation reaches a modal value (= 60 for globin) after which the poly(A) tail is relatively stable for long periods. Whilst this may not occur for all mRNA's, it argues against any general "ticketing" hypothesis. Brawerman (1976) has suggested control of translation by poly(A) by interaction of the poly(A) tail with residues at the 5' end of the mRNA. In β globin mRNA, the complete sequence shows no stretches of U residues greater than 4 and no predominance of U residues in the untranslated region at the 5' end (Baralle, 1977; Efstratiadis et al., 1977).

Moreover, poly(A) sequences are not obligatory in mRNA. Most histone mRNA contain no poly(A) sequences (Adesnik & Darnell, 1972; Greenberg & Perry, 1972; but see Levenson and Marcu, 1976), but translate faithfully both in vivo and in vitro. In addition, both in sea urchin embryos (Nemer et al., 1974; Nemer and Surrey, 1976) and in HeLa cells (Milcarek et al., 1974), a class of mRNA's lacking poly(A) sequences has been described (Nemer et al., 1974; Nemer and Surrey, 1976) accounting for upwards of 30% of all mRNA species present/

present. These species translate equally as well as poly(A) containing mRNA's.

It has also been reported that some protamine mRNAs exist both with and without poly(A) sequences (Gedamu et al., 1977). These species are equally able to direct protein synthesis.

Thus the role of the poly(A) sequence in controlling mRNA translation remains unresolved, although its presence or absence in some cases can affect the rates of initiation and elongation of protein synthesis.

4.4. Control of the interaction between mRNA and the protein synthetic machinery - kinetic factors.

4.4.1. Control at initiation step.

4.4.1.1. The Lodish Model.

Since the base sequence leading to the initiation codon of each mRNA is likely to be different, it can be envisaged that each mRNA will have a particular affinity for components which allow the initiation of protein synthesis (40S subunits, initiation factors). Consequently, the overall rate constant (K_1) for initiation will vary among mRNA's. Lodish (1974) has developed a semi-mathematical description of the effects of various conditions on the relative rates of initiation of different mRNA's involving some simplifying assumptions. Using the α and β globin mRNA's as examples, he predicts that any change in conditions liable to decrease the favourability of the formation of initiation complexes would result in a more pronounced inhibition of the initiation (and translation) of/

of the poorer initiating species. In a series of experiments designed to test this, he demonstrated a shift in the ratio of synthesis of α and β globins chains in favour of β chain synthesis when inhibition of initiation occurred. This occurred only with inhibitors which act to prevent the formation of the initial met-tRNA-40S-mRNA complex but not with inhibitors which act at initiation stages beyond this point. α chain mRNA has been demonstrated to initiate at ∿2/3 of the rate of β chain mRNA (Lodish, 1971; Lodish & Jacobsen, 1972). Thus for α and β globin mRNA's the hypothesis correctly predicted the effect of inhibition of initiation.

Systems where two major identifiable types of protein are produced, e.g. viral and cellular, internal and secreted, are especially well-suited for testing of this hypothesis. It should also be able to be successfully tested in cell-free protein synthesizing systems, where controlled variations in conditions can be made. Evidence from a number of systems has been contradictory.

4.4.1.2. Support for the Lodish Model.

In support of his hypothesis, Lodish and his coworkers have shown:

a) In reticulocyte lysates, addition of excess total globin mRNA or β globin mRNA stimulates synthesis of relatively more β globin than α globin, whereas addition of excess α globin mRNA stimulates synthesis of more α globin (Temple & Lodish, 1975). This suggested that lower translation of α globin mRNA at normal levels was not due to a lack of a specific factor.

b)/

b) In superinfection studies of f2-infected E coli with T4 phage, it was concluded that the method of inhibition of f2 RNA translation after superinfection was probably due to the higher initiation constant for T4 RNA (Goldman and Lodish, 1975). Evidence from bacterial systems where different mechanisms of protein synthesis are found, must however be treated with caution.

Further evidence for the validity of Lodish's hypothesis has come from various systems: high salt inhibition studies of vaccinia virus, immunoglobulin, collagen synthesis (Oppermann & Koch, 1976; Nuss and Koch, 1976; Koch et al., 1976), starvation high salt, and chemical inhibition studies of immunoglobulin synthesis both in whole cells (Sonenshein & Brawerman, 1976a) and cell-free systems (ibid 1976b), "cap" analogue studies with reticulocyte lysates (Suzuki, 1976).

4.4.1.3. Opposition to the Lodish Model.

Opposition to the claims of the Lodish hypothesis have often been based on messenger-specific factors (e.g. Kennedy and Heywood, 1976; Vaquero et al., 1975), whose addition to cell-free systems is claimed to direct the preferential translation of specific mRNA's. Heywood, however has admitted that his results can be interpreted on the basis of Lodish's hypothesis (Gilmore-Herbert & Heywood, 1976). Some other of his objections can be dismissed due to misunderstanding of the hypothesis, e.g. the assertion that myosin mRNA in mRNP's and polysomes, being identical by several criteria cannot be regarded

as/

as"better" (polysomal) or "poorer" (mRNP) mRNA's (Kennedy and Heywood, 1976; Heywood & Kennedy, 1976). Such partitioning of mRNA's may simply reflect the level of growth of the system. Indeed, mRNP's are degraded to yield polysomally active mRNA as development progresses (Heywood & Kennedy, 1976).

Other apparent anomalous results for the Lodish hypothesis may result from incomplete interpretation of data and use of proper controls. Bonanou-Tzedaki and Arnstein (1976) showed a temperature dependent inhibition of protein synthesis in rabbit reticulocytes or reticulocyte lysates, whose site of action was localised as being at the initiation step. While they found no change in the ratio of α:β globin chains synthesized, an increase in the ratio of free α:β globin mRNA's found in the post ribosomal supernatant suggested that α chain initiation was preferentially inhibited. A more complex series of inhibitory processes may be operating in this system. The work of Vaquero et al., (1975) on the other hand showed an inhibitory process occurring in protein synthesis but did not localise it at the initiation step. Moreover, their experimental conditions did not allow for the variation in $[K^+]$ under different conditions. One interesting exception to the Lodish theory which may prove real is the findings of Kaufman et al (1976) with poliovirus. This virus is one of the few eukaryotic mRNA's lacking a proper "cap" at its 5' terminal. Thus its interaction with 40S-mettRNA$_f$ complexes may be unlike normal mRNA interactions. Lodish has discussed many of the challenges to his hypothesis in a recent review (Lodish, 1976).

4.4.1.4. Ig production and the Lodish Model.

The studies most apposite to this project were conducted on MPC-11 myeloma cells by two groups (Nuss and Koch, 1976; Sonenshein & Brawerman, 1976). In part,their results are complementary and confirmatory. They can be summed up as follows:-

1. In whole cells, a variety of initiation inhibitors (high salt, starvation, actinomycin D) inhibited non-Ig synthesis more than Ig synthesis, and H chain synthesis more than L chain synthesis. This suggested increasing initiation constants as follows: non-Ig < H chain < L chain in general.

2. Inhibitors of elongation (cycloheximide, emetine) showed no preferential inhibition of Ig or non-Ig species, although this has been found by other workers (Stevens and Williamson, 1974).

3. In a cell-free system (wheat germ) observed effects were similar to in vivo.

These results are consistent with the Lodish hypothesis if H and L chain mRNA's have high initiation constants.

In this project results are presented which indicate a similar preferential translation of Ig mRNA in a cell-free system under conditions of inhibition of initiation. They also suggest, however, that H chain mRNA can be preferentially translated over L chain mRNA, in contradiction to the results discussed above.

4.4.2. Control of elongation/termination?

The mechanisms of elongation and termination of protein synthesis are now fairly well understood (see review by Weissbach and/

and Ochoa, 1976). No specific elongation or termination factors specific for a particular mRNA have been discovered, and it is difficult to envisage how such factors might operate. After the specificity involved in initiation (5' terminal sequence, possible specific initiation factors), the elongation process will be limited solely by the rate of attachment of specific aa-tRNA's to the mRNA-ribosome-polypeptide complex and the termination process by reactions set in motion by the appearance of a "stop" codon in the reading frame of the mRNA. Limitation of specific tRNA's has been discussed earlier but such a system would be difficult to operate in order to produce an effect on specific mRNA's.

The rate of elongation may vary instantaneously according to the rate of diffusion of the required aa-tRNA complex to the ribosome but the overall rate of elongation has been shown in cell-free systems to be constant (Hunt, 1974). It is also well established that initiation, not elongation or termination is the rate limiting step in translation.

4.5. Control of the rate of degradation of mRNA's.

The role of poly(A) sequences in preventing degradation of mRNA has already been discussed (Section 4.3.3.2.).

mRNA in prokaryotes can often be found simultaneously being transcribed, translated and degraded, the events occurring consecutively along the same mRNA from the 3' growing end where transcription is occurring, through a portion of the message being translated by ribosomes to the 5' end where degradation has set in.

In/

In eukaryotes, mRNA is usually much longer-lived. mRNA must first be transcribed, post-transcriptionally modified and released into the cytoplasm, of course, but thereafter the mRNA's often have half-lives measured in hours (Brawerman, 1974).

Most studies on mRNA degradation have involved actinomycin D blockage of all RNA synthesis but these results are open to criticism since actinomycin D in large amounts has been shown to block initiation of protein synthesis. As a consequence measurement of mRNA degradation on the basis of polysome disintegration is suspect. Experiments with pulse-chase labelling of mRNA using radioactive uridine (Murphy & Attardi, 1973) suggested half-lives of several days, whilst Penman's group (Singer and Penman, 1973) have shown at least two distinct species of mRNA in HeLa cells with half-lives of 7 hrs and 24hrs respectively. Moreover, in Xenopus oocytes, globin mRNA has been detected as being translated for several days after injection into fertilised eggs (Lane, Gurdon & Woodland, 1974).

Consequently, it is possible that selective degradation of mRNA may occur in the cell, although it has not been shown convincingly for any specific mRNA. Such selective degradation might be on the basis of labile nucleotide sequences in the mRNA which were easily attacked by nuclease action.

4.6. Conclusion.

While selection of mRNA before, during and after translation may occur, the most probable site of control is at the stage of initiation of protein synthesis. The next section will discuss control of the segregation of synthesis of proteins intended to remain/

remain within the cell and proteins due to be secreted.

5. Factors affecting the control of secretion of proteins.

5.1. Introduction.

It is now reasonably well established that proteins intended for secretion by cells are generally synthesized on membrane-associated polysomes except in abnormal conditions.(recently reviewed by Shore and Tata, 1977). It is still, however, uncertain whether membrane bound polysomes are devoted exclusively to synthesis of secreted proteins (including, for the purposes of this discussion, species which are incorporated into membranes) (reviewed by McIntosh and O'Toole, 1976). How are polysomes containing mRNA coding for secreted proteins distinguished from those polysomes concerned with non-secreted protein synthesis? Evidence has been presented for the role(s) of the mRNA, ribosomes, the membrane and the nascent polypeptide in distinguishing which polysomes become membrane-bound and has been reviewed by Shore and Tata, 1977. Membranes themselves probably contain receptors for binding ribosomes and pores for the transfer of newly-synthesized secreted polypeptides into the cell lumen. These receptors have not yet been characterised and probably do not directly determine which polysomes are bound. Evidence exists for proteins which associate with ribosomes to prevent binding to membranes in vitro but it is not unambiguous (see review of Shore and Tata, 1977). Such protein factors have not been shown to be specific for a particular class of polysomes within the cell (e.g. Blobel, 1976). Recently, the use of heterologous cell-free systems to translate mRNA has led to a growing amount of evidence that the nascent/

nascent polypeptide is the major factor determining whether a polysome becomes membrane-bound.

5.2. The "signal" hypothesis (1971).

The concept of an attachment of polysomes to membranes directed by the nascent polypeptide was first discussed in 1971 by Blobel and Sabatini and subsequently by Milstein et al., 1972. Their hypothesis aimed to explain the segregation of polysomes synthesizing secreted proteins on membranes. In their original concept, the early stages of synthesis of secreted proteins occurred on free polysomes. The primary translation product, however, contained extra amino acids at the NH_2 terminus (the "signal" peptide). When this "signal" peptide was sufficiently well clear of the ribosome, it attached to a receptor site on the endoplasmic reticulum. This site directed the transfer of the polypeptide through the membrane and into the lumen, ready for secretion.

The "signal" peptide was postulated as common amino acid sequence for all secreted proteins derived from a unique codon sequence in mRNA's for secreted proteins.

Later versions of the "signal" hypothesis (Blobel and Dobberstein, 1974a) have been modified by the discovery of precursor sequences at the NH_2 terminus of many secreted proteins. These sequences are not normally found in the mature secreted product but came to light following translation of mRNA's of secreted proteins and polypeptides in heterologous cell-free systems. Blobel and Dobberstein suggested that these precursor sequences constituted the "signal" peptides. They suggested that, after guiding the nascent/

nascent polypeptides through the membrane, such peptide sequences are proteolytically removed, possibly before the synthesis of the polypeptide is complete. Evidence for this will be discussed.

The amino acid sequencing data obtained by Schechter and his co-workers (see Fig. 1) from several Ig L chain precursors has ruled out the possibility of a unique amino acid sequence for the precursor peptide, although L chains from closely related cell lines have identical precursors (Schechter, 1977). A unique codon sequence for Ig L chain precursors, or even one allowing third base degeneracy, is also ruled out by consideration of the known amino acid variations. Ig L chains (and possibly H chains if these contain precursors) may, however, be a special case. Burstein & Schechter (1977) have suggested that the variability encountered in the L chain precursor peptides may be due to the precursor forming a part of the L chain variable region with which it is contiguous. Devilliers-Thiery et al., (1975) have shown much less heterogeneity between putative precursor sequences of several dog pancreas secreting proteins. Hence the uniqueness or otherwise of the "signal" peptide sequence has not been determined unambiguously.

Firmly established, from these studies, nevertheless, is the evidence of a high proportion of hydrophobic amino acids in the known precursor sequences. This is consistent with a role in directed transfer of secreted proteins through the membrane.

5.3/

FIG. 1.

PARTIAL AMINO ACID SEQUENCES OF THE N-TERMINI OF SEVERAL MYELOMA L CHAIN PRECURSORS[a]

	Amino acid[b]																			
NH_2 terminus	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Cell line																				
MOPC 321	M	*	T	*	T	L	L	L	W	V	L	L	L	W	V	P	*	*	T	*
MOPC 63	M	*	*	*	*	L	L	L	*	*	L	L	L	*	*	P	*	S	*	*
MOPC 41	M[c]	*	[c]M	*	A	P	A	*	I	F	*	F	L	L	L	L	F	P	*	T	*	C
MOPC 104E	M[d]	A	*	*	*	L	*	L	*	L	L	A	L	*	*	*	A	*	*	*

Legend:

a Taken from Burstein and Schechter, 1976; Burstein, Kantor and Schechter, 1976; Schechter and Burstein, 1976.

b Single letter amino acid designations as follows:-

A = Ala; C = Cys; F = Phe;

I = Ile; L = Leu; M = Met;

P = Pro; T = Thr; V = Val;

W = Trp * designates unknown residue

c Found on ∿75% of all molecules sequenced; otherwise first two residues absent

d Found on ∿10% of all molecules sequenced; otherwise first residue absent

5.3. The use of cell-free systems.

5.3.1. Cell-free systems - advantages over cellular studies.

The major advantage in most eukaryotic cell-free systems (e.g. reticulocyte lysate, wheat germ, but not Ascites cells) is that such systems do not contain large amounts of either membranes or membrane derived microsomes. This contrasts with most cells synthesizing secreted protein, which generally contain large amounts of endoplasmic reticulum. Reticulocyte lysate, derived from cells containing no visible endoplasmic reticulum is especially suitable in this respect. As a consequence of the lack of membranes, polypeptide products of cell-free systems are less prone to post-translational modification by membrane-associated synthesis. Two major processes are affected:-

i) Post-translational addition of carbohydrate residues (e.g. to Ig molecules).

ii) Post-translational cleavage of the polypeptide chain.

5.3.2. Cell-free systems - the synthesis of precursors.

The development of functional eukaryotic cell-free systems from reticulocytes (Lockard & Lingrel, 1969), wheat germ (Roberts and Paterson, 1972) and ascites cells (Aviv et al., 1971; Mathews & Korner, 1970), allowed the study of cell-free translation with mRNA's of secreted proteins.

Much work has been carried out with mRNA from secretory cells, either in an impure or fractionated form, since such cells often devote much of their protein synthetic activity towards production of a single (or small range of) proteins. This allows the possibility of/

of purification of a particular mRNA species in relatively homogenous form.

5.4.1. Existence of putative precursors to many secreted proteins.

Evidence has accumulated over recent years, through the use of cell-free systems, that many secreted proteins and polypeptide hormones are initially translated as higher MW precursors. To date much of this evidence is based on the lowered mobility on PAGE of immunologically identified cell-free products, compared to the cellularly produced material. A list of such species is presented in Table 1. A useful short review is that of Campbell & Blobel (1976).

From these reports it can be seen that the phenomenon of precursors to secreted proteins and polypeptides is widespread, occurring as a result of translation of mRNA from mammals, birds, fish and insects. It also seems to be reproducible in a number of cell-free systems, including both mammalian- and plant-derived systems. In some cell-free systems (e.g. Krebs ascites) partial processing of the precursor appears to occur. This has been attributed to the presence of membrane material in such systems. Szcześna and Boime (1976) have demonstrated that membrane material is responsible for processing in their work. The precursor sequences detected vary in size. Using only those systems from which sequence data has been obtained, viz. mouse myeloma Ig L chain, rat insulin, bovine parathyroid hormone, prolactin, dog pancreatic secretory proteins and rat liver serum albumin the range of precursor sizes is 18 - 25 amino acids. Although smaller precursor sizes have been reported (e.g. α lactalbumin 7 - 10) (Craig/

TABLE 1

CELL-FREE SYSTEM IDENTIFIED PRECURSOR-CONTAINING PROTEINS AND POLYPEPTIDES

Protein or polypeptide (aa's in precursor)	Cell-free system	Identification	Reference
a) Proteins			
Mouse Ig L chain (19-22)	WG,KA,RL,EA	M, I, S	See Table 2
Dog Pancreatic secreted proteins (≥16)	H	M, S	Devilliers-Thiery et al., 1975
Guinea pig α-lactalbumin	WG, KA	M, I	Craig et al., 1976
Rat liver serum albumin (18)	WG	M, I, S	Strauss et al., 1977
Conalbumin, (hen oviduct) (19) Ovomucoid, (hen oviduct) (23) Lysozyme, (hen oviduct) (18)	RL	M, I, S	Palmiter et al., 1977
Rabbit uteroglobulin	KA, WG	M, I	Beato & Nieto, 1976
b) Polypeptide-hormones			
Insulin (rat) (23)	WG	M, I, S	Chan et al., 1976
(carp)	WG	M, I	Rapoport et al., 1976
Growth hormone (GH)	WG	M, I	Sussman et al., 1976
Prolactin	WG	M, I	Evans & Rosenfeld, 1976
Placental lactogen	WG, KA	M, I	Boime et al., 1975,1977
Parathyroid hormone (25)	WG, KA	M, I, S	Habener et al., 1975 et seq.
Corticotropin (ACTH)	WG	M, I	Nakanishi et al., 1974

Legend

Key: 1) Cell-free systems

WG = Wheat germ

RL = Reticulocyte lysate

KA = Krebs II Ascites

H = Heterologous components

EA = Ehrlich Ascites

2) Method of identification

M = Mobility on PAGE

I = Specific immunoprecipitation

S = Sequence data

(Craig et al., 1976) these have been based on mobility data. Such measurements are of dubious validity.

An interesting feature of cell-free synthesized precursor molecules is that they are in fact often only precursors to pro-polypeptides (e.g. prolactin, proinsulin, proalbumin) which undergo subsequent proteolytic cleavage during or after secretion to yield the final active protein or polypeptide. Such pro-sequences are generally short (e.g. 5 residues for albumin) and often basic in character (e.g. pro sequences of albumin or of parathyroid hormone contain 80% and 66% basic amino acids respectively). Such findings suggest several levels of control of secreted proteins, firstly in translocation across membranes, subsequently in producing the active protein.

5.4.2. Precursors of myeloma cell products.

a) Ig L chains.

One of the most intensively investigated mRNA species has been that of the mRNA for Ig L chains. Initial studies on myeloma mRNA from MOPC 41A cells (Stavnezer & Huang, 1971) indicated the general antigenic identity of cell-free and cellularly produced L chain by immunoprecipitation. Tryptic peptide analysis, however, suggested that the N-terminal peptide differed between the cell-free and cellular products. Later workers, firstly with total mRNA from MOPC 21 cells (Milstein et al., 1972) and subsequently with MOPC 41 mRNA enriched in L chain species (e.g. Mach, Faust & Vassali, 1972) demonstrated that cell-free L chain had lower mobility (and hence higher apparent MW) than cellular L chain on analysis by PAGE.

This/

This result has been confirmed using a number of other cell-free systems and a variety of myeloma cell lines for mRNA preparation. So far mRNA from all cell lines studied has directed synthesis of L-chain-like material with higher MW's than secreted L chain. The cell lines studied are listed in Table 2.

In all cases, the cell-free product had a higher apparent MW than the cellular product. This higher MW seemed to be due to the presence of an altered N-terminus (Stavnezer & Huang, 1971; Swan et al., 1972) and seemed to be a general phenomenon in cell-free systems. It has also been observed in vivo under stringent protease inhibition conditions (Schmeckpeper et al., 1975).

The formation in vitro of a primary translation product for L chain longer than that found in the cell offered support to the "signal" hypothesis, described earlier. Since then the study of L chain mRNA translation has continued to be interpretable on the basis of this hypothesis. The evidence accumulated by study of L chain translation can be summed up as follows:-

1. L chain precursor molecules appear to exceed the length of cellular L chain by ∿1 - 2000MW units or 19 - 22 amino acid residues located at the N terminus of the molecule. Much of the work on the size and sequence has been done by Schechter and his co-workers (Schechter, 1973; 1974a; 1974b; 1975; Schechter et al., 1975; Schechter and Burstein 1976a,b; Burstein and Schechter, 1976; Burstein et al., 1976). These studies have revealed the partial sequences of the precursor segments of several myeloma L chains (MOPC 321, MOPC 41, MOPC 63, MOPC 104E). These are shown in Fig. 1. A noticeable feature of these sequences is the abundance of hydrophobic residues/

TABLE 2

TRANSLATION OF Ig L CHAIN mRNA IN CELL-FREE SYSTEMS

Cell line	L chain subgroup	Cell-free system(s)	Reference(s)
MOPC 21(P3K)	κ	RL,WG,KA	Milstein et al., 1972, Schmeckpeper et al., 1974, 1977
MOPC 41	κ	WG,KA,RL	Schechter and Burstein, 1976, Swan et al., 1972, Mach et al., 1973
MOPC 41A	κ	WG,KA,RL	Schmeckpeper et al., 1974, 1977
MOPC 321	κ	WG, KA	Schechter and Burstein, 1976a,b, Schechter, 1973
MOPC 63	κ	WG	Burstein and Schechter,1976
MOPC 104E	λ_1	WG	Burstein et al., 1976
MOPC 315	λ_2	WG, RL, KA, EA	Schmeckpeper et al., 1974, 1977, Green et al., 1976

Legend

Key: RL = Reticulocyte lysate

WG = Wheat germ

KA = Krebs II ascites

EA = Ehrlich Ascites

residues, as would be expected for a "signal" peptide which had to interact with endoplasmic reticulum. Similar hydrophobicity has been reported in precursors of proteins from other sources (De-villiers-Thiery et al., 1975; Palmiter, 1977). It would not seem unlikely that a concentration of hydrophobic residues was a common feature of precursor sequences.

2. The precursor sequence is removed due to interaction with the endoplasmic reticulum. Milstein et al., (1972) demonstrated that microsomes (with attached polysomes) from myeloma cells synthesized only mature-sized L chain. Their results from translation in the ascites system which contains small variable amounts of microsomal material also showed some processing of pre L to L chain. This has been found by other workers using ascites cells (Schechter et al., 1974; Swan et al., 1972). More convincing evidence for the role of membranes in cleavage of precursor sequences has been shown by Dobberstein & Blobel (1977). In their experiments, both heterologous ribosomes (wheat germ) and membranes (dog pancreas) were incubated in the wheat germ system with murine myeloma mRNA (MOPC 41). Under these circumstances, only mature L chain was detected. Addition of microsomes after the synthesis had been allowed to occur, however, did not process the precursor L chains to L chains, indicating that processing was closely linked to translation. Other workers have confirmed this result (Schmeckpeper et al., 1977). Moreover, the use of plant ribosomes and animal membranes indicated that the processing mechanism is widely distributed and shows functional homology between the plant and animal kingdoms.

3. Processed polypeptides are translocated through the membrane.

Again, the work of Dobberstein and Blobel (1974, 1977) has demonstrated/

demonstrated that precursor L chain synthesized in the absence of membrane fractions is labile to proteolytic digestion. Products synthesized in the presence of membrane fractions are not only processed to mature L chain but are also resistant to proteolytic digestion unless detergents, which can disrupt membranes, are present.

5.4.3. Ig H chains.

Fewer reports have been made of the synthesis of Ig H chains in cell-free systems. In part, this has been due to the nature of the cell-free systems available. As discussed earlier, the reticulocyte lysate system is hampered for translation of exogenous mRNA by the competition of large quantities of endogenous mRNA species. This problem has recently been overcome (Pelham and Jackson, 1976). The Krebs II or Ehrlich ascites system contains variable quantities of microsomal material which introduces the possible ambiguities of processing of primary translation products. They also contain significant amounts of nucleases (Jacobs-Lorena & Baglioni, 1972) and are dependent on added tRNA (Aviv et al., 1971), for mitigation of premature termination problems.

The wheat germ system, much used because of low endogenous mRNA levels and high incorporation of radioactivity, suffers from premature termination problems which become progressively worse as the length of the mRNA to be translated increases (Green et al., 1976; Sonenshein and Brawerman, 1976). This results in very low levels of synthesis of H chains. Another possible problem, especially in systems with high levels of competing endogenous mRNA, is that H chain/

chain mRNA from some myeloma cells is a relatively poor initiator of synthesis (Nuss and Koch, 1976; Sonenshein and Brawerman, 1976). Consequently, it may be translated less frequently in the presence of high levels of good initiating mRNA (e.g. globin mRNA).

Two final points to be borne in mind with cell-free synthesis of H chain concern presence of carbohydrate residues on the cellular H chain and the relatively small effect a precursor sequence would have on MW studies. In cell-free systems carbohydrate residues are unlikely to be added (see for example Cowan and Milstein, 1973). The presence of carbohydrate on H chains obviously increases their actual MW but also has a disproportionate effect on the apparent MW as estimated by PAGE. Consequently, in a cell-free system, its absence will cause H chain products to have greater mobility. This poses interpretative problems when considering evidence for the existence of precursors to H chain.

If H chain is synthesized as a precursor in cell-free systems, the effect of a precursor sequence (∿20 amino acids) on mobility of H chain (450 amino acids) is liable to be small (<5%). Such a small change in mobility might be difficult to detect by PAGE. Similar difficulties were experienced in searching for a precursor to rat liver serum albumin (Taylor and Schimke, 1973; Peterson, 1976; Strauss et al., 1977).
Nevertheless, several studies on the synthesis of Ig H chains, in cell free systems have been carried out. Both total myeloma poly(A) RNA and H chain mRNA-enriched fractions have been investigated. The cell lines, and systems used are described in Table 3.

Cowan and Milstein's work (1974) indicated that, by the criterion of PAGE, P3K cell-free products directed by mRNA contained

a/

TABLE 3

TRANSLATION OF Ig H CHAIN mRNA IN CELL-FREE SYSTEMS

Cell line	Class of H chain	H chain enriched mRNA?	Cell free system	Reference
MOPC 21(P3K)	γ_1	✓	RL	Cowan and Milstein, 1973
		X	WG	Schmeckpeper et al., 1974
		X	KA	Schmeckpeper et al., 1977
		X	RL	Schmeckpeper et al., 1977
MOPC 315	α	X	WG	Schmeckpeper et al., 1974
		X	WG	Green et al., 1976
		X	KA	Green et al., 1976
		X	KA	Schmeckpeper et al., 1977
		X	RL	Schmeckpeper et al., 1977
		✓	RL	Bedard and Huang, 1977
HPC 108	α	X	KA	Schmeckpeper et al., 1977
			RL	Schmeckpeper et al., 1977

Legend

Key: RL = Reticulocyte lysate

WG = Wheat germ

KA = Krebs Ascites

a species with a mobility slightly greater than that of marker H chains. They pointed out, however, that this may have been due to distortion caused by the presence of large amounts of carrier H chain. Polysomes or microsome directed cell-free synthesis yielded two species with mobilities slightly greater than H chains. They were not identified as H chain-like material other than by mobility. Tryptic analysis of poly(A) RNA-directed synthesis indicated the identity of the in vivo H chain and the in vitro putative H chain. They also showed the existence of an extra peptide in the in vitro synthesized H chain which may have been part of a precursor to H chain. Schmeckpeper et al., (1974, 1977) have done valuable comparisons of cell-free products of P3K mRNA in three cell-free systems. No synthesis of H chain-like material was demonstrated in the wheat germ system (Schmeckpeper et al., 1974) but a little was found in the ascites system and reticulocyte lysate system (Schmeckpeper et al., 1977). However, the data presented is difficult to interpret in mobility terms because of the use of tube gels.

MOPC 315 and HPC 108 cell lines offer the possibility of yielding less ambiguous results, since both produce α-type heavy chains, which contain a large percentage of carbohydrate. Its removal should produce a measurable change in mobility unless the existence of a precursor sequence is involved.

Comparison of the work of the several groups who have studied cell-free translation of MOPC 315 mRNA is aided by the fact that their experiments duplicate each other to some extent. With wheat germ, Green et al were unable to demonstrate production of H-chain-like material. Schmeckpeper et al., (1974) on the other hand, not only demonstrated/

demonstrated its translation but identified the α chain like product by immunoprecipitation. This α chain seemed to have a lower apparent MW than secreted MOPC 315 H chain, perhaps due to lack of carbohydrate. This was confirmed by Schmeckpeper et al., (1977) using the ascites system and the reticulocyte lysate system. Green et al., (1975) demonstrated the synthesis of 315 H and pre L chains by the ascites system using immunoprecipitation but failed to include proper controls such as internal or adjacent markers. The use by both groups of secreted H chains as markers, however, can be criticised since differences in mobility between intracellular and secreted α chains have been observed due to addition of carbohydrate residues during the final stages of secretion
Moreover secreted α chains electrophorese as diffuse bands due to variable amounts of carbohydrate residues. In contradiction to the above results, Bedard and Huang (1977) present evidence that cell-free synthesized and secreted H chain are identical, using rabbit reticulocyte lysate as cell-free system. Their gel analysis, as presented, however, is ambiguous, with blurring of the secreted H chain marker/and due to the existence of at least two cell-free species in the H chain region. No attempt was made to identify the products by immunoprecipitation leaving in doubt which species was related to H chain. Their claim that cell-free α chain had an equal MW to secreted α chain suggests poor resolution in their gel system. Cyanogen bromide digestion, moreover, did not have sufficient resolution in their hands to detect minor changes in mobility of peptide fragments. Their results should therefore be treated with caution.

The MPC 108 results of Schmeckpeper et al., (1974, 1977) show the/

the same effect and same shortcomings as their results with MOPC 315. Schechter has recently claimed that MOPC 315 α chain contains a precursor sequence (1977).

The conclusion to be drawn from the above experiments is that the question of the nature of cell-free synthesized H chain products has not yet been satisfactorily investigated. Even the relative mobilities of cellular and cell-free synthesized H chains has not been resolved, let alone the possible existence of a precursor sequence.

Results are presented in this thesis which indicate that the mobility of cell-free synthesized H chains from several myeloma cell-lines is generally higher than that of the intracellular *in vivo* H chain product, i.e. that it has a lower apparent MW as determined by PAGE.

5.5. Exceptions to the "signal" hypothesis.

The generality, although not necessarily the limited validity, of the signal hypothesis could be challenged by the demonstration of cell-free synthesis of a secreted protein, or polypeptide, not involving synthesis of a precursor sequence. This would suggest at least that an alternative mechanism of secretion existed. Several cases have been reported of non-precursor containing cell-free synthesized polypeptides corresponding to secreted proteins. Casein mRNA isolated from lactating rat mammary gland, was translated in the wheat germ system to yield 2 of the 3 major casein species (Rosen *et al.*, 1975). These were identified by mobility (equal to markers) and immune reactivity with casein antibodies. The data can be criticised, however, since casein produced *in vivo* is both glycosylated/

glycosylated and phosphorylated. This would affect the mobility of cell-free products lacking glycosylation or phosphorylation. Craig et al., (1976) reported that casein mRNA yielded three anticasein antiserum-precipitable species. These each had mobilities greater than iodinated marker proteins indicating a lower apparent MW in vitro.

Again, however, the effect of lack of glycosylation or phosphorylation on mobility was not tested. Moreover, with Ig H and L chains, iodination has been shown to alter mobilities on PAGE (P. Singer, personal communication). The interpretation of results of the translation of casein mRNA should therefore be treated with caution.

More serious evidence against the "signal" hypothesis has been the sequencing of cell-free products programmed by various hen oviduct mRNA's by Palmiter et al., (1977). Of four cell-free products sequenced, (lysozyme, ovomucoid, conalbumin and ovalbumin) three contained, as expected, a precursor sequence,whilst ovalbumin did not. The reticulocyte lysate cell-free system was used, thus eliminating the possibility of selective positive processing by membrane fragments, i.e. removal of precursor from only one of the four cell-free products. Negative processing (i.e. non removal of the ovalbumin precursor in vivo) is reportedly ruled out by the NH_2 terminal sequence of ovalbumin as sequenced by Palmiter et al., (1977) although his data is not shown. The possibility remains that ovalbumin is synthesized as a precursor but the sequence is particularly susceptible to proteolytic cleavage.

Palmiter's results suggest that the precursor peptide "signal" mechanism may not be the only mechanism whereby secreted proteins are/

are translocated across membranes (see also discussion of Ig H chains). His results, however, do not rule out the "signal" hypothesis; they merely show doubts in its complete generality.

6. Concluding remarks.

The investigation of the translation of murine myeloma cell mRNA in a heterologous cell-free protein synthesizing system offers the possibility of answering some questions concerning the nature of the control of production and secretion of Ig molecules.

Cell-free products may be characterised by several criteria and compared with their cellular analogues. Precursor sequences can potentially be identified on the basis of mobility or by examination of protease digests of the cell-free and cellular species. The effect of the absence of action of other cellular processes (e.g. carbohydrate addition) can be investigated.

The control of the relative synthesis of different polypeptide species in cell-free systems can be examined by use of controlled changes in the environment in which protein synthesis occurs. Results obtained by changing various parameters can be compared with predictions of their effects. This allows more understanding of the way control of protein synthesis may operate in myeloma cells themselves.

MATERIALS

1. General

1.1. Radiochemicals

All isotopically labelled compounds were obtained from the Radiochemical Centre, Amersham, England.

L {4,5-^{3}H} leucine	54 Ci/mmole
D {6-^{3}H} glucosamine	19 Ci/mmole
L {methyl-^{3}H} methionine	6 Ci/mmole
L {U-^{14}C} isoleucine	0.348 Ci/mmole
L {^{35}S} methionine	250 - 1050 Ci/mmole
{^{3}H} Amino-acid mixture	1mCi/ml.

(contains all aminoacids except met, cys, trp, gln, asn)

1.2. Chemicals for Liquid Scintillation Spectrometry

The chemicals for liquid scintillation spectrometry were obtained as follows:-

2,5 diphenyloxazole (PPO)	Koch-Light Laboratories Ltd., Colnbrook, England
Toluene (AR Grade)	"
Triton-X-100	Rohm & Haas (U.K.) Ltd., Jarrow, England
Protosol	New England Nuclear, Boston, Massachusetts

1.3. Enzymes

Micrococcal nuclease (S. aureus)	P-L Biochemicals, Inc., Milwaukee, Wisconsin
Creatine kinase (Sigma grade)	Sigma (U.K.) Ltd., London
Pancreatic ribonuclease A	"

Trypsin/

Trypsin TPCK	Worthington Biochemical Corp., Freehold, New Jersey

1.4. Nucleic Acids

Calf liver t-RNA	Boehringer Corporation (London) Ltd., Sussex, England

Tobacco Mosaic Virus (TMV) RNA was kindly donated by Dr. R.T. Hunt, University of Cambridge.

1.5. Photographic materials

All photographic materials were supplied by Kodak Limited, London or by Kodak Canada Ltd.

1.6. Other materials

All other chemicals or materials were, wherever possible, "AnalaR" reagents, supplied by B.D.H. Chemicals Ltd., Poole, Dorset, except for the following:-

Glutathione (reduced form)	Sigma (U.K.) Ltd., London, England
Creatine phosphate	"
Trizma base	"
Ethyleneglycol-bis-(β-amino-ethyl ether) N,N' tetraacetic acid (EGTA)	"
Cycloheximide	"
Sucrose (Grade 1)	"
Sodium deoxycholate	"
D,L Dithiothreitol (DTT)	"
2- deoxyglucose	"
Triton-X-100 (purified)	Koch-Light Laboratories Ltd., Colnbrook, England
2-mercaptoethanol	"

Acrylamide/

Table 4: Origin, History and Characteristics of Cell lines used.

Name of cell line	Tumour origin (Mouse strain, method of induction, year)	Tumour designation	Heavy chain class	Light chain class	Notes
5563 (Cl 18)	C3H female Spontaneous 1955	X5563	γ_{2a}	κ	
P1.17	BALB/c, female; Lieberman's Adjuvant 1959	Adj PC5	γ_{2a}	κ	Tissue culture line cloned 1967, Salk Institute, La Jolla California
P1.17.1	"	"	γ_{2a}	κ	Cloned 1977, University of Glasg T.R. Mosmann
P1.17.4	"	"	-	κ	Cloned 1977, University of Glasg T.R. Mosmann
SAMM 368.2	BALB/c pristane, 1976	SAMM 368	(α (γ_{2b}	(κ (κ	Cloned, 1976, University of Glasg T.R. Mosmann

Acrylamide	Koch-Light Laboratories Ltd., Colnbrook, England
Trichloroacetic Acid (TCA)	"
3 methyl-1-butanol (isoamyl alcohol)	"
N-ethylmorpholine	"
Nembutal	Abbot Laboratories, Ltd., Queensborough, Kent
Non isotopically labelled amino acids	Calbiochem Ltd., San Diego, California.
Heparin (mucous)	Evans Medical Ltd., Speke, Liverpool
NN' methylene bisacrylamide	Eastman Kodak Co., Rochester, New York

2. Tissue culture materials

2.1. Cell lines

Several murine myeloma tissue culture cell lines, adapted to culture from murine plasmacytomas were used in the course of this project. Their characterisation and derivation are described in Table 4. Production levels of these cell lines, where appropriate, are discussed in Chapter 3, Results.

2.2. Media

2.2. Standard solutions

(a) RPMI 1640 (G.E. Moore, Roswell Park Memorial Institute, Buffalo, N.Y.) wassupplied by Gibco Biocult Ltd., Paisley, Scotland, in powder form, prepared routinely by technical staff, and stored sterile in 500ml bottles at 4°C. The composition of the medium is shown on Table 5. Glutamine was omitted during preparation

and/

TABLE 5

Composition of RPMI 1640 medium (Gibco Biocult, Ltd.)

Inorganic salts	mg/litre
$Ca(NO_3)_2.4H_2O$	100.0
KCl	400.0
$MgSO_4.7H_2O$	100.0
NaCl	6000.0
$NaHCO_3$	2000.0
$Na_2HPO_4.7H_2O$	1512.0
Other components	
Glucose	2000.0
Glutathione (reduced)	1.0
Phenol red	5.0
Amino Acids	
L-Arginine (free base)	200.0
L-Asparagine	50.0
L-Aspartic acid	20.0
L-Cystine	50.0
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-/

TABLE

	mg/litre
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-Tryptophan	5.0
L-Tyrosine	20.0
L-Valine	20.0
Vitamins	
Biotin	0.2
D-Ca Pantothenate	0.25
Choline Cl	3.0
Folic Acid	1.0
i-Inositol	35.0
Nicotinamide	1.0
Para-aminobenzoic acid	1.0
Pyridoxine HCl	1.0
Riboflavin	0.2
Thiamine HCl	1.0
Vitamin B_{12}	0.005

and added separately before use.

(b) RPMI 1640 (for radioactive labelling) was prepared from powder, as described above, omitting the following constituents:- methionine, glutamine, cysteine, sodium hydrogen phosphate, etc. These components were prepared separately at 100 x normal concentration and stored sterile at $4^{\circ}C$. They were added in the appropriate combination at 1% (v/v) to the RPMI 1640 before use.

(c) Foetal Calf Serum (F.C.S.) was supplied by Gibco Biocult Ltd., Paisley, Scotland. 500ml bottles were heat inactivated by incubation at $56^{\circ}C$ for 60 minutes, then stored sterile in 100ml aliquots at $-20^{\circ}C$.

(d) Glutamine (200mM) was prepared routinely by technical staff and stored sterile in small aliquots at $-20^{\circ}C$.

(e) Penicillin/Streptomycin (10^{6} units/litre and 1% (w/v) respectively) was prepared routinely by technical staff and stored sterile in small aliquots at $-10^{\circ}C$.

(f) Trypan blue (0.1% (w/v) in PBS), was prepared from a stock solution of 1% (w/v) in PBS.

METHODS

1. Tissue culture

1.1. Maintenance of cell lines

Cells were maintained in RPMI 1640 medium, supplemented with 10% (v/v) FCS, 1% (v/v) glutamine and 1% (v/v) penicillin/streptomycin. Small volumes of cells were grown in plastic petri dishes (Falcon) whilst larger volumes of cells were grown either in 500ml glass bottles (Medical flats) or in 80oz roller bottles. Cell cultures growing in petri dishes were maintained in a 37°C constant temperature incubator with an atmosphere supplemented by 5% (v/v) CO_2. Cell cultures growing in 80oz. roller bottles were maintained in a 37°C hot room, and the bottles flushed before sealing with 5% (v/v) CO_2. The bottles were continuously rotated at constant speed. Cells were maintained at a density of between 2×10^5 and 8×10^5 cells/ml with regular checks on density and viability. Under these conditions the cells adhered loosely to the surface of their containers. Cultures were examined visually for contamination by yeast or bacteria and suspect cultures discarded.

1.2. Counting cells and estimation of viability

After shaking to dislodge adherent cells, a small volume of medium was removed aseptically and diluted with an equal volume of 0.1% (w/v) Trypan Blue stain. The solution was thoroughly mixed and a small volume expelled into a haemocytometer, and examined with illumination under a light microscope (25X). Several counting areas were examined, the cell counts totalled and averaged to give the cell density, taking account of the dilution by Trypan Blue. Cells staining/

staining dark blue were counted as dead and viability estimated by this method.

1.3. Harvesting cells

Cells were generally harvested at densities of 4×10^5 - 6×10^5 cells/ml. Cells grown in 8Ooz bottles or medical flats were dislodged by vigorous shaking whilst cells grown in Petri dishes were dislodged by repeated pipetting of the medium over the cells. The cells were centrifuged at 500x g, and were then either resuspended for use or stored at $-70^{\circ}C$ as a frozen pellet.

2. Preparation of radioactively labelled Ig

2.1. General

Radioactively labelled Ig was required for two main reasons. Firstly, Ig produced by cells labelled radioactively in tissue culture served as a source of marker heavy (H) and light (L) chains. These were used for comparison of mobilities on P.A.G.'s of cell-free synthesized myeloma messenger RNA translation products, including putative H- and L-chain-like material.

Secondly, radioactive labelling, followed by immune precipitation of Ig, allowed measurement of the amount of protein synthesis devoted to Ig in cultured cells. This could be compared with the amount of protein synthesis devoted to the production of similarly immuno-reactive material in cell-free systems stimulated by added myeloma cell polysomes or poly(A) RNA.

A small volume of cells grown up for polysome extraction was often set aside for radioactive labelling to determine the percentage

Ig production at the time of polysome extraction.

2.2. Cell labelling conditions

Cells were harvested in the mid log phase of growth (cell density $4 \times 10^5 - 6 \times 10^5$ cells/ml: cell viability >95%). Approximately 2×10^7 cells were used in a normal labelling experiment. The cells were centrifuged at room temperature at 500 x g for 10 minutes in conical tubes. The cell pellet was resuspended in 50ml of RPMI medium lacking the amino acid used for labelling (usually methionine or leucine), and centrifuged again, as described above. The washing step was repeated two times more. This step helped to deplete endogenous pools of the labelling amino acid. Cells were then suspended in Petri dishes in a small volume (∿2-3ml) of the above medium and incubated at $37^{\circ}C$ for 10 minutes in a constant temperature incubator with an atmosphere supplemented by 5% (v/v) CO_2. After this initial incubation, the radioactively labelled amino acid was added (200µci) and the incubation continued for the appropriate time. For experiments to test production of Ig by the cells, the time of labelling was 15 minutes. For experiments in which a substantial amount of secreted Ig was required, incubations were for 60 or 90 minutes. At all stages viability could be checked by the Trypan Blue staining technique described earlier and was >95% for shorter labelling periods (<30 minutes) but declined over longer periods. The incorporation of radioactive amino acid was halted by chilling the cells to $4^{\circ}C$.

2.3/

2.3. Preparation of intra- and extra-cellular Ig-containing fractions

After labelling, the cells were centrifuged at 500 x g for 5 minutes at $4^{o}C$. If labelled, secreted material was required, the supernatant from this centrifugation was carefully decanted and stored at $4^{o}C$ until required. The cell pellet was disrupted, re-suspended in 1ml 0.7% (v/v) Nonidet P-40 (NP-40) in PBS and incubated, with occasional agitation, for four minutes at $4^{o}C$, to lyse the cells. The lysate was subjected to centrifugation at 1800 x g for 10 minutes at $4^{o}C$ to pellet cellular debris and nuclei. The supernatant was decanted carefully and stored at $4^{o}C$. Small quantities of sodium azide were added to each fraction to prevent bacterial contamination. These two fractions, or immune precipitates derived from them could be used on P.A.G.'s as described elsewhere. Ig production was estimated by comparison of total TCA-precipitable material with specifically immuno-precipitable material using methods described in Section II.2.2 and using scintillation counting techniques for detection of radioactivity.

2.4. Cell labelling in the presence of 2-deoxyglucose

For experiments involving 2-deoxyglucose the following modifications to the above method were made. 10^{6} cells were used and resuspended without washing in 200µl PBS, instead of RPMI which contained high levels of glucose (11mM). The cell suspension was then supplemented with 2-deoxyglucose and radioactively labelled amino acid and incubated at $37^{o}C$ for the appropriate period in a CO_2-enriched atmosphere. Incorporation was halted and the cells centrifuged as above. The supernatant was discarded, since

2/

2-deoxyglucose blocked secretion of Ig (Melchers 1973) and the cells lysed in 200µl of 0.7% (v/v) NP40 in PBS at 4°C.

3. Preparation of polysomes from murine myeloma tissue culture cells.

3.1. Precautions against ribonuclease contamination

a. Glassware

All glassware coming into contact with solutions involved in preparing the polysomes, and with the cell lysate from which the polysomes were prepared, was soaked overnight in chromic acid (10% (w/v) potassium dichromate in 25% (v/v) H_2SO_4), washed six times with tap water, six further times with deionised water, allowed to drain and finally baked for several hours at 240°C, prior to use.

b. Other equipment

"Teflon" coated equipment was treated identically to the glassware. Centrifuge tubes and caps were soaked briefly in a dilute solution of (HCl) hydrochloric acid in deionised water, washed six times with tap water and six times with deionised water, then allowed to drain. Tubing and vessels involved in preparing sucrose gradients, and tubing and glass spectrophotometric cells involved in scanning sucrose gradients were thoroughly flushed through firstly by a dilute solution of hydrochloric acid followed by copious amounts of deionised water.

c. Experimenter precautions

All operations were carried out wearing fresh disposable plastic gloves which were replaced if thought to be contaminated. Direct contact with solutions was avoided.

d. Chemicals and preparation of solutions

All chemicals used were from bottles known to have been treated as/

as if ribonuclease-free. Chemicals were tipped from the bottles rather than removed by spatula. Weighing boats, previously unopened, were used for weighing. Liquids were dispensed using baked pipettes or sterile Eppendorf tips. Solutions were adjusted to the correct pH by addition of acid or alkali as described below and the pH measured either by testing the pH of aliquots with pH paper or by measurement of the pH of the solution on a pH meter with a carefully washed electrode. Solutions were prepared when possible on the day of the polysome extraction, or, at most, one day before and unused solutions were discarded after the extraction.

3.2. Solutions.

Most solutions used were based on solution C.

Solution C: 50mM Tris HCl (pH 7.4)
25mM NaCl
5mM Mg acetate
7mM 2-mercaptoethanol

Solution C1: as solution C with sucrose: 0.88M
heparin: 1mg/ml
cycloheximide: 100µg/ml
triton X-100: 0.08% (v/v)

Solution C2: as solution C with sucrose 0.88M
heparin: 1mg/ml
cycloheximide: 100µg/ml
triton X-100: 0.08% (v/v)
KCl: 0.2M
sodium deoxycholate (NaDOC): 0.06% (w/v)

Solution/

Solution C3: as solution C with sucrose: 1.5M

Solution C4: as solution C with sucrose: 0.5M

Solution C5: as solution C with sucrose: 1.25M

Solution D: 0.25M sucrose

3.3. Preparation of polysomes.

Polysomes were prepared by a procedure developed in this laboratory by Bennett, Fitzmaurice and Williamson (unpublished results), and it is described here with their permission. The procedure was developed from the published methods of Ascione and Arlinghaus, 1970, Buckingham et al., 1974, Palmiter, 1974, Schechter, 1974 and Shapiro et al., 1974. To approximately 100% viable myeloma cells, growing in mid-log phase was added sufficient of a concentrated solution of cycloheximide in PBS to bring the growth medium to a final concentration of 100µg/ml, in cycloheximide, to inhibit the"run-off" of ribosomes from polysomes. The resulting solution was mixed gently for two minutes, then the cells were collected by centrifugation at 500 x g for 5 minutes in 750ml centrifuge buckets. The cells were resuspended in a small volume of ice-cold PBS containing cycloheximide at 100µg/ml and collected again by centrifugation at 1000 x g for 5 minutes. Subsequent steps were carried out at 4°C whenever possible.

The cells were resuspended in four volumes of solution C1 and transferred to a Dounce homogeniser. In solution C1, heparin and Triton X-100 acted to disrupt the cell membrane, cycloheximide prevented "run-off" and heparin also acted as a ribonuclease inhibitor. The cells were ruptured with six gentle strokes from a loose fitting pestle/

pestle, then transferred to 45ml MSE polypropylene tubes and centrifuged at 48000 x g for 20 min. The supernatant was collected and was described subsequently as Fraction I of any polysome extraction. The pellet was resuspended in four volumes of solution C2 (In solution C2, cycloheximide, Triton X-100 and heparin were used as described for solution C1, whilst NaDOC was used as a further means of disrupting membranes and 0.2M KCl was used to remove tightly bound polysomes from membranes) transferred to a Dounce homogeniser and homogenised vigorously with a tight-fitting pestle. Again the homogenate was centrifuged at 48000 x g in MSE tubes for 20 min. The supernatant was collected and was described subsequently as Fraction II of any polysome extraction. At this stage Fractions I and II could be combined if necessary. Otherwise, they were routinely kept separate. Each fraction was layered on a 10ml cushion of solution C3 in a 30ml polyallomer tube and then centrifuged at 60,000 rpm for 2 hours at $4^{o}C$ in a Beckman preparative ultracentrifuge, Model L5-65, using a Beckman 60 Ti fixed angle rotor.

After centrifugation, the supernatant was decanted, the tube drained and wiped, and any contaminating material was gently washed off the polysome pellet with solution D. The polysome pellet was resuspended in a small volume of solution D by gentle homogenisation with a Dounce pestle and the solution stored at $-70^{o}C$. Polysomes derived from fractions I and II were designated Fraction I and Fraction II polysomes respectively.

3.4./

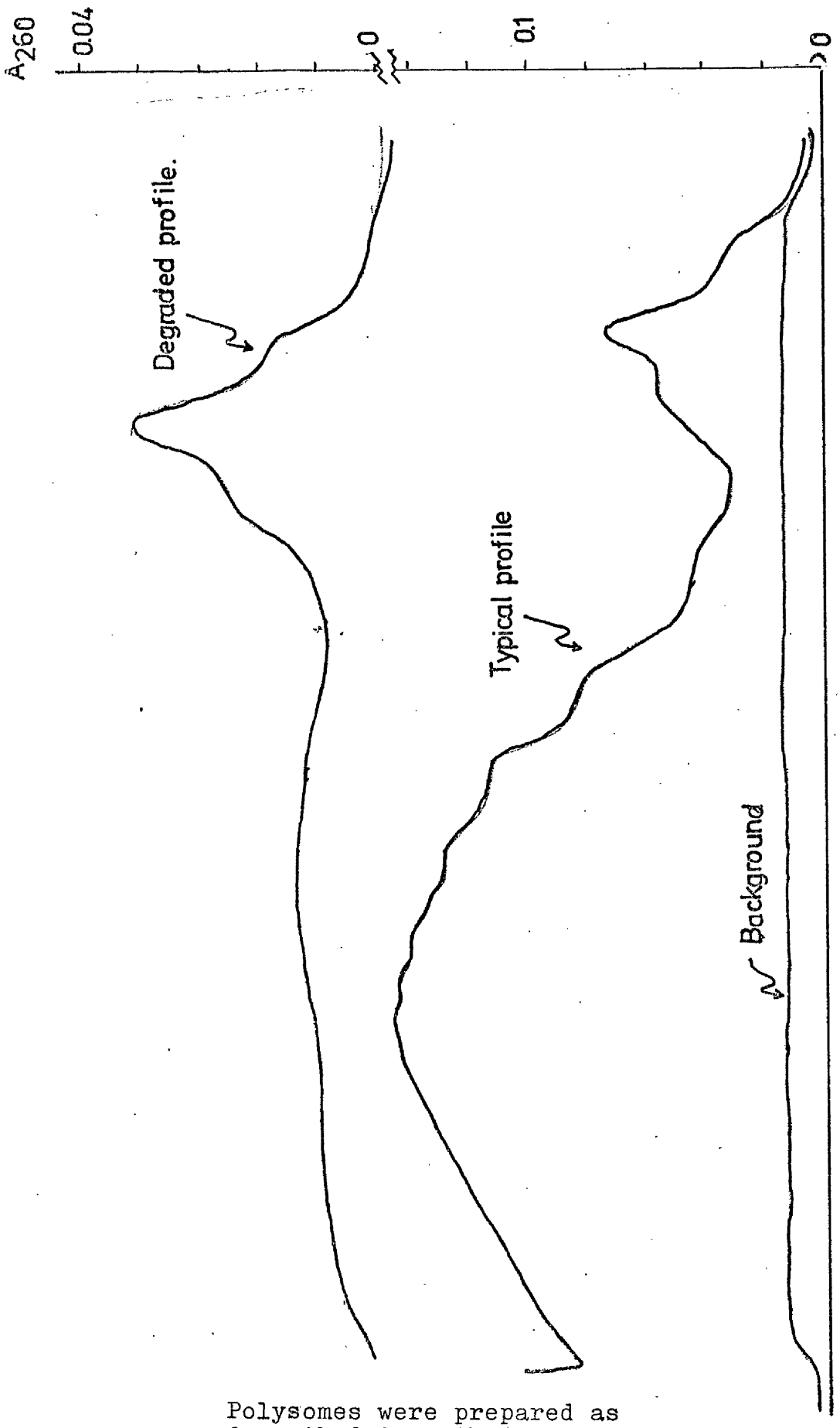
3.4. Sucrose gradient sedimentation.

To determine the integrity of polysomes produced by this method, solutions C4 and C5 were mixed on a Buchler gradient maker to produce linear 0.5 - 1.25M sucrose gradients in 13ml polyallomer tubes. A small volume of solution D, containing 10 - 20 A_{260} units of polysomes, was layered carefully on top of the gradient which was then centrifuged for 90 min at 40,000 rpm using a Beckman SW41 rotor in a Beckman L5-65 preparative ultracentrifuge.

The sucrose gradient was displaced downwards through a 1 ml flow cell and monitored continuously for absorbance at 260nm by a Pye Unicam SP 1800 Ultraviolet Spectrophotometer. The absorbance was recorded on a Unicam AR 25 Linear Recorder. The displacement of the gradient was done using an LKB Bromma 12,000 Varioperpex Peristaltic Pump set to speed 4 x 10. The eluant was discarded. A typical absorption profile of polysomes prepared from P1.17 cells, displayed on a 0.5M - 1.25M sucrose gradient, is shown in Fig. 2. P1.17 polysomes and 5563 polysomes gave essentially identical polysome profiles. The absorption profile of a sucrose gradient containing no polysomes is shown as a control. Polysomes are well resolved up to nonamers, i.e. ribosome-messenger RNA complexes containing nine attached ribosomes. This method of sucrose-gradient analysis was used to estimate the integrity of polysomes produced by the extraction procedure described above.

3.5./

Fig. 2 Sucrose gradient analysis of polysomes



Polysomes were prepared as described in Methods3.3 and displayed on sucrose gradients, as described in Method 3.4.

3.5. Polysome integrity.

The degree of degradation occurring during the isolation of polysomes was estimated in the first instance by the appearance of the polysome profiles displayed on sucrose gradients. Polysome preparations showing a low polysome:monosome ratio were judged to have been degraded to a greater or lesser extent (Figure 2.). Undegraded polysome profiles displayed:-

(a) good resolution of individual polysome sizes up to nine or more attached ribosomes

(b) a low monosome:polysome ratio

(c) a high percentage of material with size greater than nonamers

The second criterion used to estimate degradation was the ability of polysomes to stimulate the incorporation of radioactively labelled amino acids into TCA precipitable material in a cell-free protein synthesizing system (i.e. to be translatable). Polysomal material from preparations judged to be degraded on the basis of their appearance on sucrose density gradients invariably gave much lower incorporation of radioactivity per A_{260} unit of polysomes added than polysomes which yielded good polysome profiles.

4. Preparation of total polysomal RNA.

Polysomes, prepared as described above, were treated by a modification of the pH 9.0 phenol extraction method of Lee et al., 1971.

4.1. Solutions.

(a) Phenol Extraction Buffer (P.E.B.): 100mM Tris HCl, pH 9.0; 100mM NaCl, 1mM EDTA, 2% (w/v) SDS.

(b) Phenol: Chloroform: Isoamyl Alcohol (50:50:1 by volume) (P.C.I.)

4.2. Ribonuclease Precautions.

As for polysome isolation (Section 3.1.) where appropriate.

4.3. Extraction of RNA.

Polysomes were resuspended in P.E.B. at a concentration of 10 A_{260} units/ml. To this was added an equal volume of P.C.I., saturated with P.E.B., and the mixture was shaken vigorously at room temperature for 5-10 minutes. The resulting suspension was then centrifuged at 16,000 x g for 10 minutes in 30ml Corex tubes. The P.C.I. phase was removed by aspiration through the aqueous phase and the extraction repeated after addition of a similar volume of P.C.I. to the aqueous phase. After centrifugation as before, the aqueous phase was carefully removed and transferred to fresh tubes and the extraction procedure was repeated until a mirror-like interface between the aqueous and phenol phases was observed after centrifugation.

Subsequently, the aqueous phase was removed, diluted by the addition of 2 volumes of glass fibre-filtered ethanol, and was made to 0.2M in sodium acetate pH 5 by addition of a small volume of concentrated/

concentrated sodium acetate solution. This solution was allowed to stand at -20°C overnight and was then centrifuged at 16,000 x g for 10 min. The supernatant was carefully decanted and the pellet of precipitated RNA was dried with a gentle air current. The pellet of RNA was redissolved in binding buffer for application to oligo-(dT) cellulose columns. Yields of RNA were, in general, 85% - 100% of the polysome detected RNA.

5. Isolation of poly(A)-containing RNA.

Fractionation of total polysomal RNA into poly(A)- and non-poly(A)-containing species was carried out by making use of the selective retention at high salt concentrations of poly(A)-containing species by oligo(dT)-cellulose. This fractionation procedure was a modification of that described by Swan, Aviv and Leder, 1972.

5.1. Solutions.

Three solutions were used, each containing 0.01M Tris pH 7.5, 0.1% (w/v) SDS, 1mM EDTA. 0.1% SDS was present to prevent ribonuclease degradation. In addition, the solutions contained varying concentrations of LiCl as described below.

1. Loading buffer: 0.5M LiCl
2. Intermediate wash buffer: 0.1M LiCl
3. Elution buffer: No LiCl

LiCl was used as the salt in these solutions because of the greater solubility of Lithium dodecyl sulphate at low temperatures.

The/

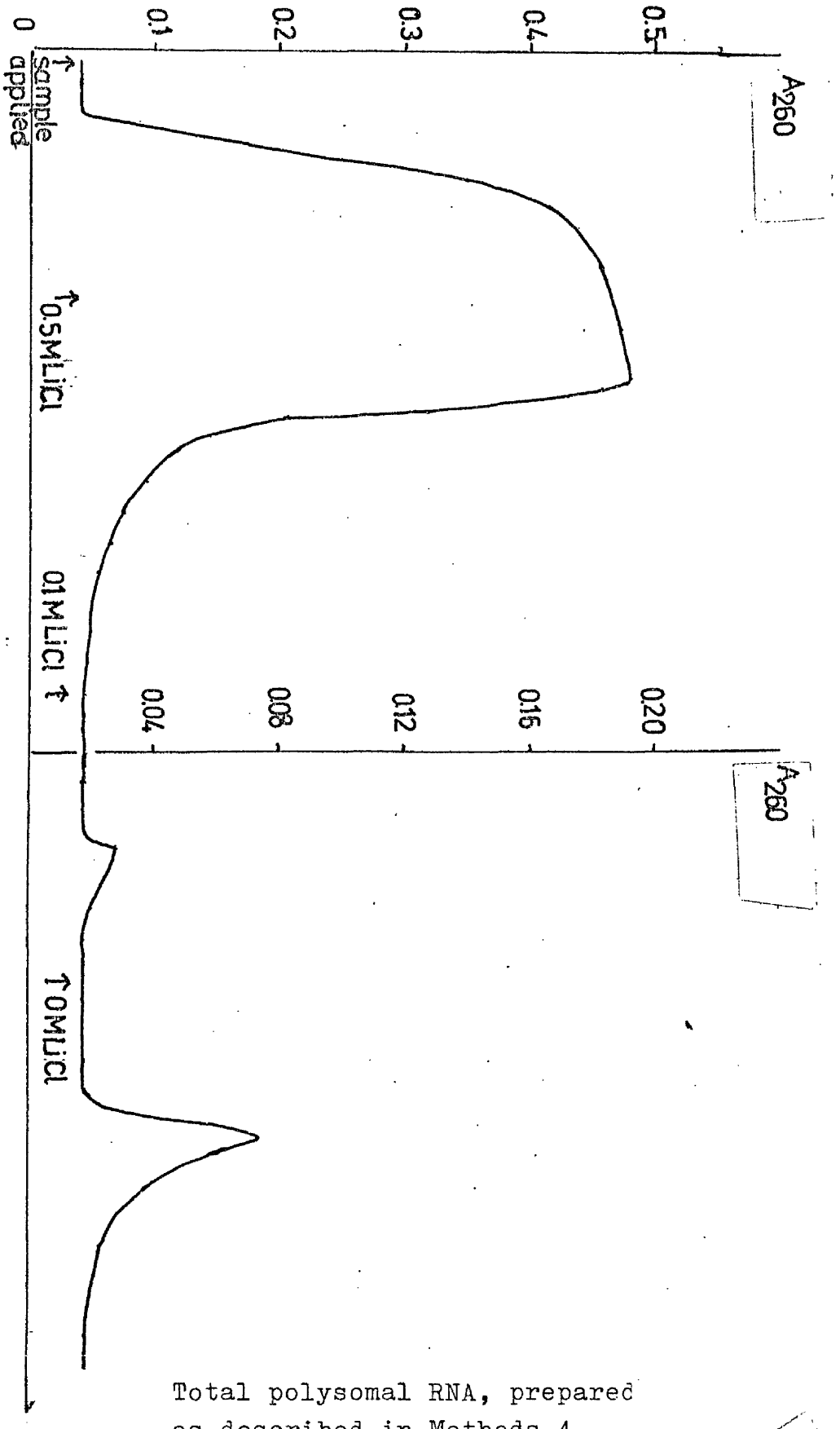
The intermediate wash solution was included in the procedure to decrease the contamination of the poly(A) RNA fraction with ribosomal RNA.

5.2. Procedure.

1g oligo dT cellulose (2.5ml), equilibrated with loading buffer was held in a 10ml column, washed with 2 column volumes of 0.1N NaOH, and re-equilibrated with loading buffer. Polysomal RNA (40-100 A_{260} units) was applied to the column in loading buffer and washed through the column with several volumes of loading buffer. The column was then washed with two volumes of intermediate wash buffer and the majority of poly(A) containing RNA species were eluted with one column volume of eluting buffer. Continuous monitoring of the optical density of the eluted volume (see Fig. 3.) indicated that approximately 3% of material applied to the column was retained and eluted after application of the elution buffer. Estimates based on the optical density of pooled bound and unbound material suggested that between 1% and 4% of total polysomal material adhered to the oligo dT column.

6./

Fig. 3 Elution profile from oligo dT cellulose column.



Total polysomal RNA, prepared as described in Methods 4, was fractionated as described in Methods 5, and the eluant monitored at 280 nm.

6. Rabbit reticulocyte lysate.

6.1. Preparation of rabbit reticulocyte lysate.

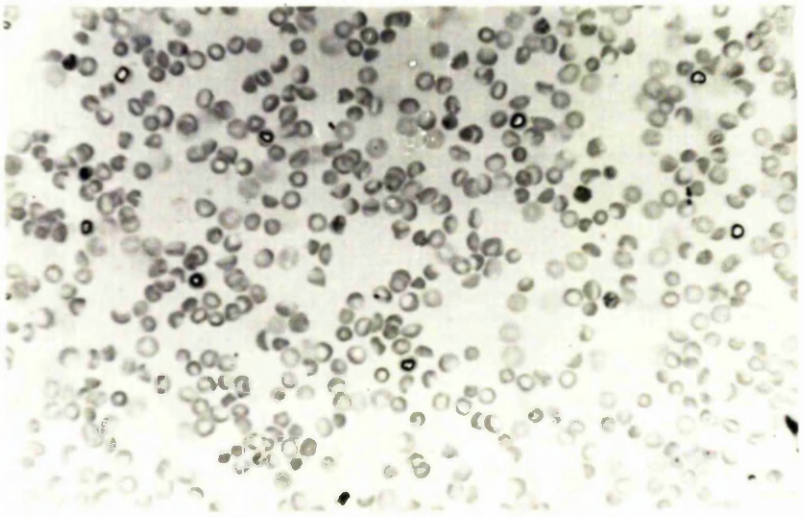
6.1.1. Induction of reticulocytosis.

Rabbit reticulocyte lysate was prepared by a modification of the method of Waxman & Rabinowitz, 1966. New Zealand White rabbits, weighing between two and four kilograms were injected subcutaneously on four successive days with a 2.5% (w/v) solution of phenylhydrazinium chloride, containing 2.5mM reduced glutathione, pH 7.0 at a dosage of 0.4ml solution/kilogram body weight/day. After resting the rabbits for two days, on the seventh day of the régime they were anaesthetized using a solution containing equal volumes of sodium pentobarbitone solution (Nembutal) and 1% (w/v) heparin solution (173 units/mg), by ear vein injection. The rabbits were bled out by cardiac puncture, the blood being collected in cooled, heparinised vessels. Whilst still anaesthetized, the rabbits were surgically opened and the aortas severed in order to ensure death. This procedure generally yielded 80-150ml of blood per rabbit.

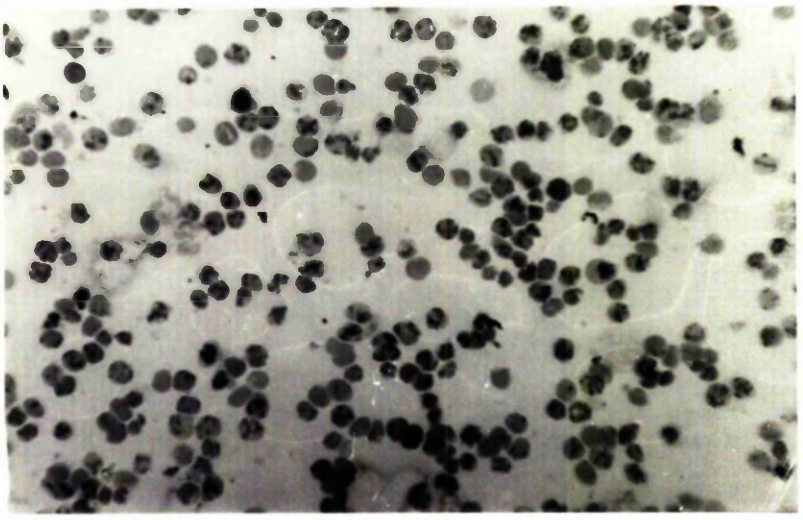
6.1.2. Estimation of the degree of reticulocytosis induced.

The extent of reticulocytosis was estimated using the staining technique developed by Brecher, 1949. A small volume of blood was mixed with an approximately equal volume of an aqueous solution of 0.5% (w/v) New Methylene Blue dye ; 1.6% (w/v) potassium oxalate, and allowed to stand for ten minutes. The mixture was then expelled on to a glass microscope slide, smeared, and dried by a gentle current of air. The smear was examined by microscope at 100X under oil immersion/

FIG. 4. COMPARISON OF NORMAL AND ANAEMIC RABBIT BLOOD.



A: Normal Rabbits.



B: Rabbits with chemically-induced anaemia.

Anaemia was induced as described in Methods6.1.1. The degree of reticulocytosis was determined as described in Methods 6.1.2.

immersion. Reticulocytes stained deep blue and appeared granular whilst erythrocytes stained light green. The degree of reticulocytosis was always greater than 50% and generally greater than 90%. In contrast, normal rabbit blood contains less than 2% reticulocytes (see Fig. 4).

6.1.3. Preparation of lysate.

All subsequent steps were performed at 4°C and with minimum delays. The rabbit blood cells were pelleted by centrifugation at 500-1000 x g. The serum was removed by aspiration and the packed cells resuspended in approximately four volumes of ice cold BSS and pelleted as described above. The supernatant was removed by aspiration and white cells, constituting a "buffy coat" layer above the red blood cells, were also carefully removed by this method. The above step was repeated two times more, after which an equal volume of deionised water was added to the packed cells and the mixture was agitated vigorously before being left on ice for three or four minutes. This step lysed the reticulocytes. The solution was then centrifuged at 16,000 x g for 10 minutes to pellet cellular debris and unbroken cells. The supernate, deep ruby-red in colour, was the final reticulocyte lysate, and was divided into aliquots of 0.8ml and stored at -70°C.

6.1.4./

6.1.4. Preparation of reticulocyte polysomes.

Reticulocyte polysomes were prepared by centrifugation through a sucrose cushion, as follows. 4-5ml of rabbit reticulocyte lysate was supplemented with solutionE(see Section 7.1.1.) to a final concentration of 25μM haemin, 12.5mM KCl, 5mM Tris HCl pH 8.2. The lysate was layered on top of a 20ml cushion of solution C3 (see Section 3.2.) in a 30ml polyallomer centrifuge tube. The remaining volume was filled with solution C (see Section 3.2.) the tube sealed and centrifuged at 60,000 rpm in a Beckman 60 Ti rotor by a Beckman L5-65 centrifuge for 120 min at $4^{o}C$. The supernatant was decanted and the polysomes were resuspended in 0.25M sucrose, 1mM MgOAc, 0.1mM EDTA. Polysome yields were 8 A_{260} units/ml retic.lysate.

6.1.5. Preparation of high salt wash factors.

Polysomes were extracted to yield high salt wash factors as described by Gross, 1977. All steps were carried out at $4^{o}C$. A 6mg/ml suspension of polysomes in the buffer described above was made 0.55M in KCl by the addition, with constant stirring, of small quantities of 1M KCl solution. The final solution was stirred for one hour. The solution was then layered carefully on top of a cushion of solution C3 (see Section 3.2.) in a 10ml polyallomer tube, and centrifuged at 60,000 rpm in a Beckman SW 60 rotor by a Beckman L5-65 ultracentrifuge for 150 minutes, in order to pellet polysomes. The high salt layer was then removed carefully and stored at $-70^{o}C$.

6.2./

6.2. Reticulocyte lysate cell-free protein synthesizing assays.

The system adopted, with some modifications, was that described by Maxwell et al., 1971. This system has been proven to be successful in translating exogenous messenger RNA, notably by Rhoads et al., 1971.

6.2.1. Solutions.

Assays performed using the system consisted of three components.

1. Reticulocyte lysate, prepared as described previously - 30µl per assay
2. RNA or polysomes in aqueous solution, prepared as described elsewhere (Methods 3, 4 and 5) -25µl per assay
3. Supplement, composition described in Table 6 -25µl per assay

6.2.2. Assay conditions.

Assays were carried out in Eppendorf 1.5ml micro test tubes, held in a water bath heated by a Haake E52 Constant Temperature Circulator to 25°C. Normally, assays were incubated for 60 min. The incubations were stopped by cooling rapidly to 4°C and were stored frozen. Measurement of the incorporation of radioactive amino acids into total protein and into specifically precipitable material were carried out following methods described in Sections 8.2. and 11.2.

7./

TABLE 6:

COMPONENTS OF RETICULOCYTE LYSATE SUPPLEMENT

Component	Percentage, by volume, of supplement	Final concentration in assay
33mM ATP, adjusted to pH 7.2 with KOH	10	1mM
6.6mM GTP, adjusted to pH 7.2 with KOH	10	0.2mM
500mM creatine phosphate, adjusted to pH 7.2 with HCl	10	15mM
19 L-(^{1}H) amino acids, each at 1.3mM	10	40µm
3.84M KCl, 102mM MgOAc (added together)	5	60mM; 1.6mM
Creatine kinase (10mg/ml)	25	60µg/assay
83µM L-(^{1}H) leucine	10	2.6µM
L-(^{3}H) leucine (1mCi/ml)	10	3.1µCi
Haemin, 60µg/ml in 5mM Tris HCl pH 7.2	10	36µM

7. The messenger dependent lysate (M.D.L.)

7.1. Preparation of M.D.L.

7.1.1. Standard solutions.

The following standard solutions were used throughout in experiments involving the M.D.L.

Solution A: 2M potassium chloride (KCl): 10mM magnesium chloride ($MgCl_2$)

Solution B: 0.2M creatine phosphate

Solution C: 19 unlabelled amino acids, each at a concentration of 0.8mM. Leucine or methionine was omitted as required.

Solution D: Creatine kinase: 0.5% (w/v) in 50% (v/v) glycerol

Solution E: 1mM haemin: 20mM Tris HCl, pH 8.2: 50mM KCl in 90% (v/v) ethanediol

Solution F: 0.1M calcium chloride ($CaCl_2$)

Solution G: Micrococcal nuclease: 1mg/ml in glass distilled H_2O (specific activity 22000 - 29000 units/mg)

Solution H: 0.5M Ethyleneglycol-bis-(β-aminoethyl ether) N,N' Tetraacetic Acid (EGTA) neutralised to pH 7.4 with KOH.

All solutions were prepared using deionised water, unless otherwise stated. All solutions were stored as small aliquots at -20°C except solutions F and H which were stored at 4°C.

7.1.2./

7.1.2. Preparation of M.D.L.

M.D.L. was prepared following the method of Pelham and Jackson, 1976. Rabbit reticulocyte lysate, prepared as described previously, and stored in 0.8ml aliquots at -70°C, was thawed rapidly to 4°C and the solutions described in the previous section added in the following order and in the volumes shown:-

800 µl rabbit reticulocyte lysate
+ 25 µl Solution E
+ 10 µl Solution D
+ 50 µl Solution A
+ 50 µl Solution B
+ 50 µl Solution C

All additions were made at 4°C.

In solution E, the presence of haemin prevented the formation of a translational inhibitor which forms rapidly in reticulocyte lysates at temperatures above 4°C (Gross and Rabinowitz, 1972). Solution D (creatine kinase) and B (creatine phosphate) provided a source of energy which was non-limiting over the period used for assays whilst Solution C provided non-limiting quantities of those amino acids not radioactively labelled. These amino acids were in addition to the endogenous pools of amino acids found in the reticulocyte lysate. Solution A adjusted the potassium and magnesium concentrations to near the optimum levels for the M.D.L. No ATP or GTP was added because studies by Balkow et al., 1975 had suggested that these triphosphates have mutually antagonistic actions in the rabbit reticulocyte lysate system, when added in large quantities, and that endogenous pools of these compounds are sufficient for normal translation/

translation.

After these additions had been made, 10µl of solution F, providing Ca^{++}, followed by 10µl of solution G, containing calcium-dependent nuclease, were added. The supplemented lysate was shaken vigorously to allow complete mixing, and allowed to incubate for 15 minutes at 20°C. This step, involving a calcium-dependent nuclease, destroyed almost all messenger activity by "nicking" whilst leaving most transfer RNA and ribosomes essentially intact functionally. The nuclease action was stopped by the addition of 5µl of solution H, which chelated calcium ions present in the solution and hence inactivated the calcium-dependent nuclease.

The M.D.L. thus prepared could either be used immediately or stored for several weeks at -70°C without appreciable loss of activity.

7.2. M.D.L. cell-free protein synthesis assays.

Unless otherwise stated, the following conditions were used in cell-free protein synthesizing assays carried out using M.D.L. Assays were performed in Eppendorf 1.5ml micro test tubes. Additions to the system (polysomes, poly(A)(+) RNA, etc.), excluding radioactively labelled amino-acids, were never greater than 20%, and normally 10%, by volume. In assays in which no additions or varying volumes of additions were made to the system (e.g. assays to determine endogeneous incorporation of radioactive amino acids) all assays were adjusted to the same volume with deionised water. Radioactive amino acids were added in a volume of 5µl. The amount of radioactivity added depended on the specific activity of the supplied solutions of amino/

amino acids and, in the case of L-(^{35}S) methionine, on the age of the batch used. In all cases, the number of pmoles of radioactive amino acid added was at least an order of magnitude lower than the endogenous pool size of that amino acid. The volume of M.D.L. normally used in assays was 50µl. This volume could be scaled up or down proportionally if desirable.

Assays were carried out at 30°C in a water bath heated by a Haake E52 Constant Temperature Circulator and the incubation time was either 40 minutes (using leucine as the labelled amino acid) or 120 min (using methionine as the labelled amino acid). Reactions were stopped by chilling the tubes rapidly to 4°C and storing the sample either at 4°C or frozen at -70°C. The former method of storage was used to prevent artefacts of freezing and thawing, such as aggregation of proteins, but led to an apparent oxidation of the M.D.L., as shown by the change in colour of the sample, after periods of two weeks or greater.

8. Detection of Radioactive Incorporation.

Incorporation of radioactively labelled compounds by cells or by cell-free systems was detected by liquid scintillation counting using a Beckman LS-333 Liquid Scintillation Counter.

8.1. General Methods of Preparing samples for Scintillation Spectrometry.

8.1.1. Samples on paper discs.

Samples prepared by precipitation on paper discs were counted in glass or plastic scintillation vials, containing 5ml of 0.4% (w/v)

2,5 diphenyloxazole (PPO) in toluene. Efficiency of counting by this method was measured as ∿9% for (^{3}H)-labelled material and ∿60% for (^{35}S)- and (^{14}C)-labelled material.

8.1.2. Liquid samples.

Liquid samples, such as redissolved immune precipitates or TCA precipitates were adjusted to a volume of 500µl with water and counted in glass or plastic scintillation vials containing 5ml of 0.4% (w/v) PPO in a toluene-triton solution (2:1 by volume). Under these conditions, measurement of the external standard gave a value of 0.318 ± 0.01 (n = 17). With this low but constant value, samples prepared by TCA precipitation and immune precipitation could be directly compared.

8.1.3. Gel slices.

a) Bisacrylamide crosslinked.

Individual gel slices, obtained as described in Section 9.1.5. were placed in plastic scintillation vials, 0.5ml of"Protosol" was added, and the vials were heated at 60°C for 60 minutes to allow digestion to occur. After cooling, 5ml of toluene:PPO solution was added to each vial and the vials examined for radioactivity by liquid scintillation counting.

b) Diallyltartardiamide crosslinked.

Gel slices were digested by addition of 0.3ml 2% periodic acid and incubated for >1 hour at room temperature. 5ml of triton/ toluene/

toluene/PPO solution was added to each vial and the vials examined for radioactivity by liquid scintillation counting.

8.2. Detection of incorporation of radioactivity by reticulocyte lysate assays or M.D.L.s.

8.2.1. Paper disc/toluene/PPO detection method.

For all estimates of incorporation of radioactively labelled amino acids into polypeptides by cell-free systems, where comparison with immunoprecipitates was not carried out, the following protocol was used, adapted from the method of Bollum, 1968.

From each cell-free assay, duplicate 5µl aliquots were spotted on Whatman 3MM 2.5cm filter paper discs and dried at room temperature. The discs were placed in a perforated basket and processed successively through the following solutions:-

1. 10% (w/v) TCA at $4^{\circ}C$ for several minutes to precipitate protein and RNA.
2. 5% (w/v) TCA at room temperature for 10 minutes to wash out excess free labelled amino acid.
3. 5% (w/v) TCA at $100^{\circ}C$ for 10 minutes to discharge amino acids from tRNA.
4. 10% (w/v) TCA: 98% formic acid: 30% (w/v) H_2O_2 (2:1:1) at room temperature for 60 minutes to bleach coloured components.
5. Ethanol:diethyl ether (1:1) at room temperature for 10 minutes followed by
6. Diethyl ether at room temperature for 10 minutes, these last to dry the discs of water.

All/

All solutions, except 5 and 6, contained 10mM unlabelled amino acid. All solutions, except 3, were stirred continuously using a magnetic stirrer. After step 6, the discs were dried under a heat lamp, then placed in glass or plastic scintillation vials, each containing 5ml toluene:PPO and their radioactivity measured by scintillation counting as described in Section 8.1.1. Duplicate samples generally varied by 5% or less. Latterly, duplicate blank paper discs were included in the washing procedure to estimate free radioactive amino acids adhering non-specifically to the discs. Counts detected on these discs were subtracted from the assay disc results.

8.2.2. Triton/toluene/PPO detection method.

This method allowed direct comparison of total TCA precipitable and specifically precipitable material. 5µl aliquots of M.D.L. or reticulocyte lysate were treated with fresh acid-acetone solution (1% (v/v) HCl in acetone), to precipitate and decolourise protein. The precipitated material was washed once with 5% (w/v) TCA, resuspended and boiled for 10 minutes in 5% (w/v) TCA, then washed with ethanol-diethyl ether (1:1 by volume) and ether. The dried precipitate was dissolved in 9M deionised urea and aliquots treated as described in Methods 8.1.2.

8.3. Fluorographic detection of radioactivity (Bonner and Laskey, 1974).

8.3.1. Polyacrylamide gels.

Detection of radioactively labelled proteins in polyacrylamide slab/

slab gels was both awkward and time consuming to perform using the slicing, solubilising and counting methods described for tube gels. Moreover, the advantages of increased resolution and comparability of adjacent gel wells were decreased using these methods. Consequently, the PPO impregnation technique developed by Bonner and Laskey, 1974 was used.

Slab gels (approximately 40mls liquid volume) were soaked with gentle agitation in two successive 500ml volumes of dimethyl sulphoxide (DMSO) for 1 hour each. This replaced water present in the gel with DMSO, in which PPO is soluble. This differed slightly from the method of Bonner and Laskey in that the ratio of DMSO volume to gel volume is lower but the washing time is longer to compensate. The gel was then immersed in four volumes of a 20% (w/w) solution of PPO in DMSO and gently agitated for 1 hour. This step is shorter than in the published method since experience showed a longer time to be unnecessary and long washing times increased diffusion of proteins. To precipitate PPO within the gel, the last solution was decanted and the gel flushed continuously for 1 hour with H_2O. Gels were dried on to Whatman 3MM paper under vacuum.

Fluorography was carried out using Kodak RP Royal "X-Omat" or, where stated, Kodak Xomatic H Medical X-ray film. The gel was held in contact with the X-ray film between two glass plates. The gel, film and plates were placed in a light-safe bag and stored at $-70^{o}C$. When exposed for the required period they were developed by standard methods.

8.3.2. Thin layer chromatographic plates.

After separation of labelled species on thin layer chromatographic plates (either polyamide sheets for dansylation experiments or silica gel sheets for tryptic mapping), PPO was introduced into the sheets as follows. Ascending chromatography was performed in the same direction as used for the second dimension separations performed previously on the sheets, using a solution of 7% (w/v) PPO in acetone or ether. The sheets were then dried at room temperature and fluorography carried out as described for dried polyacrylamide gels.

8.3.3. Quantitative analysis of Fluorographs.

Fluorographs of PAG's were quantitatively analysed by densitometry using a Joyce-Loebel scanning densitometer.

9. S.D.S. polyacrylamide gel electrophoresis (PAGE).

9.1. Polyacrylamide tube gels.

The composition of polyacrylamide tube gels and buffers was that described by Summers et al., 1965 with some slight modifications.

9.1.1. Standard Solutions.

Components are shown with their final gel concentrations in parentheses, where appropriate.

a) Solution A: 0.334M (0.1M) sodium phosphate buffer pH 7.2:

0.166/

0.166% (v/v) (0.05% v/v) N N N'N' Tetramethylethylene diamine (TEMED): 0.33% (w/v) (0.1% (w/v)) SDS: 6M (6M) urea.

b) Solution B: 16.6% (w/v) acrylamide: 0.45% (w/v) NN' methylene bisacrylamide: 6M urea.

These solutions were filtered, stored in brown bottles at 4°C and used within two months. Various proportions of these solutions were mixed with fresh 6M urea as described below to produce gels containing different acrylamide concentrations.

% acrylamide in gel	Solution A	Solution B	6M urea
5	3	3	4
6.6	3	4	3
7.5	3	4.5	2.5
10	3	6	1.0

Alternatively, in some experiments, diallyltartardiamide was used as crosslinking agent instead of bisacrylamide. Polymerisation was catalysed by the addition to the gel solution of 0.2% (w/v) ammonium persulphate.

c) Running buffer

0.1M sodium phosphate buffer pH 7.2 containing 0.1% (w/v) SDS.

9.1.2. Pouring gels.

Polyacrylamide tube gels were formed in 7.5cm gel tubes (0.7cm internal diameter). The bottom of the tube was sealed with Parafilm, the gel solution carefully added to about 0.5cm from the top of the tube and a small volume of deionised water carefully layered on top of the gel solution to provide a flat surface to the gel and to exclude/

exclude oxygen. After polymerisation, the parafilm was removed and the water decanted. The gels were placed in a standard tube gel electrophoresis apparatus and the lower and upper chambers filled with running buffer.

9.1.3. Sample preparation.

Samples for electrophoresis were dissolved in 2% SDS, reduced at 37°C for 30 min by addition of Dithiothreitol (DTT) to 50mM, and alkylated at room temperature for 30 min by addition of iodoacetamide to 120mM. After boiling, the samples were mixed with a small volume of bromophenol blue dye solution in 50% (v/v) glycerol and carefully layered on top of the gel.

9.1.4. Electrophoresis conditions.

Tube gels were electrophoresed at 7.5mA/gel until bands formed by the bromophenol blue tracking dye had reached within 1cm of the end of the gel. Gels were removed from their tubes and either sliced immediately or stored frozen at -70°C, until required.

9.1.5. Gel slicing.

Gels were sliced by pressing through an array of 100 parallel razor blades arranged 1mm apart. The gels were best sliced whilst semi-frozen. Individual slices were then removed from the slicer and placed in glass or plastic scintillation vials.

9.2/

9.2. Polyacrylamide slab gels.

The composition of polyacrylamide slab gels and buffers was that described by Laemmli, 1970 for tube gels, with some slight modifications.

9.2.1. Standard solutions.

a) Separating gel solutions.

Solution 1: 30% (w/v) acrylamide: 0.8% (w/v) NN'methylene bisacrylamide. Before use, the acrylamide was recrystallised from chloroform in order to remove impurities. After making solution 1, it was stirred slowly for one hour with 10% (w/v) "Amberlite" resin to deionise the solution, followed by filtration through Whatman No. 1 filter paper to remove the resin. The deionised solution was stored at $4^{\circ}C$ in a brown, stoppered glass bottle for no longer than two months.

Solution 2: 1.5M Tris HCl, pH 8.8: 0.13% (v/v) NNN'N' Tetramethylethylene diamine (TEMED). This solution was stored at $4^{\circ}C$. The TEMED concentration is 30% higher than that described by Laemmli. This ensured more rapid polymerisation of the gel.

Solution 3: 0.4% (w/v) sodium dodecyl sulphate (SDS). This solution was stored at $4^{\circ}C$.

Solution 4: 15-20mg ammonium persulphate, per 10ml of solution 3. This solution was made immediately before use and was not stored.

b) Stacking gel solutions.

Solution 5: as solution 1 above

Solution 6: 0.65M Tris pH 6.8 (adjusted with concentrated HCl)

Solution/

Solution 7: 10% (w/v) SDS.

c) Other solutions.

Solution 8: Reservoir buffer: 0.125M Tris - 0.96M glycine pH 8.8; 5% (w/v) SDS. This solution was a five times concentrated stock solution. It was stored at room temperature and diluted immediately before use.

Solution 9: Sample buffer: 0.08M Tris HCl, pH 6.8; 2% (w/v) SDS; 0.1M DL-Dithiothreitol (DTT); 10% (v/v) glycerol; 0.001% (w/v) Bromophenol Blue dye. This solution was made in 50ml quantities and stored at -20°C, frozen as small aliquots. This solution differed from that of Laemmli in two respects. The molarity of Tris in the solution was 20% higher which improved buffering capacity. The reducing agent used in the solution was DTT instead of 2-mercaptoethanol; DTT was a more stable reducing agent.

9.2.2. Preparation of gel solutions.

a) Separating gel.

Shown below are the proportions of Solutions 1,2 and 4 required to make gels containing various percentages of acrylamide.

	Percentage acrylamide					
Solution	3%	5%	7.5%	10%	12.5%	15%
1. (acrylamide)	0.4	0.66	1.0	1.33	1.66	2.0
2. (Tris-TEMED)	1.0	1.0	1.0	1.0	1.0	1.0
4. (SDS-persulphate)	1.0	1.0	1.0	1.0	1.0	1.0
H_2O	1.6	1.33	1.0	0.66	0.33	0

All/

All solutions were at room temperature when mixed. Solution 4 was added last and the complete gel solution was de-aerated for approximately 60s in a stoppered side-arm flask attached to a water pump. The solution was immediately poured into the gel apparatus and kept oxygen-free as described below (Section 9.3.4.) until the solution had polymerised.

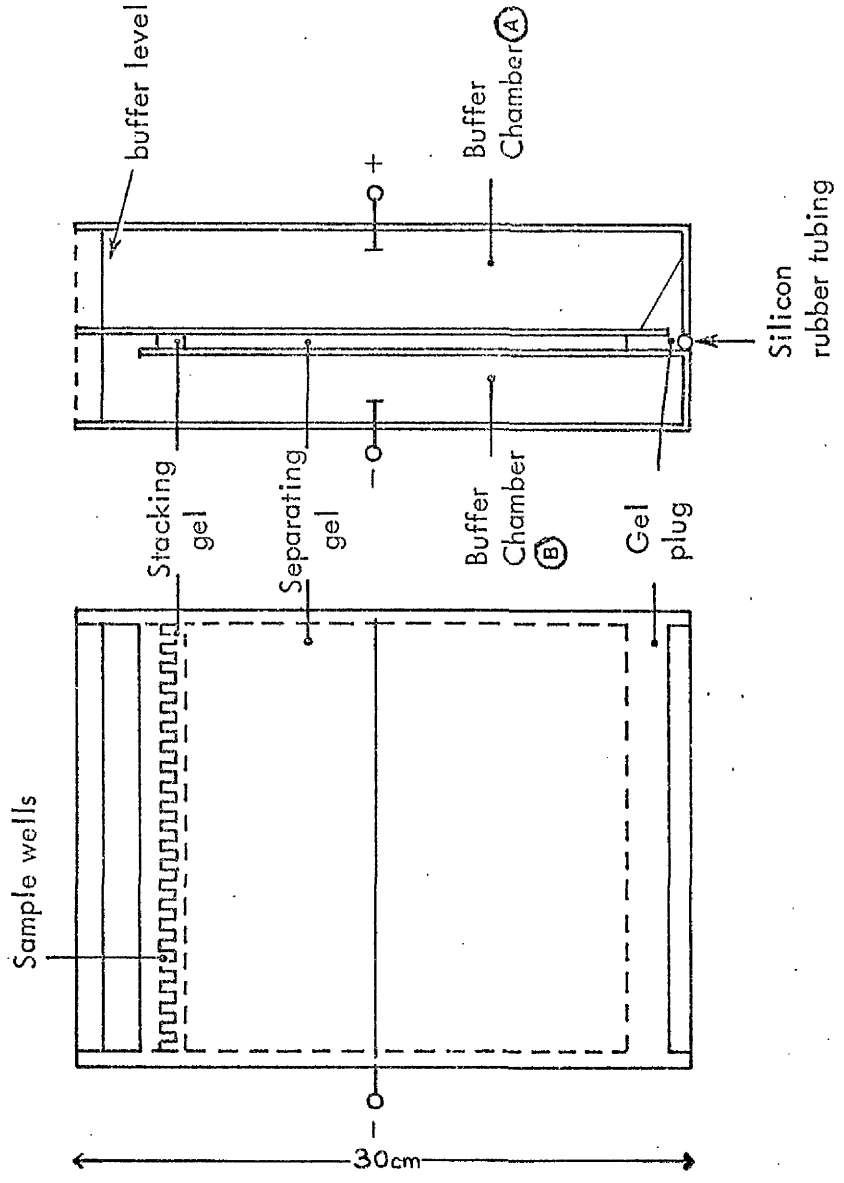
b) Stacking gel.

Stacking gel (6% acrylamide) was prepared by mixing solutions 5,6 and 7 with H_2O in the proportions 8:4:1:69. To this solution was added TEMED to 0.1% (w/v) and ammonium persulphate to 0.1% - 0.2% (w/v). After agitation to dissolve the ammonium persulphate, the gel solution was poured into the apparatus as described below (Section 9.2.4.) and polymerised in the presence of a "Teflon" comb to produce wells for loading samples.

9.2.3. Polyacrylamide slab gel apparatus.

The apparatus used for running of slab gels was designed by Dr. T.R. Mosmann with whose permission this description is given. The apparatus (Fig. 5.) consisted of two plastic reservoir chambers A & B, separated by silicon rubber tubing held compressed in place by Bulldog clips. The first chamber (A) had a slit along its inner side i.e. that side held clamped to the other chamber. This slit allowed the passage of current from a platinum electrode in the chamber into the gel. The second chamber (B) had a cut-away section at the top of its inner side providing access to the gel for current from a platinum electrode in this chamber. Thus a free path for current existed/

Fig.5: Polyacrylamide slab gel apparatus.



existed from the electrode in one chamber via the gel to the electrode in the other chamber. The silicon rubber tubing when compressed provided an enclosed space 1.5mm wide between the two reservoir chambers in which to pour the gel and also acted as an effective seal against leakage of the gel solution from between the two plates.

9.2.4. Pouring the gel.

The polyacrylamide slab gel was poured in several stages as follows:

a) The gel apparatus was set up with the silicon tubing in place. The space enclosed by the silicon tubing was open at the top at the cutaway section of the reservoir chamber, B, and included the slot at the bottom of the other reservoir chamber, A.

b) The apparatus was tilted at approximately 30 degrees to the vertical with reservoir chamber A uppermost and about 40ml of separating gel solution was poured into chamber A. This formed a triangular wedge of liquid over the slot in chamber A, and a small volume of liquid was drawn up by capillary action into the gel space. The solution was kept deoxygenated by continuously flushing chamber A with nitrogen until polymerisation had occurred.

c) The apparatus was returned to the vertical and, via the cutaway section of chamber B, approximately 90ml of separating gel solution was poured into the gel space, taking care to avoid trapping bubbles of air in the solution between the plates. This solution (80-100ml) reached to about 2 or 3cm below the cutaway section of chamber B. On top of this solution was inserted the reverse, flat/

flat edge of the "comb" later used for forming wells in the stacking gel (see Fig. 5.) in order to provide a smooth upper edge to this gel solution and also in order to prevent contact with the atmosphere. After polymerisation, this comb was removed and the top of the gel flushed several times with reservoir buffer to remove unpolymerised gel solution.

d) About 20ml of stacking gel solution was made and some of this solution used to fill the gap between the top of the separating gel and the bottom edge of the cutaway section of chamber B. At this point, the "comb" was inserted with serrated edge downwards, taking care to avoid trapping air bubbles in the spaces between the "teeth" of the "comb". Polymerisation was allowed to occur and the "comb" removed. The spaces left were flushed several times with reservoir buffer to remove unpolymerised gel solution.

e) Both chambers were then filled with 1X reservoir buffer, taking care not to displace the gel wedge in chamber A. The gel apparatus was then ready for loading of samples and electrophoresis.

9.2.5. Preparation of samples.

Solution 9, the sample buffer was mixed with samples to be electrophoresed in a ratio of 9:1. Samples were in several forms. M.D.L. assays were either mixed untreated with sample buffer, or after initial precipitation with 5% (w/v) trichloroacetic acid (TCA). Immuno-precipitates were normally re-dissolved in 9M deionised urea before mixing with sample buffer.

After mixing samples with sample buffer, the resulting solutions were boiled for 1-2 minutes in order to completely dissolve and/

and dissociate the proteins. Even this procedure had difficulty in dissociating immune complexes. This often led to aggregated material remaining in sample wells and not entering the gel.

Final volumes of sample loaded were 30-50µl depending on the capacity of the wells produced by the "comb". The samples taken up into a length of thin nylon tubing (Portex) attached to a Finn pipette with tip, adjusted to the correct volume. Samples, expelled slowly at the bottom of the well, formed sharp well-defined layers due to the presence of glycerol in the sample buffer. The tubing was then carefully removed. In those wells in which no sample was being loaded, a volume of sample buffer, equal to the volume of the other samples, was added.

9.2.6. Electrophoresis conditions.

After loading all samples into their wells the gel and apparatus were placed in a 4°C cold-room and attached to a Shandon VOKAM SAE 2761 Power Pack. The samples were electrophoresed at a constant current of 15mA, generally overnight. The electrophoresis was carried out at 4°C to minimise overheating problems in the gel. The heat produced was nevertheless sufficient to prevent the SDS in the reservoir buffer from precipitating from solution.

9.2.7. Elution of polypeptides from polyacrylamide gels.

Fluorographs of dried, PPO-impregnated gels were matched with the appropriate gels and radioactively labelled bands of interest were marked/

marked out on the gels. Such areas of the gel were then carefully excised, the backing paper removed and were chopped into small pieces. These pieces were then resuspended in 1 ml 0.1% (w/v) SDS, 0.05M Tris, pH 8.2, 0.01M DTT and incubated overnight at 37°C. The gel pieces swelled up and absorbed some of the buffer. The remaining buffer was removed and lyophilised. A small aliquot was set aside for estimation of the radioactivity released and was analysed in triton/toluene/PPO by scintillation counting as described in Section 8.1.2.

10. Preparation and use of dextran gel (Sephadex) columns.

10.1. Materials.

The following grades of Sephadex beads were used in this project:- G-10 fine, G-25 fine, G-25 coarse, G-100. Both home-made and commercially manufactured columns were used. Commercially produced columns were supplied by Pharmacia (G.B.) Ltd. Where necessary and applicable, scrupulous ribonuclease precautions were employed, as described in Section 3.1. Otherwise columns and attachments were thoroughly washed with deionised water before use.

10.2. Preparation and pouring of gels.

For each grade of Sephadex used, the beads were swollen for the period recommended by the manufacturer either at room temperature or heated in a boiling water bath. The beads were swollen/

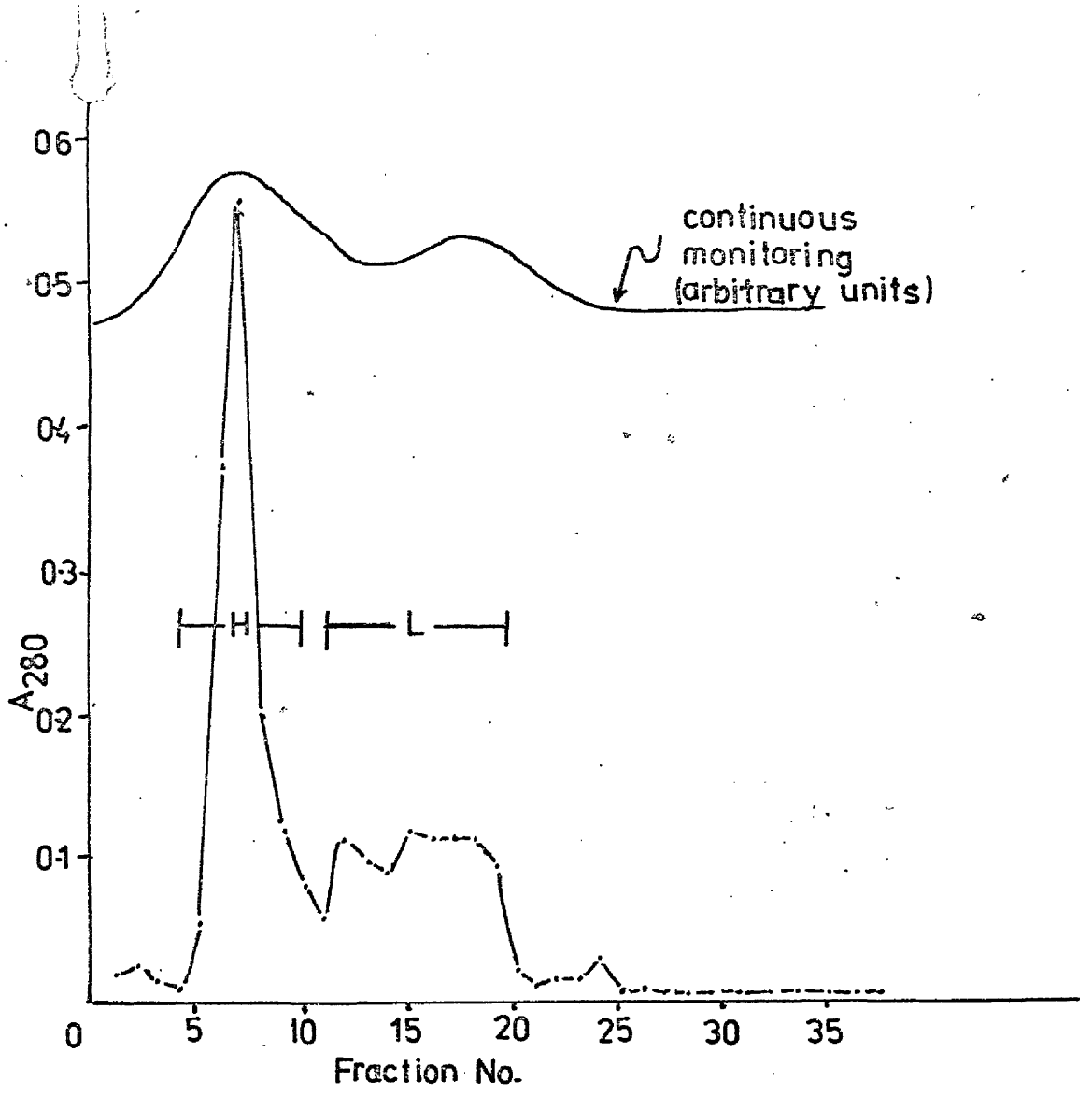
swollen in deionised water. After swelling, the gel slurry was carefully poured into the column, so as to avoid trapping bubbles, and the gel allowed to settle. The gel was then equilibrated with the buffer required by passing several column volumes of buffer through the gel. The columns were then used immediately. Gravity flow was used with pressure heads recommended by the manufacturers.

10.3. Separation of Ig H and L chains on G-100 Sephadex.

500ml G-100 Sephadex, prepared as described in Section 10.2, was equilibrated with 6M deionised urea in 20% neutralised formate buffer (1N formic acid + 0.2N NaOH) under a constant pressure head of 15cm. Reduced and alkylated Ig in urea/formate buffer was loaded in the column by gravity feed and washed through the column with urea/formate buffer under a constant pressure head of 15cm. Eluant was monitored continuously at 280nm by an LKB Spectrophotometric scanner/recorder and collected in 3ml fractions. A typical eluant profile is shown in Fig. 6. The first peak to elute was H chain, and the second L chain. Appropriate fractions were pooled and protein precipitated, when required, by acetone precipitation. Recovery of A_{280} absorbing material varied between 60 and 90% of loaded absorbance units. Radioactively labelled Ig was added in the presence of excess unlabelled Ig. This unlabelled Ig was normally the immune complex by which the radioactively labelled Ig had been isolated. Recoveries of radioactively labelled Ig (by cpm) were generally lower than recoveries of A_{280} absorbing/

FIG. 6: Separation of Ig H and L chains by Sephadex G-100 chromatography.

Ig was reduced and alkylated, as described in Methods 9.1.3., then loaded on to a column of G-100 Sephadex, pre-equilibrated with 6M urea/20% neutralised formate buffer (Methods 10.3.). The sample was loaded in this buffer. Eluant was continuously monitored for absorbance at 280nm, and collected in 3ml fractions. The fractions were independently monitored at 280nm. H and L chain fractions were taken as indicated and pooled.


0·6
0·5
0·4
0·3
0·2
0·1
A_{280}
continuous
monitoring
(arbitrary units)
H
L
0
5
10
15
20
25
30
35
Fraction No.

absorbing material (30 - 50% cpm loaded).

Small aliquots of each eluant fraction were dissolved in triton/toluene/PPO as described in Section 8.1.2. and radioactivity measured by scintillation spectrometry.

10.4. G-10, G-25 Sephadex.

Use of these gels will be described in the appropriate section of Results. (Results, Section 15).

11. Immune precipitation techniques.

11.1. Antisera.

a) Antisera directed against mouse immunoglobulin.

Antisera directed against murine myeloma Ig species were raised in rabbits by standard methods. Antisera were stored sterile at 4°C and centrifuged before use to remove aggregated material. Antisera against 5563 Ig and P1 Ig were prepared in this way.

b) Antiserum directed against rabbit immunoglobulin.

Antiserum directed against rabbit immunoglobulin was raised in goats by standard methods. Antiserum was stored sterile at 4°C and centrifuged before use to remove aggregated material.

c) Normal rabbit serum (NRS).

Normal rabbit serum was obtained from whole rabbit blood, after removal of blood cells and fibrinogen by clotting. It was stored sterile at 4°C and centrifuged before use to remove aggregated material.

d) /

d) Other antisera.

Antisera raised against SAMM 368 H (α) chain, H (γ) chain and L (κ) chain were kindly donated by Dr. T.R. Mosmann.

11.2. Precipitation techniques.

11.2.1.Direct.

Techniques used here were adapted from those of Rhoads et al., 1973. The sample which contained immunoprecipitable material was diluted fifty-fold in PBSA containing Triton X-100 (1%(v/v)) and NaDOC (1%(w/v)). 5µl of rabbit anti-mouse antiserum (or an equal volume of normal rabbit serum) was added to the solution and interaction allowed to occur at 37°C for 30 minutes, followed by incubation overnight at 4°C. The solution was then layered over discontinuous layers of 0.5M sucrose and 1M sucrose in PBSA/Triton/DOC in an Eppendorf micro test tube. The tube was centrifuged at 16,000 x g at 4°C for ten minutes in a Sorvall HB-4 rotor. Immune complexes were centrifuged to the bottom of the 1M sucrose layer. The entire tube was frozen at -70°C and the tip of the tube, containing the immune complexes, was cut off using a hot scalpel blade. The tip was treated in one of two ways:-

(i) The tip was washed with several volumes of ice cold PBSA to remove the immune complex pellet followed by centrifugation of the PBSA to re-pellet the material.

(ii) Alternatively, the tip was washed with a small volume (50µl) of deionised 9M urea to dissolve pelleted immune complexes and/

6 μCi / μl. 1300 Ci / mmole.

420 000 cpm. 1 μCi 2.22 x 10^6 dpm

10^6 dpm.

$$\frac{0.5\ \mu Ci}{1300 \times 10^6\ \text{mmoles}}$$

4×10^{-10} mmoles.

4×10^{-13} moles.

0.4 pmoles / 30 μl.

1.2 pmoles / ml.

500ci/mi.

5.27 x 10^6

1 x 10^6

40,000 x 6 = 240,000

5

1200,000

1.2×10^6 = .5 μCi

and the urea solution was transferred to a fresh Eppendorf tube.

This method involved fewer losses and was used latterly.

11.2.2. Indirect.

The sample was diluted fifty-fold as described for direct immune precipitation and 5µl of rabbit anti-mouse antiserum was added. Reaction was allowed to proceed at room temperature for 30 minutes. A volume of goat anti-rabbit Ig sufficient to precipitate all rabbit-mouse Ig complexes was added (100 - 140µl) and incubation continued overnight at $4^{o}C$. The visible immune precipitates thus formed were treated in either of two ways:-

(i) as described for direct immune precipitation above

(ii) The assays were centrifuged to pellet the immune precipitates. Supernatant was removed by aspiration and the pelleted material disrupted. 1 ml of ice-cold PBSA (containing unlabelled amino acid where necessary) was added, the pellet resuspended and centrifuged once more to produce a pellet. The supernatant was removed by aspiration and this washing procedure repeated two times more. The final washed pellet was dissolved in 0.1M NaOH, if optical density measurements were required, or in deionised 9M urea for all other purposes.

The rationale for the various volumes of antiserum, washings, etc. described above is described in Results.

12. N-terminal amino group analysis of proteins/

12. N-terminal amino group analysis of proteins.

12.1. Dansylation procedure.

Determination of the N-terminal residue of proteins was carried out following the procedure of Gray, 1972. 50 - 250µg acetone- or TCA-precipitated proteins were dissolved, with boiling, in 50µl of 1% (w/v) SDS in a small glass test tube. To this solution was added 50µl of redistilled N-ethylmorpholine, followed by 75µl of a freshly prepared solution of 1-dimethylaminonapthalene-5-sulphonyl chloride (dansyl chloride) (25mg/ml) in dimethyl formamide. The reaction was allowed to proceed at room temperature for one hour, or until the characteristic yellow colour of dansyl chloride had disappeared. The protein was then precipitated by the addition of 0.5ml of acetone, pelleted by centrifugation and dried. 6N HCl (ARISTAR) containing 1mM DTT was used to redissolve the protein and the tube was then evacuated and sealed. Hydrolysis of peptide bonds was promoted by incubation for 12 hours at 105°C. Thereafter, the tube was opened and the HCl driven off under vacuum against sodium hydroxide pellets. The residue was redissolved in a small volume of ethanol (10 - 20µl) in preparation for analysis on polyamide layer sheets.

12.2. Chromatography of dansylated amino acids on polyamide layer sheets.

Separation of dansylated amino acids was carried out by chromatography in two dimensions on polyamide layer sheets after the method described by Woods and Wang, (1967), with some slight modifications, as described below. 0.5µl of a mixture of dansylated amino acids in ethanol was applied to one side of a 10cm x 10cm polyamide sheet in

a/

a compact spot (approx.1mm in diameter) about 1cm from each edge. To the reverse side, and at the same position as the spot containing marker amino acids, approximately 5µl of dansylated protein hydrolysate in ethanol was applied in a compact spot. Samples were applied with thin glass tubing drawn out, by heating, to capillary size. Chromatography in the first dimension was carried out using 2% (v/v) formic acid as solvent. After allowing the solvent front to reach 0.5cm below the upper edge of the sheet, the sheet was thoroughly dried at room temperature, then chromatographed in the second dimension using toluene:glacial acetic acid (9:1 v/v) as solvent. Toluene was preferred to benzene because of benzene's known carcinogenic properties. No significant difference in mobility was observed between samples run with the two solvents.

The solvent front was allowed to reach 0.5cm below the upper edge of the sheet, then the sheet was dried in a fume hood, using a current of air, and examined under ultraviolet light. Dansylated amino acids could be detected by their characteristic fluorescence and dansylated residues from the protein sample could be identified by comparison of their mobilities with the marker dansylated amino acids on the other side of the polyamide layer sheet. Radioactively labelled residues were detected as described in Section 8.3.2.

12.3. Non-N-terminal dansylated derivatives.

Three major types of fluorescent spot not resulting from N-terminal dansylation would be detected even with proteins known

to/

to contain blocked N-terminal residues.

1. Dansyl OH. This was the major breakdown product from the decay of dansyl chloride and whilst much of it was removed at the acetone precipitation stage, a certain amount was carried over to the final separation on polyamide sheets. It separated as an extended spot with a mobility of ∿ 0.6 in the first dimension solvent and with little or no mobility in the second dimension solvent. It could easily be distinguished since it characteristically fluoresced with a light blue colour, whereas dansylated amino acids fluoresced light green.

2. Dansyl chloride reacts not only with free N-terminal NH_2 groups but also to a greater or lesser extent with many of the amino acid side chains. The most reactive side groups are the ε amino group of lysine and the ionised phenolic group of tyrosine. Both these side chains are dansylated, if accessible, under the same conditions used for N-terminal dansylation and appear generally as much stronger fluorescing spots under ultraviolet light than the N-terminal dansylated amino acid due to the relatively high molar ratio of lysine and tyrosine residues to N-terminal amino acids in most proteins. Such side chain dansylated amino acids had characteristic mobilities quite different from the same amino acids dansylated at a free N-terminus.

13. Proteolytic cleavage by trypsin.

13.1. Conditions for proteolysis.

Peptide mapping of Ig H and L chains and their putative precursors/

precursors was carried out using TPCK trypsin as cleavage enzyme. Standard techniques were employed. 50 - 500µg of acetone or TCA-precipitated protein (including the appropriate carrier when dealing with small amounts of radioactively labelled protein eluted from gels) were redissolved or at least finely resuspended in 200µl 0.5% (w/v) ammonium bicarbonate solution. To this was added sufficient of a 1mg/ml solution of trypsin in 1mM HCl to give an enzyme:substrate ratio of 1:20. The digestion sample was incubated at 37°C for 4 hours, whereupon a further equal quantity of trypsin was added and the digestion continued at 37°C for 12 hours. The digested sample was then lyophilised and the residue redissolved in a small volume (∿20µl) of electrophoresis buffer, prior to separation of peptides as described below.

13.2. Separation of proteolytically generated peptides.

Peptides generated by trypsin cleavage were separated by a combination of high voltage electrophoresis, followed by ascending chromatography, on silica gel plates (Camlab Ltd., Cambridge).

Lyophilised tryptic cleavage products were re-dissolved in 0.5% (v/v) pyridine, 5% (v/v) glacial acetic acid in a volume sufficient to give a concentration of 100µg/5µl. 5µl of this solution was applied to a silica gel sheet approximately 2cm from each edge. The sample was applied in small amounts and dried after each addition. 0.5µl methyl green tracking dye was applied close to one edge of the sheet. The sheet was then wetted carefully with electrophoresis buffer (0.5% (v/v) pyridine, 5% (v/v) glacial/

glacial acetic acid, as above) as follows. Two sheets of Whatman 3MM paper were laid in parallel on the sheet, one on each side of the spots, with a 1cm gap between their edges and the sample and tracking gels. Electrophoresis buffer was then evenly applied across the paper and allowed to permeate the silica gel plate, approaching the spots from either side with the two solvent fronts finally flowing together at the positions of the sample and tracking spots. The silica gel plate was then placed in a high voltage electrophoresis tank and electrophoresed at 20mA for 5 hours. Cotton gauze, soaked on electrophoresis buffer provided electrical contact between the plate and the buffer reservoirs. After electrophoresis, the plate was thoroughly dried and chromatographed at right angles to the direction of electrophoresis using butanol: acetic acid:water:pyridine (15:3:12:10), until the solvent front had reached within 0.5cm of the upper edge of the plate (approximately 2 hours). The sheet was thoroughly dried and stained for peptides or further treated for fluorography, both described below.

13.3. Detection of peptides.

13.3.1. Ninhydrin - cadmium acetate.

a) Standard solutions.

Solution A: 1% (v/v) indanetrione hydrate (ninhydrin) in acetone

Solution B: 1% (w/v) cadmium acetate in 30% (v/v) glacial acetic acid.

b) Detection/

b) Detection methods.

15ml of solution B and 100ml solution A were mixed immediately prior to use and the resulting solution was sprayed lightly and evenly over the sheet. The sprayed sheet was heated in an oven at 100°C for a few minutes during which time the N-terminal groups of the peptides react to impart colours varying from yellow to red to the peptide spots. The sheet was examined and the spots marked on the reverse side.

13.3.2. Fluorographic detection of radioactivity on cellulose sheets.

Radioactively labelled peptides were detected by application of techniques described in Section 8.3.2. After fluorography, PPO impregnated cellulose sheets could be treated with ninhydrin -cadmium acetate as described above to visualise all tryptic products.

RESULTS

1. Polysome yields using the method of Bennett et al.

Extractions of polysomes from myeloma cells were made as required according to the procedures described (Methods Section 3). The yields of these preparations are shown on Table 7. Polysome yields varied from extraction to extraction but several general points were observed.

a) The 260:280nm ratio of various polysome preparations was found to have an average value of 1.61 ± 0.126(n=21). Variation in this value can be explained by the degree of contamination of polysome pellets by protein, DNA, etc.

b) The yield of polysomes extracted varied widely and had an average of 57.8 ± 23.2(n = 10) A_{260} units/10^9 cells. Variations depended on the state of the cells, and of the thoroughness of the extraction procedure. Yields by other workers are as follows:-

	Tissue	A_{260}/g wet wt*	260/280
Palmiter, 1974	Slime mold	18.2	1.69
	Silk moth	30	1.71
	Chicken Brain	33.5	1.55
	Chicken Kidney	86	1.78
	Chicken Liver	191	1.78
	Chicken Oviduct	111	1.73
	Rabbit reticulocytes	89	1.90
Schechter, 1974	Murine myeloma cells	170-180	1.69

* 1g wet weight of tissue ≡ 10^9 cells

Hence/

TABLE 7: Polysome yields using the method of Bennett et al.

Date	Cell line	No. of cells	Polysome yield (A_{260} units)	$A_{260}:A_{280}$	A_{260} units/ 10^9 cells
10/2/76	P1	n.d.	I 57 II 27	1.676 n.d.	?
6/4/76	P1	1.2×10^9	I 72 II 43	1.64 1.74	96
31/3/76	5563	1.2×10^9	I 20 II 20	n.d. n.d.	33.3
26/5/76	5563	2.7×10^9	I 110 II 65.5	1.47 1.64	65
	P1	2.3×10^9	I 43 II 94.5	1.72 1.43	60
2/9/76	P1	4×10^9	I 182 II 140	1.46 1.555	80
	5563	6×10^9	I 300 II 174	1.64 1.63	80
27/1/77	P1	4.2×10^9	I 94 II 25	1.51 1.66	28
	5563	1.2×10^9	I 38 II 31	1.49 1.55	60
12/2/77	P1	2.4×10^9	I 42.5 II 33	1.33 1.78	31.5
	5563	1.5×10^9	I 30 II 34	1.80 1.62	45

Legend: Polysomes were prepared as described in Methods Section 3. I and II refer to first and second extractions as described in Methods 3.3. Yields of A_{260} units/10^9 cells are based on the total A_{260} units of polysomes extracted.

Hence the yields obtained by this method are within the range obtained by other workers using similar extraction methods in a range of tissues. The average yield, however, is rather low compared to that found from the myeloma cell line used by Schechter (1974). This may be due to a difference in synthetic capacity between Schechter's myeloma cells and those used in this project. More likely, in view of the variability in the polysome yields between different experiments in this work, it may be due to insufficiently vigorous attempts to extract the polysomes from the cells.

2. Translation of myeloma polysomes in the reticulocyte lysate.

Initial studies on cell-free translation using the reticulocyte lysate system were carried out. Pl.17 polysomes, isolated as described in Methods Section 3, were initially donated by Dr. L. Fitzmaurice and Mrs. J. Bennett, although latterly, Pl.17 polysomes were prepared by the author. Several preparations of Pl polysomes were investigated for their ability to stimulate incorporation of radioactivity into antibody-precipitable material. Data obtained from these assays is collected in Table 8. Some of the polysomes used had been isolated by specific precipitation from a total myeloma polysome mixture. This was done using antibodies directed against the nascent myeloma protein H and L chains present on the polysomes after the method of Schechter, 1974. Those polysomes remaining after such precipitation were also tested for activity. Several general observations were made.

Specifically precipitable radioactivity increased in a roughly linear/

TABLE 8: Incorporation of L(^{3}H) leucine by reticulocyte lysate containing various P1.17 polysome fractions.

Legend:

P1.17 polysomes, in the amounts shown were incubated in 80µl reticulocyte assays (Expts. 1 and 3) or 160µl,assays (Expt. 2) comprising polysomes, supplement and lysate in the ratio 5:5:6 (by volume). Assays were incubated as described in Methods 6.2.2. Incorporation of radioactivity into TCA-precipitable material was measured as in Methods 8.1.2., while specific precipitations were carried out as in Methods 11.2.2.(a) and radioactivity measured as in 8.1.2. In some assays, various levels of pactamycin were present.

TABLE 8: Incorporation of L(^{3}H) leucine by reticulocyte lysate containing various Pl.17 polysome fractions.

Description		µg polysomes /assay	TCA-precipitable counts/assay ($X10^{-3}$)	Specifically precipitable counts/assay ($X10^{-3}$)	% age specifically precipitable	Notes
Pl.17 total polysomes (20/10/75)	1	0	404.7	1.68	0.41	1,2,3 refer to separate experiments
		29	287.5	15.36	5.34	
		88	412.6	27.58	6.68	
		146	385.0	22.16	5.75	
	2	0	256.4	1.26	0.49	
		293	487.1	16.83	3.45	
	3	88	55.1		0	0.08µM pactamycin
		88	272.6	4.8	1.76	0.08µM pactamycin
Pl.17 total polysomes (22/10/75)	1	0	349.8	1.68	0.48	
		13	118.4	4.44	3.75	
		39	202.7	14.54	7.17	
		65	277.0	29.52	10.65	
	2	0	256.4	1.26	0.49	
		124	357.2	18.85	5.28	
Pl.17 specifically precipitated polysomes (22/10/75)		0	404.7	1.68	0.41	
		10*	157.2	8.58	5.46	
		30*	246.8	26.52	10.74	
		50*	279.7	28.14	10.06	
Pl.17 non-precipitated polysomes (22/10/75)	1	0	349.8	1.68	0.48	
		18	131.4	7.08	5.4	
		54	236.4	13.68	5.78	
		90	231.6	21.6	9.3	
	2	0	256.4	1.26	0.49	
		178	507.8	16.1	3.17	
Pl.17 total polysomes (10/2/76)		0	990.72	3.5	0.35	
		61.7	234.7	13.4	5.7	
		29.3	289.4	12.07	4.15	

* - estimate

linear manner in response to increased amounts of polysomes added to the system (Fig. 7). Only in one case (Fig. 7A) did an increase in the amount of polysomes added lead to a decrease in specifically precipitable radioactivity. This may have been due to the saturation of the system with polysomes. The amount of radioactivity specifically precipitated/µg polysomes added showed evidence in some cases of being constant. It was clear, however, that higher levels of polysomes decreased the net specific incorporation/µg polysomes added,possibly due to limiting availability of substrate.

Another phenomenon observed in the reticulocyte lysate was the tendency for total incorporation of radioactivity into TCA-precipitable material to be reduced by the addition of exogenous polysomes. Two possible explanations presented themselves. The polysomes could have contained traces of inhibitory substances,(e.g. cycloheximide, heparin),from the polysome isolation stage. This was thought unlikely since the inhibition of total incorporation seemed to be alleviated as the amount of polysomes (and hence also the amount of"inhibitory"material) added to the system was increased. Total TCA-precipitable incorporation of radioactivity, however, seldom reached the level of incorporation achieved by reticulocyte lysate alone. The alternative explanation was that the presence of large quantities of exogenous polysomes in the reticulocyte lysate inhibited the incorporation of radioactivity by the lysate due to competition between endogenous and exogenous polysomes for limiting substrate or factors. Such competition might inhibit processes such as re-initiation of translation of messages. Addition of increasing quantities of polysomes might increase incorporation caused by "run-off"/

FIG. 7: Immunoprecipitable and total incorporation of L-(^{3}H) leucine by reticulocyte lysate in response to the addition of P1.17 polysomes.

Reticulocyte lysate, supplemented as described in Methods 6.2.1. was incubated with 5µCi L-(^{3}H) leucine and varying amounts of P1.17 polysomes of 20/10/75 or 22/10/75 (Methods 3.3.). Total TCA-precipitable incorporation of radioactivity was measured as described in Methods 8.2. Specific immunoprecipitations were performed as described in Methods 11.2.2., and the immunoprecipitates isolated (Methods 11.2.2.). No normal rabbit serum control precipitations were used. For explanation of the specific precipitation of P1 mRNA-containing polysomes see text (Results 2).

a) Total and antibody precipitable incorporation with P1.17 total polysomes (20/10/75).

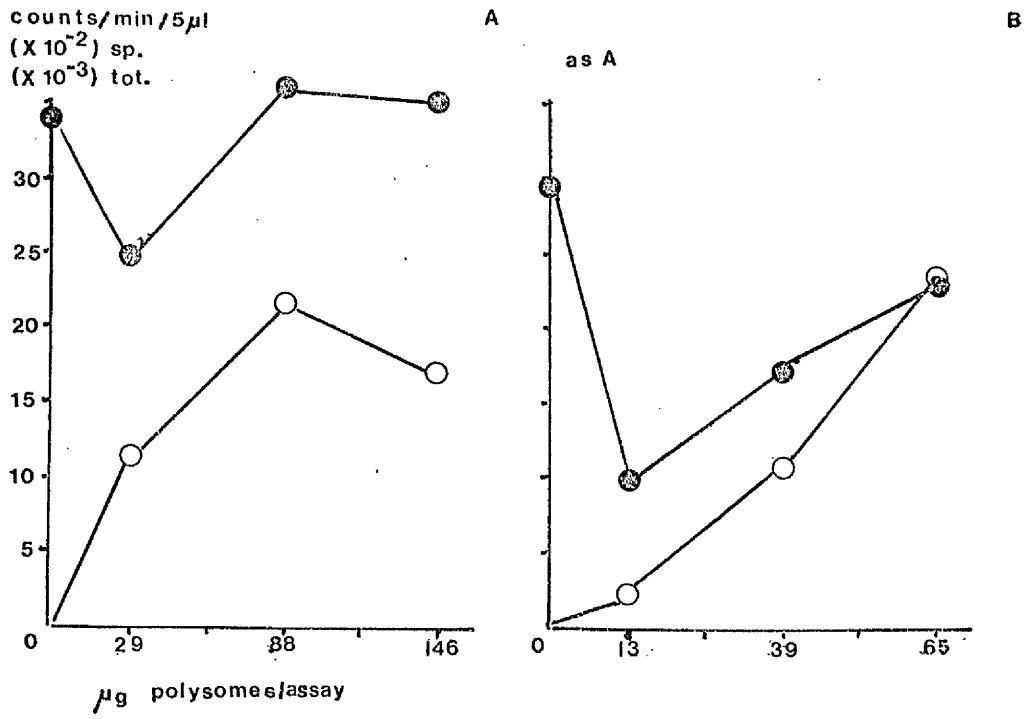
b) Total and antibody precipitable incorporation with P1.17 total polysomes (22/10/75).

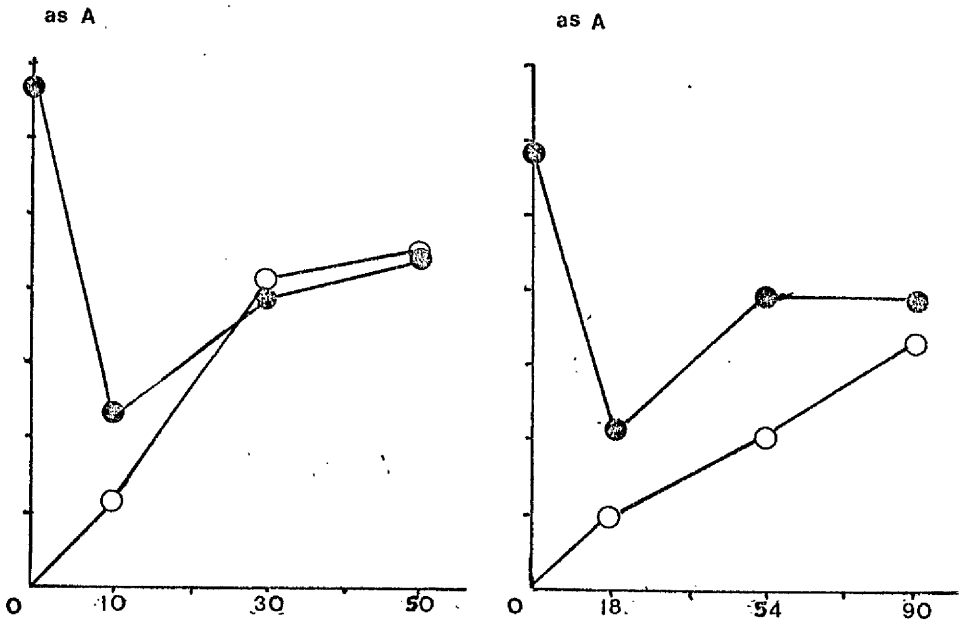
c) Total and antibody precipitable incorporation with P1.17 specifically precipitated polysomes (22/10/75).

d) Total and antibody precipitable incorporation with P1.17 non-precipitated polysomes (22/10/75).

—●— Total incorporation of radioactivity

—○— Specifically precipitable incorporation of radioactivity.


counts/min/5µl
(X 10⁻²) sp.
(X 10⁻³) tot.
A
B
as A
30
25
20
15
10
5
0
29
88
146
0
13
39
65
µg polysomes/assay


C
D
as A
as A
0
10
30
50
0
18
54
90

off" of existing message-attached ribosomes without necessarily removing blocks on processes such as initiation. A similar effect has been noted by other workers (e.g. Milstein et al., 1972,using polysomal RNA).

The effect of the initiation inhibitor pactamycin was investigated. A high concentration of pactamycin (0.8μM) inhibited TCA-precipitable incorporation of radioactivity by 87%. It also reduced specifically precipitable radioactivity to background levels. This was in agreement with the known effects of pactamycin. At high concentrations, it inhibits both initiation events and elongation reactions. (Goldberg et al., 1973).

At tenfold lower concentrations of pactamycin, incorporation of radioactivity into TCA precipitable material in the presence of exogenous polysomes was found to be 34% less than the levels of incorporation found in the absence of pactamycin. The incorporation of radioactivity into specifically precipitable material fell to 20% of levels found in similar assays with no pactamycin present.

These results suggested that initiation and re-initiation of protein synthesis accounted for a substantial proportion of the cell-free products in the reticulocyte lysate, particularly those directed by exogenous polysomes.

Polyacrylamide gel analysis of both total products and specifically precipitable material from these,and subsequent,assays was carried out. As the data in Fig. 8 demonstrated, specifically precipitable material was separated on gels mainly into two peaks of radioactivity. These peaks had the approximate mobilities of Pl Ig H and L chains, as judged by comparison with both internal markers/

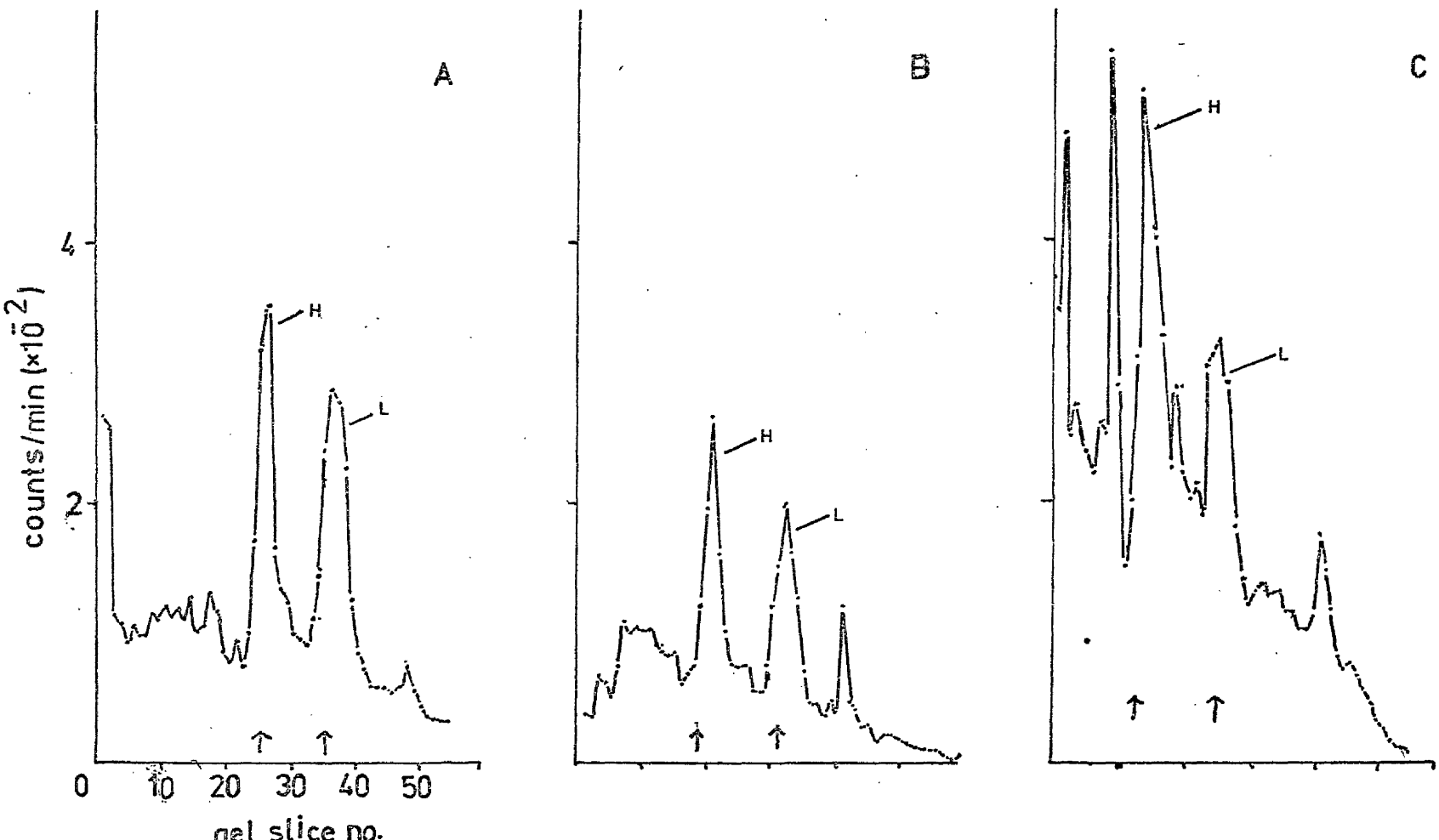
FIG. 8: PAGE analysis of immunoprecipitable material from reticulocyte lysate assays containing Pl.17 polysome fractions - identification of H and L chain products.

Samples of immunoprecipitates of the L(^{3}H) leucine-labelled products of assays containing various Pl.17 polysome fractions were dissolved in 8M urea:2% (w/v) SDS, containing ∿1000c.p.m. of L(^{35}S) methionine-labelled Pl.17 protein and reduced and alkylated as described in Methods 9.1.3. The samples were applied to 10% (w/v) SDS-phosphate polyacrylamide tube gels, using NN' methylene bisacrylamide as cross-linker (Methods 9.4.). PAGE was carried out as described in Methods 9.1.4. Gels were then sliced (Methods 9.1.5.) and solubilised (Methods 8.1.3.) and radioactivity detected as described in Methods 8.1.3. Channel settings in the liquid scintillation spectrophotometer were adjusted to exclude ^{3}H energy range counts from one channel, and to cover the ^{3}H energy spectrum in the other. Spill-over of ^{35}S counts into the "^{3}H only" channel was measured as X2 the counts detected in the "^{14}C only" channel and subtracted from the counts in the "^{3}H only" channel. The graphs show only the ^{3}H counts detected. The positions of (^{35}S)-labelled H and L chains are indicated by arrows.

A. Total Pl.17 polysome fraction - specifically precipitable products.

B. Specifically-precipitated Pl.17 polysome fraction - specifically precipitable products.

C. Non-precipitated Pl.17 polysome fraction - specifically precipitable products.


A
B
C
H
L
H
L
H
L
counts/min (×10⁻²)
4
2
0
10
20
30
40
50
gel slice no.

markers and external markers, radioactively labelled with L-(^{35}S) methionine. Relative mobilities of marker H and L chains and precipitated putative H and L chains were as follows:-

Chain	$\overline{Rm}$	S.D. = σ	n
Hm	0.355	± 0.026	5
H_{c-f}	0.380	± 0.035	8
L_m	0.524	± 0.018	5
L_{c-f}	0.535	± 0.032	8

On gels with diallyltartardiamide (DATD) as cross linker (data not shown) the relative mobilities of marker and cell-free putative H and L chains were as follows:-

Chain	$\overline{Rm}$	S.D. = σ	n
H_m	0.475	-	2
H_{c-f}	0.509	± 0.016	3
L_m	0.625	-	2
L_{c-f}	0.635	± 0.033	3

These figures suggested that the material specifically precipitated by anti-Pl antiserum from the cell-free system was Pl heavy and light chain or their cell-free precursors. No direct controls were used in the assays in question to determine non-specific precipitation, e.g. replacing anti-Pl antiserum with normal rabbit serum. Two pieces of evidence, however, suggest that background precipitation was low. Firstly, in reticulocyte lysate assays containing no added polysomes/

polysomes, total incorporation of radioactivity into TCA-precipitable material was often up to two times the total incorporation in the presence of polysomes. In both these assays and in assays containing exogenous polysomes a major proportion of synthetic activity was devoted to translation of endogenous reticulocyte mRNA's. Nevertheless, specific antiserum precipitated at least 3 times as much radioactivity and usually about 15 times as much radioactivity from assays containing exogenous polysomes as from assays containing only reticulocyte polysomes. Moreover, polyacrylamide gel analysis of samples of these reticulocyte lysate background precipitates yielded no detectable peaks of radioactivity (data not shown).

Secondly, it could be argued that exogenous polysomes themselves promote some non-specific aggregation resulting in trapping of radioactivity in immune precipitates. In one assay of exogenous polysomes, however, the presence of pactamycin allowed total incorporation of radioactivity similar to another control assay. While the pactamycin assay contained 2 x as many exogenous polysomes as the control, specifically precipitable radioactivity was only 30% of the control. Samples of the pactamycin precipitate, moreover, gave no peaks of radioactivity corresponding to H and L chain markers on a polyacrylamide gel (data not shown).

The ratio of radioactivity in H and L chain-like material varied from gel to gel and from assay to assay. Using only information from assays invdving total polysomes, the following ratios were obtained:-

	Ave	S.D.=σ	n
$H:L_m$	1.50	± 0.26	4
$H:L_{c-f}$	1.05	± 0.11	6

(H:L ratio based on known amino acid composition (Parkhouse, 1969) would be 2.28 using L-(^{35}S) methionine as label and 2.19 using L-(^{3}H) leucine as label.)

This suggested that the ratio of synthesis of cell-free putative H and L chains was different from the ratio of the secreted H and L chains used for markers. These more closely approximated the ∿2:1 ratio of radioactivity in H and L chains expected from amino acid composition data. This expected ratio assumes that either

i) Both chains are synthesized from the same amino acid pool, or

ii) their separate amino acid pools rapidly equilibrate with added radioactive amino acids to the same specific activity.

It also assumes that H and L chains are secreted in equal numbers, i.e. only as H_2L_2 molecules.

The rather large variation in H:L ratio between apparently identical samples could have arisen from the relatively small but persistent problem of aggregation of radioactive material at the top of the gels. This could randomly affect how much each of H and L chains were electrophoresed into the gel. The difference in the H:L ratio between secreted marker Ig and cell-free system-produced putative H and L chain material was, however, significant.

When total TCA-precipitable material from reticulocyte lysate assays containing or lacking Pl.17 polysomes was analysed by PAGE, striking differences were observed (Fig. 9). TCA precipitates of reticulocyte lysate alone displayed one broad major peak of radio-

activity/

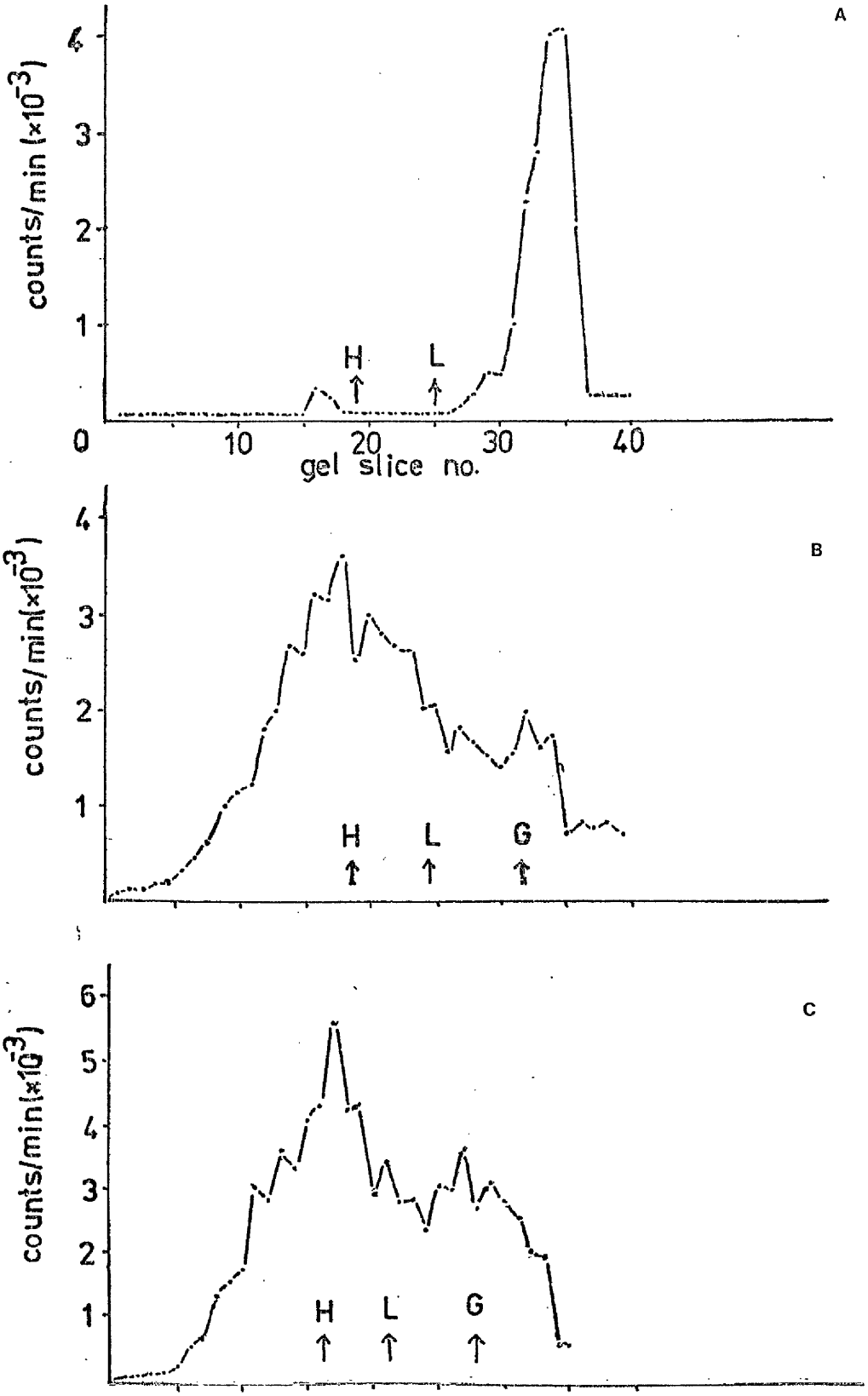
FIG. 9: PAGE analysis of TCA-precipitable products from reticulocyte lysate assays containing various Pl.17 polysome fractions.

5µl aliquots of reticulocyte lysate assays containing various Pl.17 polysome fractions were precipitated as described in Methods 8.2. The precipitates were re-dissolved in 20µl 8M urea:2% (w/v) SDS and reduced and alkylated (Methods 9.1.3.). The samples were applied to 10% (w/v) SDS-phosphate polyacrylamide tube gels with NN' methylene bisacrylamide as cross-linker (Methods 9.1.1.) and electrophoresed as described in Methods 9.1.4. Gels were sliced (Methods 9.1.5.), then solubilised and radioactivity measured by liquid scintillation spectrophotometry (Methods 8.1.3.). The positions of marker Pl.17 H and L chains and of globin are indicated by arrows.

A. Endogenous products of reticulocyte lysate.

B. Products of Pl.17 total polysomes in reticulocyte lysate.

C. Products of Pl.17 non-precipitated polysomes in reticulocyte lysate.


A
counts/min (×10⁻³)
4
3
2
1
0
H
L
10
20
30
40
gel slice no.
B
counts/min(×10⁻³)
4
3
2
1
H
L
G
C
counts/min(×10⁻³)
6
5
4
3
2
1
H
L
G

activity with a mobility of 0.87 - 0.89. This was assumed to be globin, the major protein product of reticulocytes (see Fig. 9A). It will be shown later (Fig. 23) that reticulocyte lysates synthesize other proteins in addition to globin. In contrast, PAGE of assays containing exogenous polysomes revealed much more complex radioactive profiles (see Figs. 9B,C). Radioactive material was found across the entire gel. The globin peak was generally much reduced in terms of actual counts and as a proportion of total counts. This suggested that added myeloma polysomes were being translated in competition with, and in addition to, endogenous reticulocyte material. Nothing could be clearly stated about the polypeptides synthesized since the resolving power of the method used to analyse the gels (slicing) was insufficiently great.

These gels illustrated the major disadvantage of the reticulocyte lysate cell-free system, viz. the non-quantifiability of the effect of exogenous mRNA due to competing endogenous mRNA.

Methods of removing the effect of endogenous mRNA were considered.

3. Polysome-depleted reticulocyte lysate.

Reticulocyte lysate was centrifuged in the presence of 25μM haemin at 40,000 rpm for 120 min at 4°C. This step led to the formation of a polysome pellet which was separated from the supernatant and resuspended, as for myeloma polysomes, in 0.25M sucrose. When the effect of adding various polysome fractions to the polysome depleted reticulocyte lysate was investigated the results shown in Table 9 were obtained. These results demonstrated several points. Firstly/

TABLE 9: Incorporation of radioactivity by polysome-depleted reticulocyte lysate in the presence of Pl.17 polysomes.

Polysome fraction added	Nature of lysate	Specific counts/assay	TCA precipitated counts/assay
0	Normal	1331	912736
0	Depleted	1363	337920
1.42 A_{260} Pl I polysomes	Normal	13392	234744
1.42 A_{260} Pl I polysomes	Depleted	1849	336384
0.675 A_{260} Pl II polysomes	Normal	12072	289416
0.675 A_{260} Pl II polysomes	Depleted	2507	288576
Reticulocyte polysomes (5% of pellet)	Depleted	2271	1231104

Legend:

13ml of reticulocyte lysate, containing 25µM haemin was centrifuged at 40,000 rpm in a Beckman L5-65 ultracentrifuge, using a Beckman SW41 rotor. Centrifugation was for 120 min at 4°C. The polysome depleted supernatant was decanted and stored at -70°C until use. The polysome pellet was re-suspended in 500µl 0.25M sucrose and stored at -70°C until use. Assays with depleted lysate were performed as with normal lysate (Methods 6.2.2.). Total TCA precipitable radioactivity and specifically immunoprecipitable radioactivity were measured by standard methods (Methods 8.1.2. and 8.2.2.).

Firstly, the polysome removal step had been partially successful in that activity (incorporation of radioactivity) had decreased by 63%. Background specific precipitation of normal and depleted reticulocyte lysate remained approximately constant. Addition of exogenous polysomes to depleted lysate led to no significant change in total incorporation by the lysates. Addition of exogenous polysomes to normal lysate, on the other hand, led to an inhibition of total incorporation of 68 - 75%. Specific precipitation of radioactivity from depleted lysates suggested that only a low level of myeloma polysome translation was occurring. By comparison, specifically precipitable material from normal lysates containing myeloma polysomes contained 9 X and 10 X as much radioactivity as the appropriate background precipitation. That the depleted lysate could respond to added polysomes was demonstrated by the recovery in the total TCA-precipitable incorporation of radioactivity upon addition of a fraction of the reticulocyte polysomes removed from the lysate by centrifugation.

4. Messenger dependent lysate (M.D.L.)-optimisation of conditions.

4.1. Incorporation of radioactivity.

The M.D.L. was developed by Pelham and Jackson (1976) as a cell-free system which combined a high level of stimulation by exogenous messenger RNA with low levels of endogenous incorporation. Moreover, the reticulocyte lysate cell-free system, from which it was developed, has been shown to exhibit a high degree of fidelity in translating exogenous message. The system must nevertheless be tested with each new species of message added, in order to ensure optimal translation/

translation conditions.

M.D.L. was first tested for incorporation with exogenous mRNA. Reticulocyte lysate was treated with ribonuclease, subsequently inactivated, as described in Methods 7.1.2. Using L-(^{35}S) methionine as label, a comparison was made of the incorporation characteristics of reticulocyte lysate, of M.D.L. and of M.D.L. containing tobacco mosaic virus (TMV) RNA. TMV RNA was kindly donated by Dr. R.T. Hunt. Incorporation of radioactivity into TCA-precipitable material was measured over a time course. As the data in Fig. 10 demonstrated, M.D.L. alone showed only a low level of background incorporation (approximately 9% of the incorporation by reticulocyte lysate after 40 min incubation). Reticulocyte lysate, in contrast, gave a steady, almost linear increase in incorporation of radioactivity over the time course of the experiment. M.D.L. containing TMV RNA showed a steady increase in incorporation over most of the time course of the experiment although the increase in incorporation deviated from linearity after 25 min. This was possibly due to limiting factors, such as availability of amino acids. Over the first 25 minutes of the assay the rate of incorporation stimulated by TMV RNA in M.D.L. was twice as great as the rate of incorporation in the reticulocyte lysate. This suggested that the M.D.L. was a very efficient system for translating exogenous mRNA. A comparison of M.D.L. and reticulocyte endogenous products separated by PAGE is shown in Fig. 20.

4.2. Determination of optimal conditions for translation of myeloma mRNA.

L-(^{35}S) methionine was used as the radioactively labelled amino acid/

FIG. 10: Time course of incorporation of radioactivity by reticulocyte lysate and M.D.L.

Supplemented reticulocyte lysate, prepared as described in Methods 7.1.2., was divided into two portions, one of which was nuclease-treated to prepare M.D.L. (Methods 7.1.2.). 50µl assays of either reticulocyte lysate or M.D.L. were incubated in the presence of 10^6 cpm L-(^{35}S) methionine at 30°C for 40 minutes. One assay contained in addition 2µg TMV RNA. 5µl aliquots were removed at various times during the incorporation and treated to allow measurement of total radioactivity incorporated into TCA-precipitable material as follows:- each 5µl aliquot was diluted to 1.5ml with 1ml distilled H_2O + 0.5ml 0.5N NaOH: 10% (w/v) H_2O_2: 10mM unlabelled methionine. This was incubated at 37°C for 15 min. 1 ml of 25% (w/v) TCA was added and precipitation allowed to occur for > 30 min. This solution was then Millipore filtered and washed c 8% (w/v) TCA. The filters were dried and counted for radioactivity in toluene/PPO.

FIG. 11: Time course of incorporation of L-(^{35}S) methionine by M.D.L. containing P1.17 polysomes in the absence and presence of unlabelled methionine.

50µl assays,prepared as described in Methods 7.1.2., were incubated with 29µg P1.17 polysomes in the presence of 3µCi L-(^{35}S) methionine. Incubation took place either in the absence or presence of 50 pmoles unlabelled methionine. Single 5µl aliquots were removed at intervals and treated on paper discs as described in Methods 8.2.1. M.D.L. assays, containing no added polysomes were incubated under otherwise identical conditions to the above assays. Radioactivity incorporated by these assays remained almost constant at 4500 ± 500 counts/min/5µl (no added methionine) or 3950 ± 380 counts/min/5µl (50 pmoles unlabelled methionine). These figures were subtracted from each time point as appropriate.

Fig 10

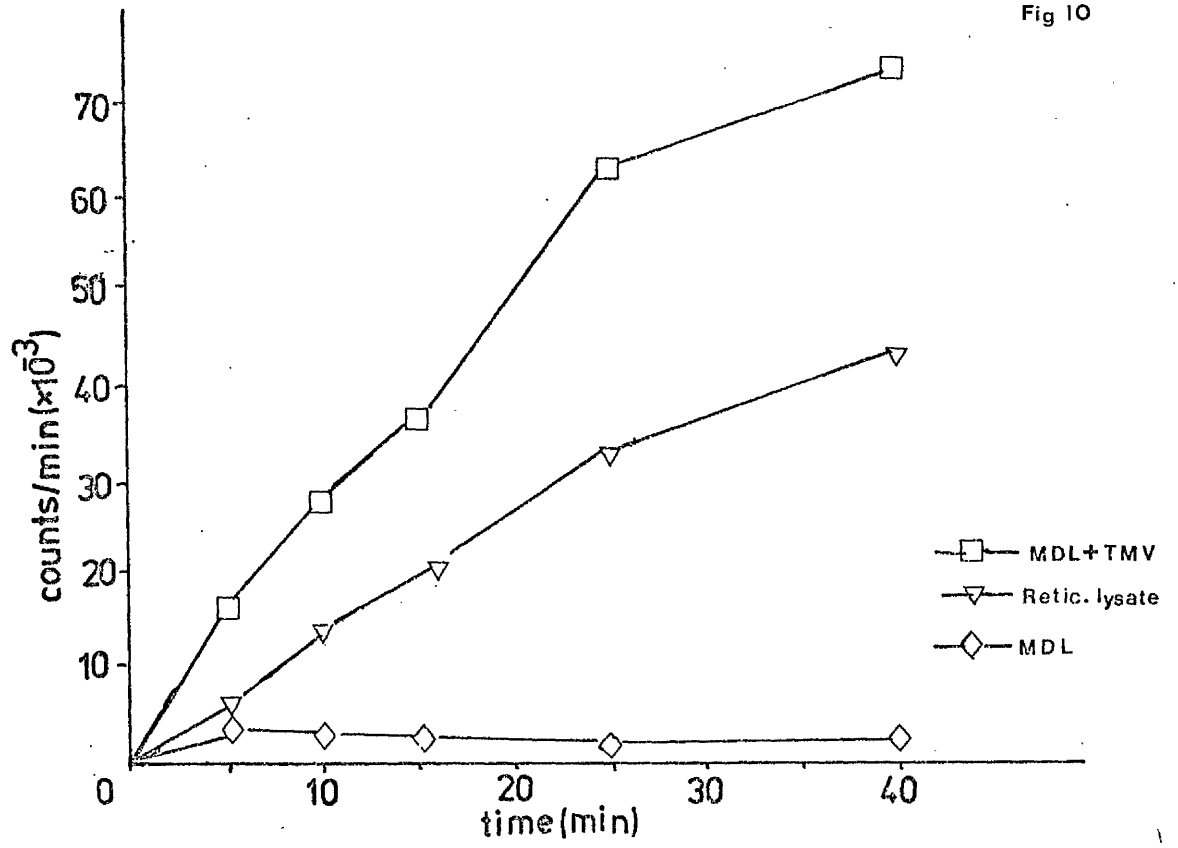
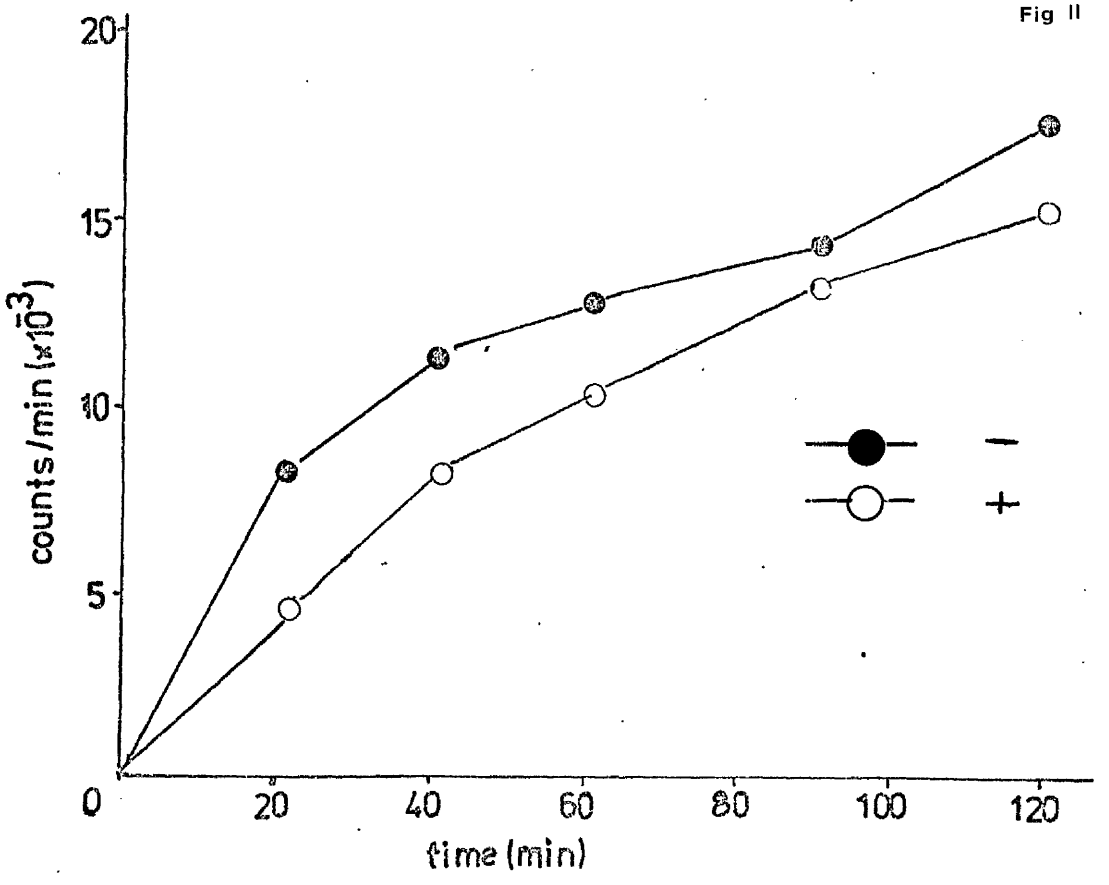

counts/min (×10⁻³)
70
60
50
40
30
20
10
0
10
20
30
40
time (min)
MDL+TMV
Retic. lysate
MDL

Fig II


counts/min (×10⁻³)
20
15
10
5
0
20
40
60
80
100
120
time (min)
−
+

acid of preference in M.D.L. studies. In assays where other amino acids (L-(^{3}H) leucine, L-(^{3}H) methionine, L-(^{14}C) isoleucine, (^{3}H) amino acid mixture) were used, their use will be noted in the text or figure legends.

L-(^{35}S) methionine offered several advantages as a labelling amino acid:-

i) High specific activity of the radioactively labelled amino acid

ii) Higher E_{av} for β-particle emission made fluorography more sensitive

iii) The relatively small number of methionine residues in the Ig molecules under study provided the possibility of simple unambiguous peptide mapping

The incorporation of L(^{35}S) methionine by the M.D.L., stimulated by myeloma polysomes,was investigated over a time course of 120 min. (see Fig. 11). This data suggested that incorporation was continuous with methionine as the labelled amino acid over the time course studied. The rate of incorporation, however, decreased steadily after 20-40 min. Assays were generally incubated for 120 min. The inclusion of 50 pmoles of unlabelled methionine in the assay reduced overall incorporation by only 20% on average, suggesting that a substantial pool of endogenous methionine existed in the M.D.L. (see Results 4.3).

The concentration of K^{+} ions required for optimal translation of either myeloma poly(A) RNA or myeloma polysomes was found to be between 90 and 100mM (Fig. 12). Subsequent assays were carried out at this level of K^{+} ions.

The/

FIG. 12: (K^+) optima for incorporation of L-(^{35}S) methionine in M.D.L.'s containing Pl.17 polysomes or Pl.17 poly(A) RNA.

(i) Poly(A) RNA. To normal 50µl M.D.L. assays (Methods 7.2.1.) various concentrations of KCl solution were added in volumes of 5µl. 1µg Pl.17 poly(A) RNA, 50 pmoles unlabelled methionine + 2.5µCi L-(^{35}S) methionine were then added (10µl) and incubations carried out as in Methods 7.2. 5µl aliquots were removed from each assay in duplicate after incubation and TCA-precipitable radioactivity determined as in Methods 8.2.1. The average counts/min/5µl were plotted. Assays containing no added poly(A) RNA (but otherwise identical) were run simultaneously and TCA-precipitable radioactivity was measured.

(ii) Polysomes. M.D.L. was prepared containing no added KCl. Various concentrations of KCl solution were added to 50µl aliquots of M.D.L. in a volume of 5µl. Pl.17 polysomes and L-(^{35}S) methionine were added in a volume of 10µl and assays incubated as described in Methods 7.2. TCA-precipitable radioactivity in 5µl duplicate aliquots was measured as described in Methods 8.2.1.

KEY: -⊕- MDL + poly(A) RNA
-○- MDL alone.
-△- MDL + polysomes.

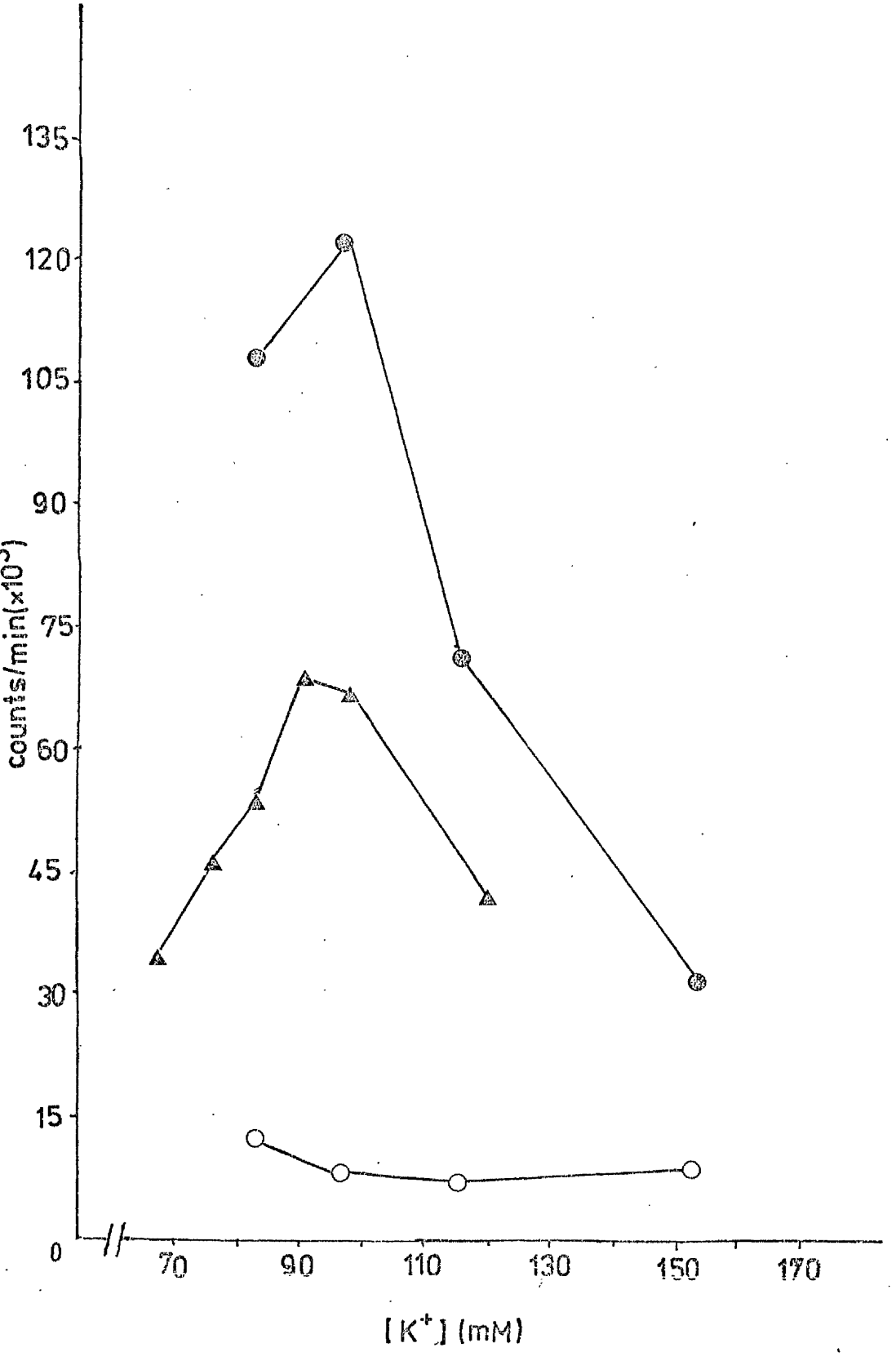
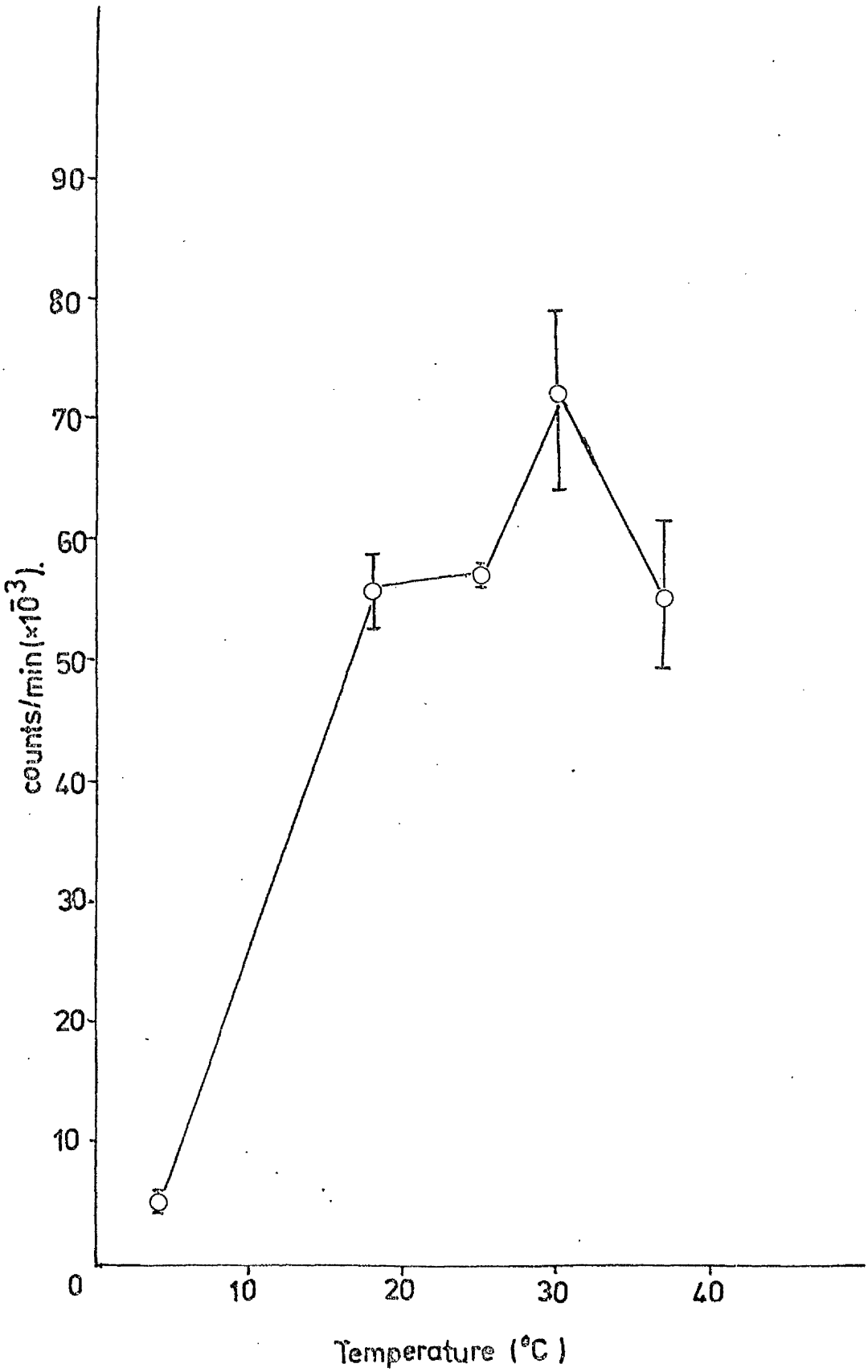

135
120
105
90
75
60
45
30
15
0
counts/min(x10^{-3})
70
90
110
130
150
170
[K$^+$] (mM)

FIG. 13: Temperature optimum for incorporation of L-(^{35}S) methionine in M.D.L. containing Pl.17 polysomes.

M.D.L. assays, containing Pl.17 polysomes were incubated with 5μCi L-(^{35}S) methionine at various temperatures for 120 min. (Methods 7.2.). 5μl duplicate aliquots were removed from each assay and radioactivity incorporated into TCA-precipitable material was measured as described in Methods 8.2.1.


90
80
70
60
50
40
30
20
10
0
counts/min (×10⁻³).
10
20
30
40
Temperature (°C)

The optimum temperature for incubation of M.D.L. assays was found to be 30°C, as shown in Fig. 13.

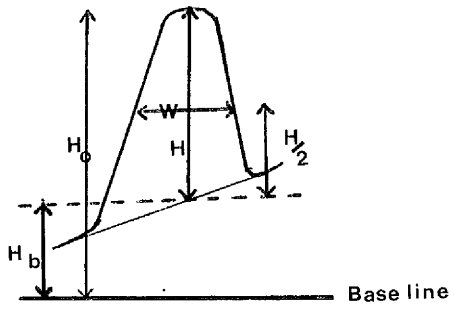
4.3. Endogenous levels of methionine.

The endogenous pool size of methionine was of interest since this knowledge was required for meaningful calculations of the number of rounds of translation occurring in the M.D.L.

Two methods of calculating this number were used. Firstly, a sample of M.D.L. was analysed on a JLC-5AH Amino Acid Analyser (this work was carried out by Mr. Robert Blackie). The appropriate section of the analyser trace indicated that the endogenous methionine pool size was 400 pmoles/50µl assay (Fig. 14). This value was calculated as follows, using the HW method recommended by the manufacturers of the analyser (Japan Electron Optics Laboratory).

a) Standard.

5nmoles of each amino acid. Peaks were analysed as follows.



$$H = H_o - H_b$$

$$\frac{H \times W}{S} = C_{1\,nmole}$$

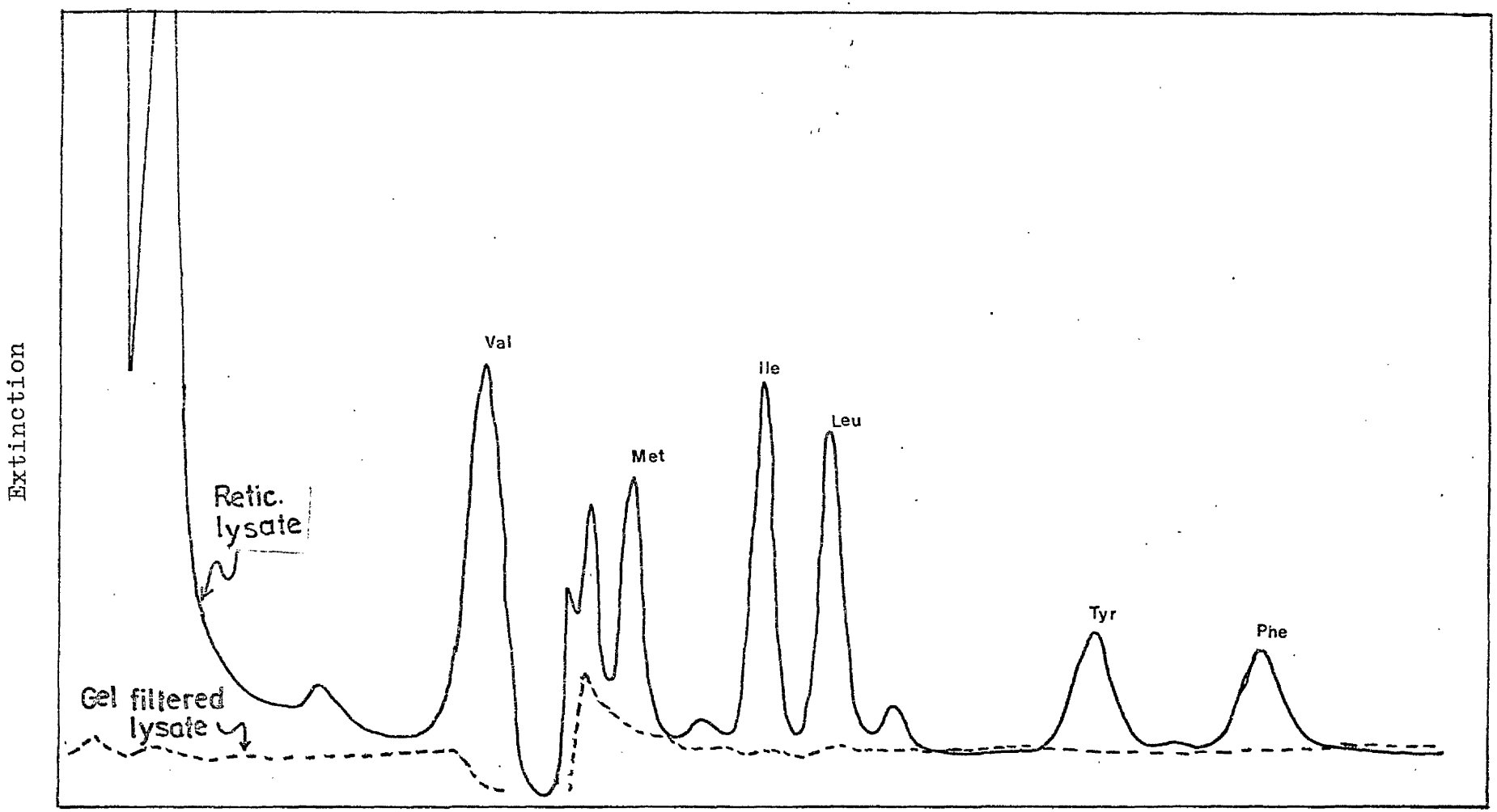

Extinction
Val
Ile
Leu
Met
Retic. lysate
Gel filtered lysate
Tyr
Phe
Vol. eluant.

FIG. 14: Comparison of amino acid analyses of normal reticulocyte lysate and reticulocyte lysate passed over G-25 Sephadex.

In each case 1 ml of reticulocyte lysate was precipitated by the addition of concentrated TCA solution to a final concentration of 10% (w/v). The precipitated material was centrifuged to a pellet and the supernatant analysed by a JLC-5AH amino acid analyser (this work was performed by Mr. R. Blackie). The number of pmoles of methionine present was calculated as described in Results 4.3.
Only the portion of the amino acid analyser trace containing the methionine peak is shown.

b) Unknown

Calculate HXW as above. $\frac{HXW}{C}$ = No. of nmoles of amino acid in sample.

A second method of detecting the methionine pool size was used. This involved dilution of the radioactively labelled methionine in the assay by the addition of known amounts of unlabelled methionine. When sufficient unlabelled methionine has been added to reduce the incorporation of radioactivity in the assay by 50%, this amount of unlabelled methionine should equal the endogenous pool plus radioactive methionine. In this experiment, the incorporation of a second amino acid, labelled with a different radioactive isotope was measured to ensure that the decrease in incorporation of radioactivity was not due to a decrease in protein synthesis. L-(^{14}C) isoleucine was used for this purpose. This experiment (see Fig.15) suggested that the endogenous methionine pool was about 700 pmoles/50µl assay calculated as follows:-

No. of pmoles L-(^{1}H) methionine required to reduce incorporation by 50% = 1540

= x + y

where x = added L-(^{3}H) methionine = 830 pmoles

y = endogenous methionine

$\therefore$ 1540 = 830 + y

$\therefore$ y = 710 pmoles

Note:
L-(^{3}H)-methionine was used in order that the higher energy emitter (L-(^{14}C)ile) could be used as an unambiguous indication of the amount of protein synthesis. (By selection of channels on the scintillation counter, all ^{3}H energy range counts were excluded from counting of ^{14}C).

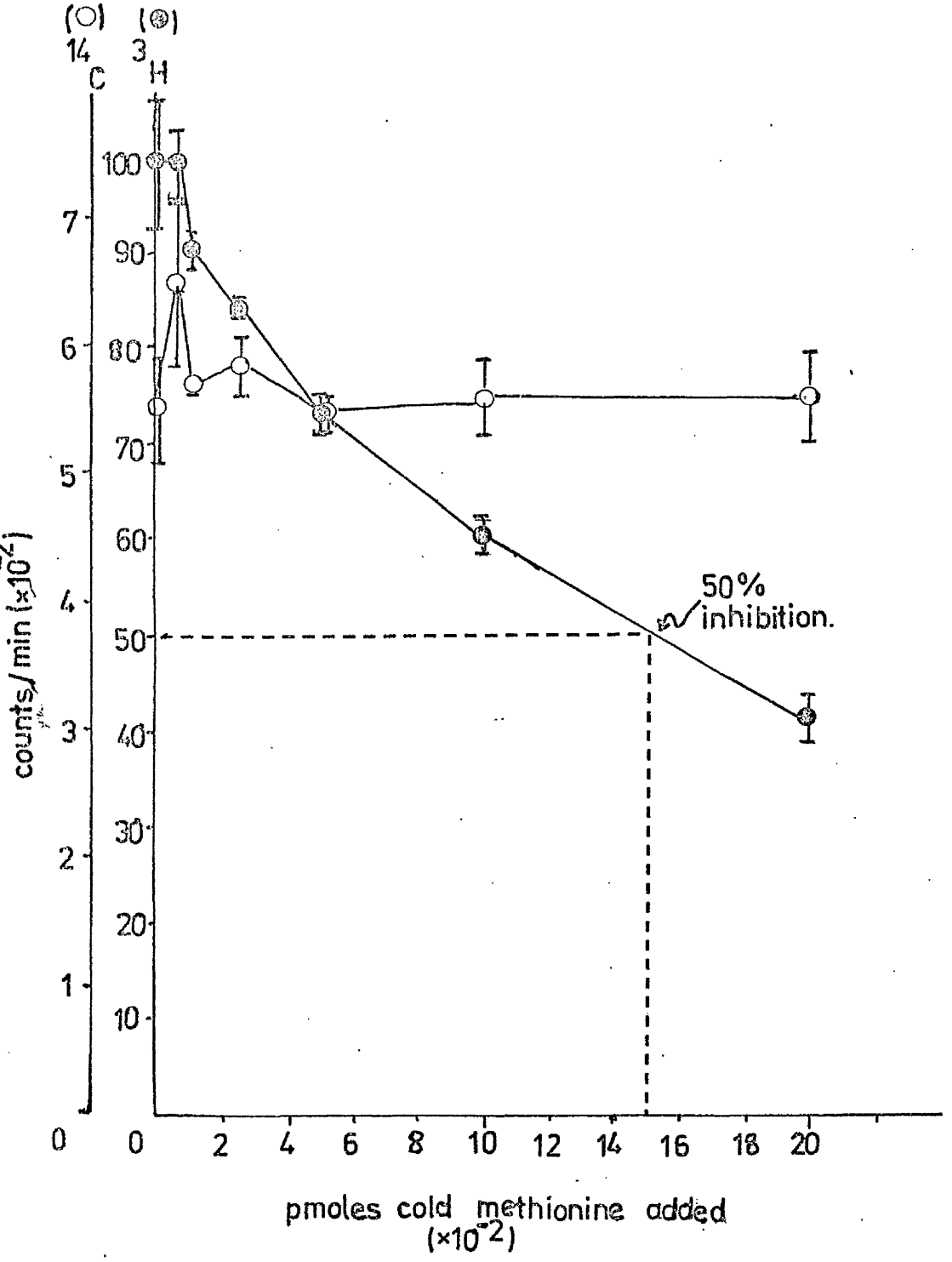

(○) (●)
14 3
C H
100
90
80
70
60
50
40
30
20
10
7
6
5
4
3
2
1
0
counts/min (×10⁻²)
50% inhibition.
0 2 4 6 8 10 12 14 16 18 20
pmoles cold methionine added
(×10⁻²)

FIG. 15: Estimation of the size of the endogenous unlabelled methionine pool in M.D.L. assays by double-labelling.

To normal reticulocyte lysate assays, prepared as Methods 7.1.2., containing 5μCi L-(^{3}H) methionine and 0.25μCi L-(^{14}C) isoleucine were added serial dilutions of unlabelled methionine, initially at a concentration of 2000 pmoles/5μl, in a volume of 5μl. Each assay was performed in duplicate. Incubation conditions were as described in Methods 7.2. After incubation, duplicate 5μl aliquots from each assay were prepared for liquid scintillation counting as described in Methods 8.2.1. Counting channels were selected to exclude all ^{3}H energy range counts from one channel, and to include both ^{14}C and ^{3}H energy range counts in the other channel. The level of ^{14}C counts in the wide channel was estimated by measurements of radioactivity in an assay containing only L-(^{14}C) isoleucine as label. 30% of ^{14}C counts were detected in the restricted channel. Therefore, assuming quenching to be constant, the number of counts detectable in the ^{3}H and ^{14}C channel attributable to ^{14}C was approximately 2 x the number of counts detectable in the ^{14}C-only channel. This correction was made when arriving at the number of counts detected due to the incorporation of L-(^{3}H) methionine. The endogenous pool size was calculated as described in Results 4.3.

This figure was of the same order of magnitude as that determined by amino acid analysis but varied significantly enough to require explanation. The following explanations for the difference can be made.

a) Amino acid analysis detects only free amino acids; tRNA-bound amino acids are precipitated from solution before analysis. The double-label dilution experiment theoretically measures both free and bound amino acids.

b) Amino acid analysis results may also be low due to oxidation of methionine to methionine sulphone, which appears elsewhere on the amino acid analysis trace.

c) The use of L-(^{3}H) methionine for measurement of the endogenous methionine pool size in the dilution experiment introduced a source of error. Inaccuracy of measurement of the volume of L-(^{3}H) methionine added (5µl) by for example 10% (0.5µl) would result in an under- or over-estimation of the endogenous pool size by ± 80 pmoles.

As a result of these qualifications, the figure of 700 pmoles methionine/50µl was taken as the more accurate and used in all subsequent calculations. This generally involved a correction of the specific activity of the added methionine by a factor of $\sim 10^{2}$-fold, using the equation

$$\text{Specific activity in assay} = \frac{700}{\text{no. of pmoles L-(}^{35}\text{S) met added}} \times \frac{\text{Assay volume}}{50} \times \text{specific activity L-(}^{35}\text{S) methionine}$$

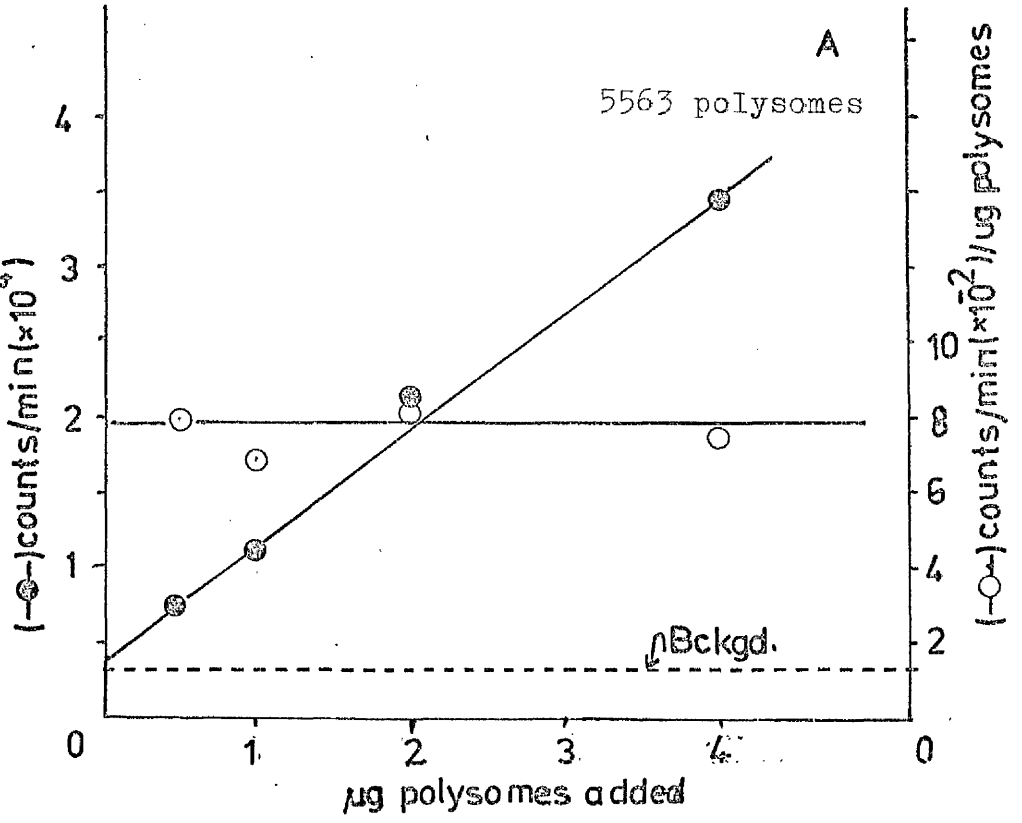
4.4. Dose response to myeloma mRNA.

The M.D.L. was tested with serial dilutions of both polysomes and poly(A) RNA to determine the effect of increasing amounts of mRNA being added to the system. This could be used to discover whether levels of polysomes and poly(A) RNA added to the system were saturating. As can be seen from Fig. 16, 5563 polysomes showed no saturation over the range examined (0.5 - 4.3 µg/assay), the effect of increasing quantities of polysomes added being a linear increase in incorporation of radioactivity (10^6 cpm added/assay). The level of incorporation/µg polysomes added remained fairly constant over the range of concentrations studied. Pl.17 polysomes, however, showed a rather pronounced levelling off of incorporation at higher concentrations and even at the lower end of the concentration range studied (2.0 - 15.6µg/assay), the level of incorporation/µg polysomes was not constant. In this experiment, maximum stimulation by polysomes resulted in incorporation of 83% of the added radioactivity. The plateau effect could therefore be attributed to lack of substrate rather than saturation by mRNA. Consequently, within the concentration ranges studied there was found no saturation of the M.D.L. by myeloma polysomes, except at the highest levels.

Using poly(A)-containing RNA from two cell lines, increasing concentrations of RNA again produced a linear increase in incorporation of radioactivity, with a constant level of incorporation/µg poly(A) RNA added (see Fig. 17). The incorporation of radioactively labelled substrate/µg added RNA varied by a factor of 20 - 100 between polysomes and poly(A) containing RNA. Since poly(A) containing RNA is generally accepted to constitute a small fraction (1 - 5%) of the RNA in polyribosomes (see Methods), this was not unexpected. Incorporation by polysomes, although showing marked variations, due to differences in/

FIG. 16: Dose response of M.D.L. with various polysome fractions.

5µl M.D.L. assays containing 10^6 counts/min of L-(^{35}S) methionine, were incubated with serial dilutions of Pl.17I, or 5563 II polysomes, for 40 min at 30°C. Incorporation of radioactivity into TCA-precipitable material was measured as described in the Legend to Fig. 10. Incorporation of radioactivity in the absence of added polysomes is shown as a straight line parallel to the abscissa. Calculations of counts/min incorporated/µg polysomes added were based on the net incorporation in the presence of polysomes.


A
5563 polysomes
(—●—)counts/min(×10⁻⁴)
(—○—)counts/min(×10⁻²)/µg polysomes
Bckgd.
µg polysomes added

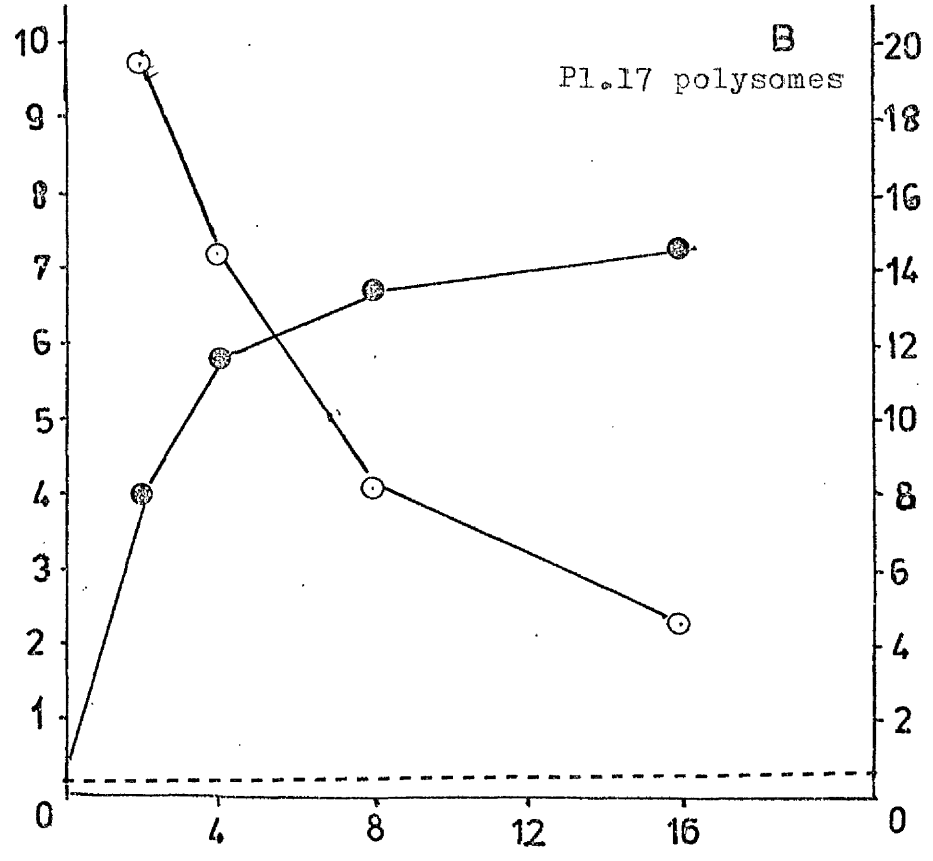
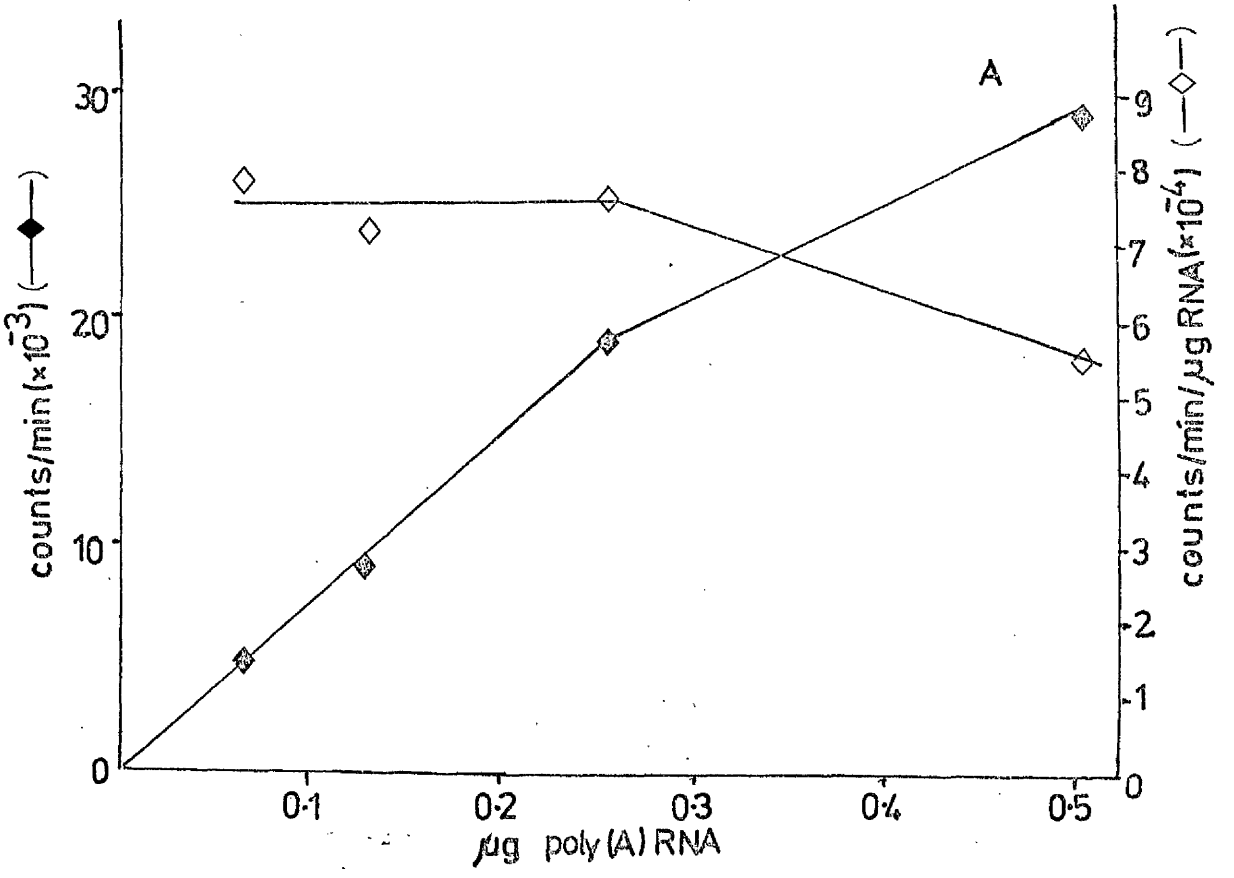

B
Pl.17 polysomes

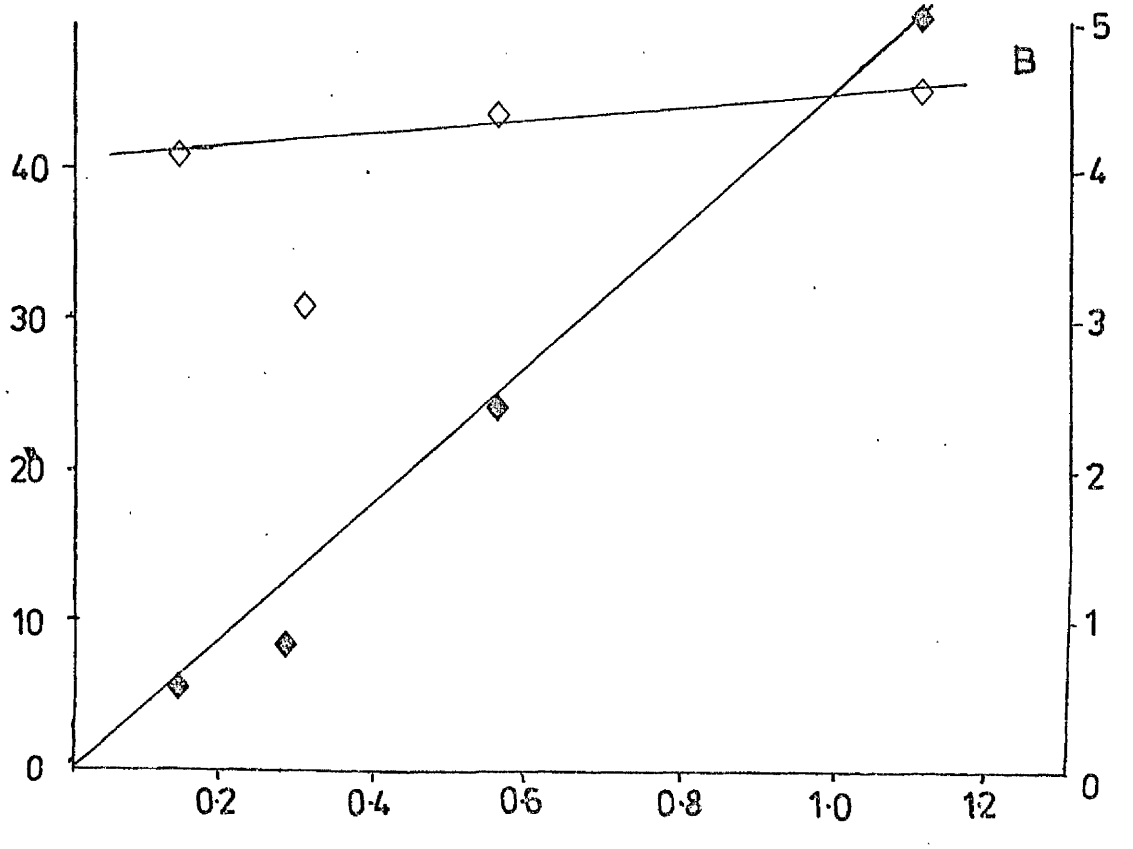
FIG. 17: Dose response of M.D.L. to the addition of Pl.17 or 5563 poly(A) RNA.

50µl M.D.L. assays, containing 10µCi L(^{35}S) methionine (5µl) were incubated in the presence of serial dilutions of either Pl.17 poly(A) RNA or 5563 poly(A) RNA under standard conditions (Methods 7.2.). Duplicate 5µl aliquots were treated and measured for radioactivity incorporated into TCA-precipitable material as described in Methods 8.2.1. Background incorporation of 7400 counts/min was subtracted from each assay. The calculation of incorporation/µg poly(A) RNA added is based on the incorporation with background incorporation subtracted.

A: Pl.17 poly(A) RNA

B: 5563 poly(A) RNA


A
counts/min (×10⁻³) (—◆—)
counts/min/µg RNA (×10⁻⁴) (—◇—)
µg poly (A) RNA
0·1
0·2
0·3
0·4
0·5


B
0·2
0·4
0·6
0·8
1·0
1·2

in incubation time, volume of assay, amount of radioactive substrate added, gave incorporation in the range 10^4 - 10^5 cpm/µg polysomes added. Incorporation by poly(A) RNA was in the range 10^6 - 10^7 cpm/µg poly(A) RNA added. Assays of polysomes contained 10 - 30 µg polysomes/50µl M.D.L., whilst assays of poly(A) RNA contained 0.1 - 1.5µg poly(A) RNA/50µl M.D.L.

4.5. Myeloma mRNA in the M.D.L. - rounds of translation.

Several calculations were made on the number of rounds of translation of poly(A) RNA, occurring in the M.D.L. Calculations involved several assumptions which are listed below:-

(i) Endogenous methionine levels were 700 pmoles/50µl (see Section 4.3)

(ii) Efficiency of detection of L(^{35}S) methionine by scintillation counting was 60%.

(iii) The poly(A) RNA used in the M.D.L. was essentially free from non-translatable RNA, e.g. ribosomal RNA.

(iv) All mRNA molecules present in poly(A) RNA were undamaged and capable of translation.

(v) The average MW of mRNA molecules present was 4.5×10^5.

(vi) The average protein contained 6 methionine residues (Parkhouse, 1969)

Of these assumptions, (i) is based on experiments described in Section 4.3. Assumption (ii) was based on experiments with L(^{35}S) methionine on paper discs in toluene/PPO scintillation fluid. Assumptions (iii) and (iv) are both idealised cases which would tend to underestimate the number of rounds of translation/active mRNA molecule. Assumption (v) is based on an average of an L chain mRNA/

mRNA 1150 - 1200 nucleotides long (750 coding nucleotides, 200 non-translated nucleotides, 200 nucleotides in the 3' poly(A) tail) and an H chain mRNA 1750 - 1800 nucleotides long (1350, 200, 200 nucleotides as before). These mRNA's represent medium length mRNA's, hence giving a reasonable average MW. They should also constitute a significant proportion of the cellular mRNA species. Assumption (vi) is based on an average of the number of methionine residues found in several IgG H and L chains since these are major proteins synthesized in the system.

With these assumptions, the following calculations were made, using the assays giving highest incorporation of L(^{35}S) methionine.

1. 0.31µg P1.17 poly(A) RNA, translated in 50µl M.D.L., containing 4.73µCi L(^{35}S) methionine.

Counts/min incorporated/assay with P1.17 poly(A) RNA = 3.56×10^6

" " " " no additions = 0.397×10^6

Net incorporation directed by P1.17 poly(A) RNA= 3.16×10^6 cpm/assay

Assuming 60% efficiency of detection of radioactivity,

Actual net incorporation by P1.17 poly(A) RNA = 5.27×10^6 dpm/assay

Specific activity of L(^{35}S) methionine = 500Ci/mmole

= 1.1×10^6 dpm/pmole

pmoles L(^{35}S) methionine incorporated/assay = $\frac{5.27 \times 10^6}{1.1 \times 10^6}$

= 4.79 pmoles

Also, pmoles L(^{35}S) methionine added = 9.46

and pmoles unlabelled endogenous methionine/assay = 700

$\therefore$ Ratio of (^{32}S) : (^{35}S) = $\frac{700}{9.46}$

$\therefore$/ = 74

$\therefore$ Total pmoles methionine incorporated/assay = 74 x 4.79

= 354.46

Assuming 6 methionine/average protein

No. of pmoles protein made = 59.08

Now, No. of µg poly(A) RNA added/assay = 0.35

Ave. MW of mRNA = 4.5×10^5

$\therefore$ No. of pmoles mRNA/assay = $\frac{3.5 \times 10^{-7}}{4.5 \times 10^5}$

= 0.77

$\therefore$ No. of pmoles protein synthesized/pmole mRNA added = 76

= No. of rounds of translation/mRNA molecule

2. Using 5563 poly(A) RNA, the best figure obtained was 43.6 rounds of translation/mRNA molecule.

The best and worst Pl.17 results varied by about a factor of 4 whilst those of 5563 varied by a factor of 7. It should be emphasised that although the figures quoted above are the best results obtained, they are probably underestimates. As such, they nevertheless indicate the high activity of the system in re-initiation of protein synthesis. The most comparable results published are those of Palmiter (1974) who obtained values of between 56 and 110 rounds of translation of globin mRNA and between 18 and 45 rounds of translation for ovalbumin mRNA in reticulocyte lysate (18 using the above method).

5. Cell-labelling experiments/.

5. Cell-labelling experiments.

An accurate knowledge of the percentage of the protein synthetic capacity of myeloma cells devoted to Ig production was essential in order to assess the significance of the level of immunoreactive material synthesized by the M.D.L. cell-free protein synthesizing system in response to exogenous messenger-containing material (polysomes or poly(A) RNA).

5.1.1. Pl.17 cells labelled with L-(^{3}H) leucine.

Pl.17 cells were incubated in RPMI medium lacking leucine with 250μCi L-(^{3}H) leucine and lysed as described in Methods, Section 2.3. Percentage Ig production was measured as 16.27% after labelling for 5 minutes and 14.6% after labelling for 90 minutes. Second immune precipitations indicated that almost all immunoprecipitable material was removed by a single immunoprecipitation. The results are shown on Table 10 together with results of a labelling of 5563 cells carried out simultaneously. Total products and specific precipitates separated by PAGE are shown in Fig. 18.

5.1.2. Pl.17 cells labelled with L-(^{35}S) methionine.

Pl.17 cells were incubated in RPMI medium lacking methionine with L-(^{35}S) methionine and lysed as described in Methods, Section 2.3. Percentage Ig production was measured as 17.4% after incubation for 60 minutes (compare with figures using L-(^{3}H) leucine). Results are shown in Table 11, together with results of a labelling of 5563 cells carried out simultaneously. Total products and immunoprecipitates are shown in Fig. 19.

5.2.1./

TABLE 10: Radioactive labelling of Pl.17 and 5563 cells with L-(^{3}H) leucine.

Cell Line	Description of sample	Volume precipitated (µl)	Total TCA precipitable counts/min	Specifically precipitable 1 counts/min	N.R.S. precipitable1 counts/min	Specifically precipitable2 counts/min	N.R.S. precipitable2 counts/min	%age production
Pl.17	5' cell lysate	50	158818	21541	1089	5683	-	16.27
Pl.17	90' cell lysate	10	175130	26211	695	607	504	14.6
Pl.17	90' secreted	50	26687	11380	204	97	226	42
5563	5' cell lysate	50	307927	24026	1460	1870	922	7.6
5563	90' cell lysate	10	139617	9404	752	674	367	6.4
5563	90' secreted	50	10671	4100	228	454	111	39.5

TABLE 10: Radioactive labelling of P1.17 & 5563 cells with L-(^{3}H) leucine

Legend:

2×10^{7} P1.17 or 5563 cells (>90% viable) were incubated with 200μCi L-(^{3}H) leucine as described in Methods for 5 min or 90 min. After incubation, cell lysate was prepared from both incubations of each cell line and supernatant retained from the 90 min labelling of both cell lines. Duplicate immunoprecipitation reactions, using 5μl anti-5563 antiserum (or 5μl normal rabbit serum), followed by 100μl anti-rabbit Ig antiserum were performed on duplicate aliquots of the various fractions, and the immunoprecipitates isolated (Methods 11.2.2(b)). After the first immunoprecipitation, a second immunoprecipitation was carried out on the supernatant remaining and the immunoprecipitate isolated as before. Total TCA-precipitable material was obtained as described in Methods 8.2.2. Samples of both TCA- and immuno-precipitates were measured for radioactivity as described in Methods 8.1.2. Percentage production was calculated as

$$\frac{\text{Total Specific counts/min} - \text{total NRS counts/min}}{\text{TCA counts/min}} \times 100$$

TABLE 11: Radioactive labelling of Pl.17 and 5563 cells with L-(^{35}S) methionine.

Cell fraction	Total TCA cpm/5µl	Sp ppte I cpm/5µl	NRS I cpm/5µl	Sp-normal I cpm/5µl	Sp ppte II cpm/5µl	NRS II cpm/5µl	Sp-normal II cpm/5µl	Total Sp pptble cpm/5µl	% production or % precipitable
Pl cell lysate	331684	59591	1936	57655	n.d.	n.d.	n.d.	57655	17.4
Pl secreted	25478	30300	1886	28414	9081	1230	7851	36265	"100"
5563 cell lysate	384574	47984	6717	41267	n.d.	n.d.	n.d.	41267	10.73
5563 secreted	39465	28412	2076	26336	10625	1077	9548	35884	90

n.d. = not determined

Cells were labelled with 200µCi L-(^{35}S) methionine, as in Methods 2.2. Incubation was for 60 min. Cell lysates and supernates were prepared as in Methods 2.3. Immunoprecipitation reactions and TCA precipitations were carried out on 5µl aliquots. After the initial immunoprecipitations, the supernatant remaining was subjected to a further immunoprecipitation. Radioactivity was measured as described in Methods 8.1.2.

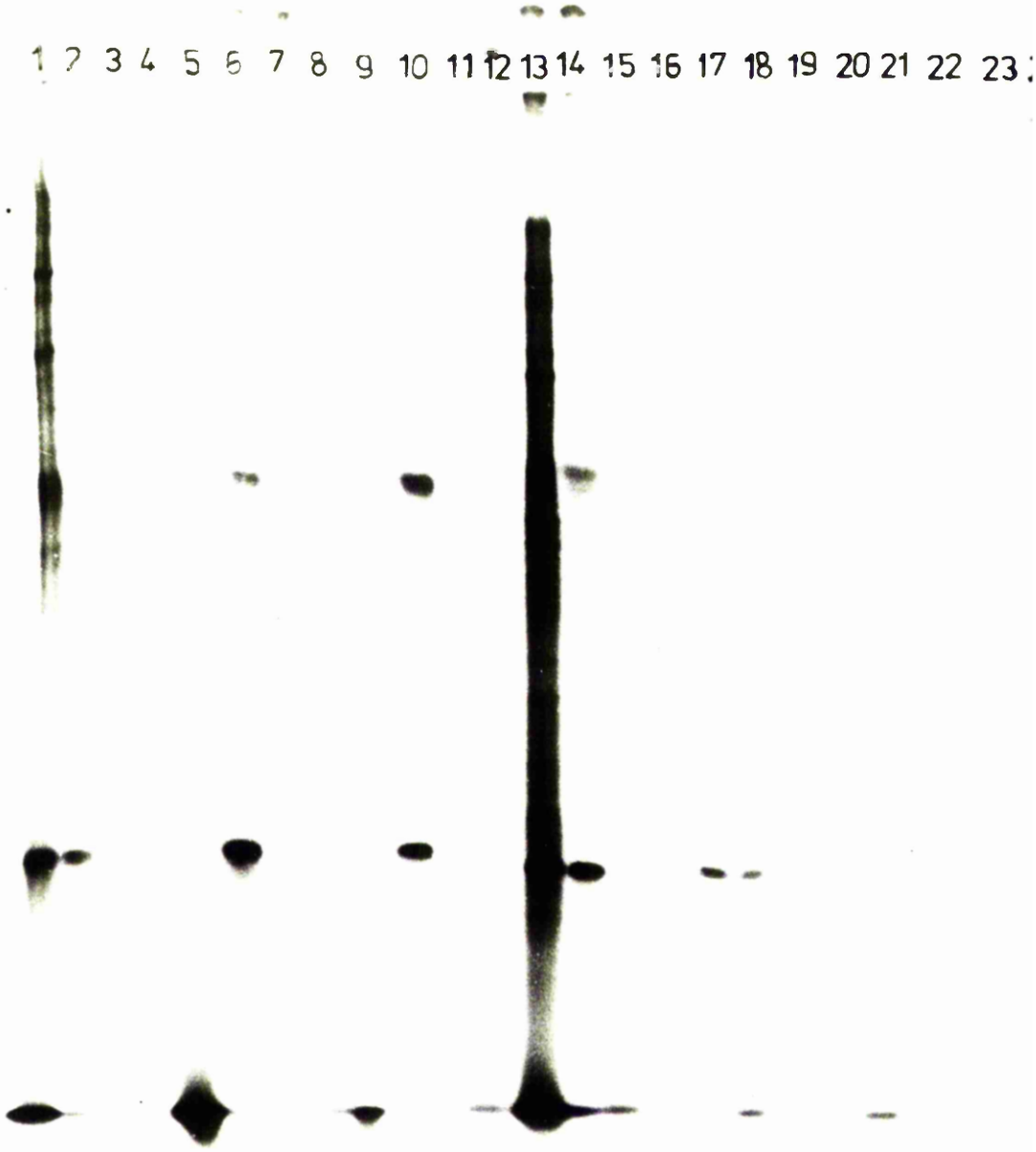

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23

FIG. 18: PAGE analysis of 5563 and P1.17 cellular products, radioactively labelled with L-(^{3}H) leucine.

(see Table 10).

5563 or P1.17 cells (10^{7} cells/labelling) were labelled with L-(^{3}H) leucine as described in Table 10. Aliquots were subjected to indirect immunoprecipitation as described in Methods 11.2.2.(b). Immunoprecipitates were washed, re-dissolved in 9M urea, and small aliquots were analysed by PAGE and fluorography (Methods 9.2.5. and 8.3.1.). Equivalent amounts of both cell lysate and cell supernatant fractions were similarly analysed. In some wells, samples were lost at TCA precipitation. Normal rabbit serum controls were performed to measure non-specific precipitation.

Well	Description
1	P1.17 5' cell lysate - total TCA products
2	P1.17 5' cell lysate - 1st specific ppn.
3	P1.17 5' cell lysate - 2nd specific ppn.
4	P1.17 5' cell lysate - NRS ppn.
5	P1.17 90' cell lysate - total TCA products
6	P1.17 90' cell lysate - 1st specific ppn.
7	P1.17 90' cell lysate - 2nd specific ppn.
8	P1.17 90' cell lysate - NRS ppn.
9	P1.17 90' secreted - total TCA products
10	P1.17 90' secreted - 1st specific ppn.
11	P1.17 90' secreted - 2nd specific ppn.
12	P1.17 90' secreted - NRS ppn.

13/

Well	Description
13	5563 5' cell lysate - total TCA products
14	5563 5' cell lysate - 1st specific ppn.
15	5563 5' cell lysate - 2nd specific ppn.
16	5563 5' cell lysate - NRS ppn.
17	5563 90' cell lysate - total TCA products
18	5563 90' cell lysate - 1st specific ppn.
19	5563 90' cell lysate - 2nd specific ppn.
20	5563 90' cell lysate - NRS ppn.
21	5563 90' secreted - total TCA product
22	5563 90' secreted - 1st specific ppn.
23	5563 90' secreted - 2nd specific ppn.
24	5563 90' secreted - NRS ppn.

5.2.1. 5563 cells labelled with L-(^{3}H) leucine.

5563 cells were incubated in RPMI medium lacking leucine with L-(^{3}H) leucine and lysed as described in Methods Section 2.3. Percentage Ig production was measured as 7.6% after labelling for 5 minutes and as 6.4% after labelling for 90 minutes. Second precipitations indicated that almost all immunoprecipitable material was removed by a single immunoprecipitation. The results are shown on Table 10. (see also Fig. 18).

5.2.2. 5563 cells labelled with L-(^{35}S) methionine.

5563 cells were incubated with L-(^{35}S) methionine and lysed as described in Methods, Section 2.3. Percentage Ig production was measured as 10.73% after labelling for 60 minutes (compare with figures for L(^{3}H) leucine labelling). Results are shown in Table 11. (see also Fig. 19).

5.2.3. 5563 cells labelled with L-(^{35}S) methionine in the presence of varying concentrations of 2-deoxyglucose.

5563 cells were incubated with L-(^{35}S) methionine in PBSA in the presence of varying concentrations of 2-deoxyglucose as described in Methods,2.4. Percentage Ig production in the absence of 2-deoxyglucose was measured as 7%. The percentage Ig production in the presence of varying concentrations of 2-deoxyglucose is shown in Table 12 (see also Fig. 35).

Incorporation of radioactivity into total TCA pptble. material decreased to a constant level of 20% of uninhibited values, whilst specifically pptble. radioactivity levels continued to decrease as the concentration of 2-deoxyglucose increased.

TABLE 12: Radioactive labelling of 5563 cells in the presence of 2-deoxyglucose.

{2-deoxyglucose} (μM)	Total TCA cpm/25μl	% age of control	Sp pptble cpm/25μl	NRS cpm/25μl	Sp-normal cpm/25μl	% age of total	% age of control
0	445700	100	51000	19950	31100	7.00	100
10	142400	32	14450	9300	5150	3.62	16.55
20	96700	21.7	16200	9350	6900	7.11	22.08
40	115000	25.8	14000	8100	5900	5.12	18.93
60	95700	21.5	10300	6600	3800	3.93	12.09
75	92900	20.8	10800	8100	2700	2.93	8.74

6 samples of 10^6 5563 cells were radioactively labelled with 200μCi L-(^{35}S) methionine as described in Methods 2.4. in the presence of varying concentrations of 2-deoxyglucose. Incubation was for 90 min. Cell lysates were prepared as described in Methods 2.3. and immunoprecipitation reactions (Methods 11.2.2.) and TCA precipitations carried out on 25μl aliquots. Radioactivity was measured as in Methods 8.1.2.

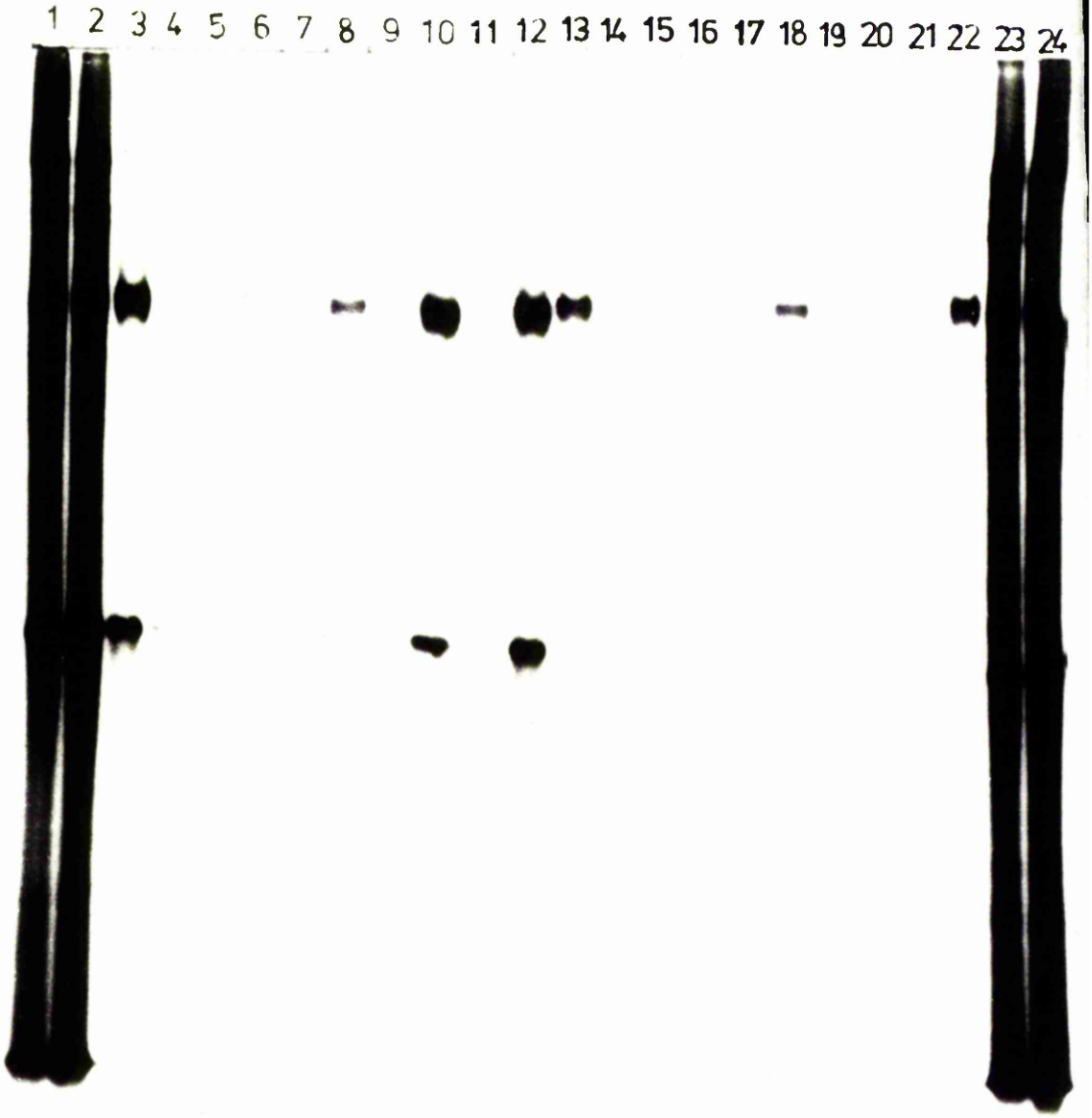

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

FIG. 19: PAGE analysis of 5563 and Pl.17 cellular products radioactively labelled with L-(^{35}S) methionine (see Table 11).

5563 and Pl.17 cells were labelled with L-(^{35}S) methionine as described in Table 11. Direct and indirect immunoprecipitation was carried out on cell lysates and cell supernatant material as described in Methods 11.2.1. and 11.2.2(b), respectively. Total labelled material and fractions of immunoprecipitable material were analysed by PAGE and fluorography as described in Methods 9.2.5. and 8.3.1., respectively.

Well	Description
L	Pl.17 5' cell lysate - total products
2	Pl.17 90' cell lysate - total products
3	Pl.17 90' secreted - total products
4	Pl.17 90' cell lysate - indirect ppte. (1/25 ppte).
5	Pl.17 90' cell lysate - NRS ppte. (1/25 ppte).
6	Pl.17 90' cell lysate - direct ppte. + Oμl 5563 (1/2 ppte).
7	Pl.17 90' cell lysate - NRS ppte. + Oμl 5563 (1/2 ppte).
8	Pl.17 90' cell lysate - direct ppte. + 2.5μl 5563 (1/2 ppte).
9	Pl.17 90' cell lysate ; NRS ppte. + 2.5μl 5563 (1/2 ppte).
10	Pl.17 90' cell lysate - direct ppte. + 5μl 5563 (1/2 ppte).
11	Pl.17 90' cell lysate - NRS ppte. + 5μl 5563 (1/2 ppte).
12	Pl.17 90' secreted - total products
13	5563 90' secreted - total products
14	5563 90' cell lysate - indirect ppte (1/25 ppte).
15	5563 90' cell lysate - NRS ppte (1/25 ppte).
16	5563 90' cell lysate - direct ppte + 5μl 5563 (1/2 ppte).

17/

Well	Description
17	5563 90' cell lysate - NRS ppte + 5µl 5563 (1/2 ppte).
18	5563 90' cell lysate - direct ppte + 2.5µl 5563 (1/2 ppte).
19	5563 90' cell lysate - NRS ppte + 2.5µl 5563 (1/2 ppte).
20	5563 90' cell lysate - direct ppte 0µl (1/2 ppte).
21	5563 90' cell lysate - NRS ppte 0µl 5563 (1/2 ppte).
22	5563 90' secreted - total products
23	5563 90' cell lysate - total products
24	5563 5' cell lysate - total products

5.3. SAMM 368 cells labelled with L-(^{35}S) methionine.

SAMM 368 cells were incubated with L-(^{35}S) methionine and lysed as described in Methods, Section 2. Percentage Ig production after 15 minutes and 90 minutes was measured using three specific rabbit antisera directed against SAMM 368 α-heavy chains, γ-heavy chains and κ-light chains respectively. The anti-α antiserum showed some cross-reactivity with γ-heavy chains (Dr. T.R. Mosmann, personal communication). The precipitation with anti-κ antiserum was not complete. Results are shown in Table 13. Total products and specific precipitates are shown in Fig. 20.

6. Relative production of H and L chains in cultured cells.

6.1. Introduction.

Comparison of cell-free synthesized putative H and L chains with cellularly secreted H and L chains of Pl.17 had revealed significant differences in the relative production of H and L chains. This conclusion was based on the relative amounts of radioactivity detected in H and L-chain like material separated by PAGE (Results Section 2). This difference might reflect a difference in H:L chain ratio between intracellular and secreted material under normal culture conditions. Alternatively, it may have been an artefact caused by trapping of material at the top of polyacrylamide gels. Both these possibilities were investigated.

The H:L chain ratio, both intra- and extracellular in Pl.17 myeloma cell cultures was investigated. Several different methods of solubilisation of immune precipitates were also used to test whether/

TABLE 13: Radioactive labelling of SAMM 368 cells with L-(^{35}S) methionine.

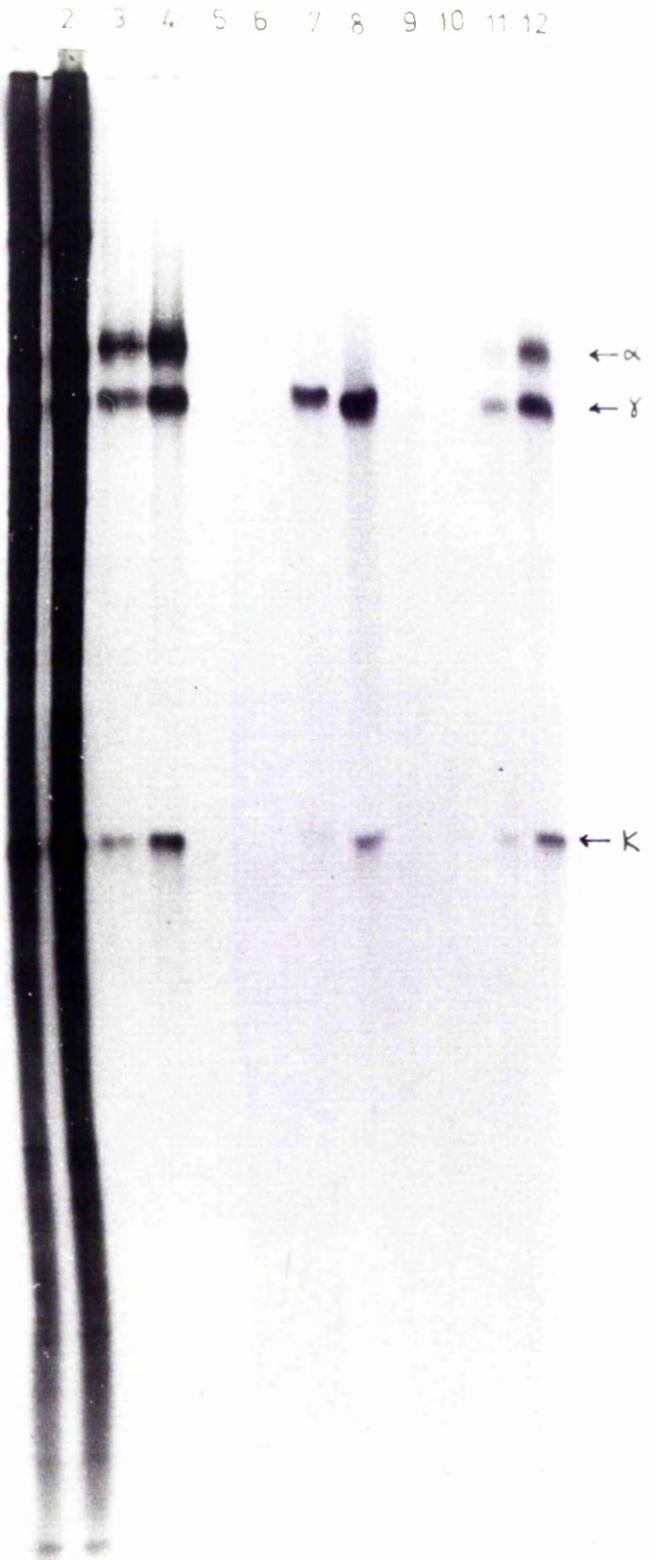
Time of labelling	Total TCA pptble cpm	cpm pptble by anti α	% of total	anti γ	% of total	anti κ	% of total	NRS	% of total
15 min	229000	35200	15.37	14600	6.40	20800	9.09	3000	1.32
90 min	412000	66600	16.17	32500	7.90	42900	10.40	4600	1.13

SAMM 368 cells were radioactively labelled with 200μCi L-(^{35}S) methionine as described in Methods 2.2. Cell lysates were prepared of cells labelled for 5 minutes and 90 minutes (Methods 2.3 Immunoprecipitation reactions were carried out as in Methods 11.2.2. Radioactivity in immune and TCA precipitates was measured as in Methods 8.1.2.

FIG. 20: PAGE analysis of SAMM 368 cellular products radioactively labelled with L-(^{35}S) methionine (see Table 13).

SAMM 368 cells were labelled with L-(^{35}S) methionine as described in Table 13. Indirect immunoprecipitation was carried out on cell lysate aliquots as described in Methods 11.2.2(b). Total labelled material and equivalent fractions of immunoprecipitable material were analysed by PAGE and fluorography, as described in Methods 9.2.5. and 8.3.1., respectively.

Well	Description
1	SAMM 368 15' cell lysate - total products
2	SAMM 368 60' cell lysate - total products
3	SAMM 368 15' cell lysate - anti α indirect ppte.
4	SAMM 368 60' cell lysate - anti α indirect ppte.
5	SAMM 368 15' cell lysate - anti γ indirect ppte.
6	SAMM 368 60' cell lysate - anti γ indirect ppte.
7	SAMM 368 15' cell lysate - anti κ indirect ppte.
8	SAMM 368 60' cell lysate - anti κ indirect ppte.
9	SAMM 368 15' cell lysate - NRS indirect ppte.
10	SAMM 368 60' cell lysate - NRS indirect ppte.


2 3 4 5 6 7 8 9 10 11 12
←α
←γ
←K

whether the problem of trapped radioactivity could be improved by better solubilisation. The possibility that a pool of light chain existed, not totally precipitable with antiserum directed against whole Pl Ig molecules, was investigated using an antiserum directed against L(κ) chains. Finally, conditions of both normal growth and growth under the inhibiting effect of cycloheximide were investigated.

Cells were labelled for a long period (4.5 hours) to allow a substantial amount of secretion of Ig and also to ensure steady-state synthesis of Ig. Viability of cells was measured at appropriate points. Cells remained 100% viable during pelleting and resuspension and until the addition of radioactively labelled methionine. After incubation, cell viability had dropped to 70%.

Labelling of cells was followed by preparation of cell lysates and cell supernatant as described in Methods 2.2. and 2.3. Immune precipitate reactions were carried out on small volumes of cell lysate (60µl) and on 10fold greater volumes of cell supernatant (600µl) as described in Methods 11.2.2. Radioactivity in each precipitate was detected by liquid scintillation spectrometry using triton/ toluene/PPO as the solvent/fluor system. Results are shown in Table 14.

6.2. Incorporation of L-(^{35}S) methionine into specifically precipitable material.

Antiserum (5µl), directed against Pl Ig precipitated all immunoreactive material from cell lysate samples, both in the absence and presence of cycloheximide. This was suggested by graphical methods (data not shown)

TABLE 14: Comparison of incorporation of radioactivity into specifically immunoprecipitable material by cultured cells in the absence and presence of cycloheximide.

Volume and nature of antiserum	Cells labelled normally		Cells labelled in presence of cycloheximide		cpm cyc / cpm no cyc	cpm αPl / cpm ακ a) no cycloheximide b) cycloheximide present	
	counts/ppte ($X10^{-4}$)	counts/ppte -NRS ($X10^{-4}$)	counts/ppte ($X10^{-4}$)	counts/ppte -NRS ($X10^{-4}$)		a)	b)
a) secreted							
5µl αPl	36.8 ± 9.7	35.4 ± 9.7	27 ± 4.7	26 ± 4.7	0.735	3.8	3.01
1µl αPl	19.3	-	12.5	-	0.65		
0.2µl αPl	6.82	-	5.02	-	0.735		
5µl ακ	10.66± 1.3	9.3 ± 1.3	9.66 ± 1.3	8.63 ± 1.3	0.91		
5µl NRS	1.36	0	1.02	0	0.76		
b) cell lysate							
5µl αPl	14.3 ± 4.6	11.8 ± 4.6	9.08 ± 0.66	7.19 ± 0.66	0.61		
1µl αPl	11.2	-	6.84	-	0.61		
0.2µl αPl	3.7	-	-	-			
5µl ακ	16.3 ± 2.1	13.8 ± 2.1	7.35 ± 1.6	5.46 ± 1.6	0.39	0.85	1.3
5µl NRS	2.5	0	1.89	0	0.76		

TABLE 14: Comparison of incorporation of radioactivity into specifically immunoprecipitable material by cultured cells in the absence and presence of cycloheximide.

Legend:

2×10^7 cells were incubated with 170μCi L-(^{35}S) methionine in either the absence or presence of cycloheximide (200μg/ml) for 4.5 hours, as described in Methods 2.2. and 2.3. After incubation, viability was ∿70%. Triplicate or duplicate indirect immunoprecipitates, using the antisera noted, were performed as described in Methods 11.2.2. 60μl aliquots of cell lysate and 600μl aliquots of cell supernatant were immunoprecipitated.

Abbreviations:

NRS = Normal Rabbit Serum

αPl = Rabbit antiserum against Pl.17 Ig

ακ = Rabbit antiserum against mouse K L chains

, plotting radioactivity precipitated against volume of antiserum added, and corroborated by the much higher level of radioactivity in specifically precipitated material from cell medium using the same amount of antiserum. Both intra- and extracellular Ig would be expected to have similar specific activity, implying that the higher level of radioactivity precipitated extracellularly was due to the presence of more protein. Extracellular precipitation was not necessarily quantitative but must have been approaching complete precipitation of immunoreactive material, as judged by the shape of the titration curve obtained

6.3. Effect of cycloheximide on incorporation into specifically precipitable material.

Cycloheximide (0.2µg/ml) lowered the level of incorporation of radioactivity into specifically precipitable material by approximately 25% in extracellular material and by 40% in intracellular material in this experiment. (Table 14) The only major exception to this was the reduction of anti-κ precipitable material by 60% intracellularly but by only 10% extracellularly. The difference between intra-and extracellular inhibition may not be significant. Cycloheximide at the level used, however, did not block protein synthesis or secretion completely.

6.4. Comparison of precipitation by αPl and ακ antisera.

As Table 14 demonstrated, anti-κ antiserum precipitated considerably/

considerably less secreted material than did anti-Pl antiserum, approximately by a factor of 3, both in the presence & absence of cycloheximide. The presence of cycloheximide reduced the relative difference in precipitable radioactivity by approximately 20%. With intracellular material, anti-κ antiserum precipitated more material than anti-Pl antiserum in cells grown under normal conditions, but in the presence of cycloheximide, this was reversed.

6.5. Methods of solubilisation of immune precipitates.

Samples of immune precipitates of intra- and extracellular material from cells grown normally were reduced and alkylated in each of the following buffers before separation by PAGE:-

1. 8M urea in 0.2M Tris HCl pH 8.5
2. 8M urea, 2% (w/v) SDS in 0.2M Tris HCl pH 8.5
3. 2% (w/v) SDS in 0.2M Tris HCl pH 8.5

Samples were reduced and alkylated as described in Methods, 9.1.3., acetone precipitated and redissolved in 1OM urea before loading on the gels. As can be seen from Figs. 22 A-F, secreted material was resolved with least trapping using 8M urea/0.2M Tris pH 8.5 for reduction and alkylation, although of the three solvents, only 8M urea/2% (w/v) SDS/0.2M Tris pH 8.5 showed significant trapping of material. Intracellular specific precipitates were resolved with least trapping using 2% (w/v) SDS in 0.2M Tris HCl pH 8.5, although none of the three solutions eliminated trapping problems completely. Estimations of H:L chain radioactivity in extracellular material varied from 2.54 to 2.79. Estimates of the ratio of/

FIG. 22: PAGE analysis of immunoprecipitates of intra- and extracellular material from P1.17 cells, radioactively labelled with L-(^{35}S) methionine.

Cells were incubated with 170μCi L-(^{35}S) methionine as described in Methods 2.2., for 4.5 hours. Cell lysates and cell supernatant were prepared (Methods 2.3.) and immunoprecipitation was carried out (Methods 11.2.2.). The immunoprecipitates were re-dissolved, after 3 washes with 0.7% (v/v) NP-40 in PBS, in several Tris HCl buffer solutions (see below). Reduction and alkylation (Methods 9.1.3 preceded application to 7.5% SDS-phosphate polyacrylamide tube gels with NN' methylene bisacrylamide as cross-linker (Methods 9.1.1.). Electrophoresis, gel slicing and liquid scintillation counting were carried out (Methods 9.1.4., 9.1.5. 8.1.3., respectively).

A: Secreted immunoprecipitable material in 2% SDS: 0.2M Tris HCl pH 8.5

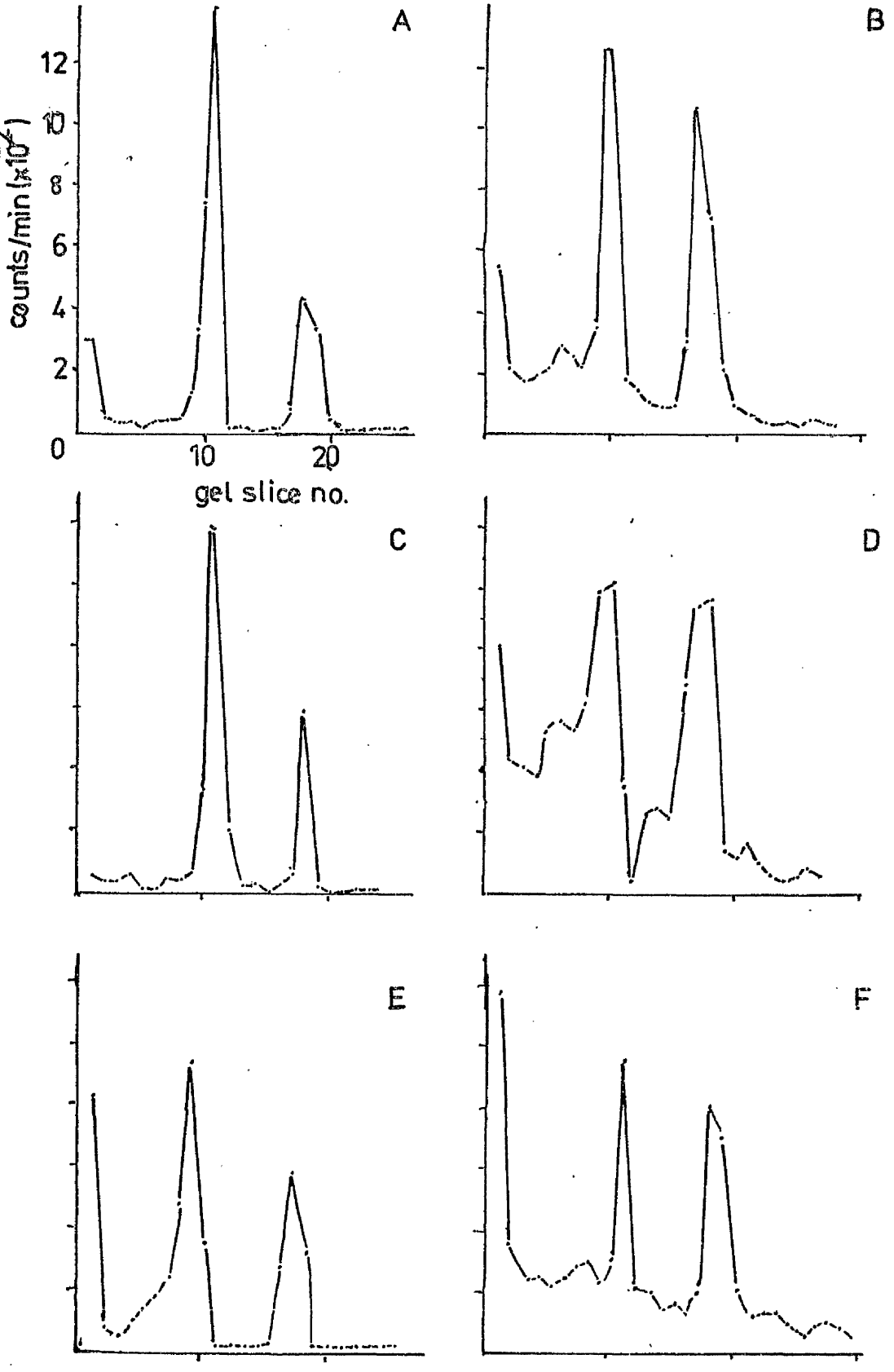
B: Intracellular immunoprecipitable material in 2% SDS: 0.2M Tris HCl pH 8.5

C: Secreted immunoprecipitable material in 8M urea: 0.2M Tris HCl pH 8.5

D: Intracellular immunoprecipitable material in 8M urea: 0.2M Tris HCl pH 8.5

E: Secreted immunoprecipitable material in 8M urea:2% SDS: 0.2M Tris HCl pH 8.5

F: Intracellular immunoprecipitable material in 8M urea: 2%SDS: 0.2M Tris HCl pH 8.5


A
B
C
D
E
F
counts/min (×10⁻²)
12
10
8
6
4
2
0
10
20
gel slice no.

of H:L chain radioactivity intracellularly may be more affected by trapping problems, but an average of 0.99 was obtained.

The cells were radioactively labelled with L-(^{35}S) methionine. Previous studies by other workers (Parkhouse, 1969) have shown the H chain of Pl to contain 8 methionines whilst the L chain contained 3.5 methionines. Hence on a molar basis, the ratio of radioactivity in H and L chains (assuming balanced production) would be 2.66 (assuming 3 L chain methionine residues) or 2 (assuming 4 L chain methionine residues). This former figure was in agreement with the ratio found on gels of secreted Pl Ig. Intracellular Ig, however, showed a much lower H:L chain ratio suggesting the presence of a large intracellular pool of L chain. This pool may be produced either by unbalanced production of H and L chains or by selective degradation of H chains. The pool was estimated, on a molar basis to be 130% above the level for balanced H and L chain production. Such imbalances in H and L chain production have been described by other workers (Stevens and Williamson,1974). Similar findings with specifically precipitable material from reticulocyte lysate containing myeloma polysomes(although using a different labelled amino acid) suggested that cell-free translation was representative of cellular events (see Section 2).

6.6. Effect of cycloheximide on Ig synthesis relative to total protein synthesis.

The effect of cycloheximide on the synthesis of Ig relative to total protein synthesis was investigated. Pl.17 cells were labelled/

labelled for either 30 min or 180 min with 250μCi L-(^{3}H) leucine in the presence or absence of cycloheximide at 0.2μg/ml. Lysates were prepared and both TCA precipitable and specifically precipitable material was measured for radioactivity. As the results in Table 15 show, the synthesis of antigenically active material was inhibited to approximately the same extent as total protein synthesis by cycloheximide. This is in agreement with the results of other workers (Nuss and Koch, 1976; Stevens and Williamson, 1974, both using emetine).

7. Translation in Messenger-Dependent Lysate.

After optimisation of the M.D.L. system for translation of myeloma mRNA, investigation of the products of translation in M.D.L. was carried out. This was done mainly by electrophoretic analysis on polyacrylamide slab gels.

7.1. Comparison of endogenous products of reticulocyte lysate and M.D.L.

Samples of radioactively labelled reticulocyte lysate assays and M.D.L. assays were analysed by PAGE in order to investigate the effect of nuclease action on reticulocyte lysate. They were also used to look for endogenous radioactively labelled species present in the M.D.L. Such species might interfere with interpretation of results obtained using myeloma polysomes or poly(A) RNA. As Fig. 23 shows, the reticulocyte lysate made several proteins (Well2), the most prominent of which being proteins of apparent MW 64,000,and 18,000/

TABLE 15: Effect of cycloheximide on relative synthesis of TCA-precipitable and specifically precipitable material in Pl.17 cells.

Description	0.2µg/ml cycloheximide	TCA precipitable counts/min($X10^{-3}$)	% age change	Specifically precipitable counts/min(10^{-3})	NRS precipitable counts/min($X10^{-3}$)	Specific-NRS counts/min(10^{-3})	% age change
Cell lysate 30 min	-	28.3	- 79	4.66	0.68	4.0	- 74
Cell lysate 30 min	+	6.2		2.77	1.73	1.04	
Cell lysate 180 min	-	311.68	-71	13.72	1.30	12.41	-58
Cell lysate 180 min	+	76.73		7.71	2.49	5.22	
Secreted 180 min	-	34.13	-61	9.46	2.56	6.90	-68
Secreted 180 min	+	13.20		3.89	2.56	1.33	

TABLE 15: Effect of cycloheximide on relative synthesis of TCA-precipitable and specifically precipitable material in Pl.17 cells.

Legend:

Aliquots of 4 x 10^7 Pl.17 cells were incubated as described in Methods 2.2. for either 30 min or 180 min. 250μCi L-(^{3}H) leucine was present in each incubation. Incubation was performed in either the presence or absence of 0.2μg/ml cycloheximide. Cell lysates and cell supernatant were prepared (Methods 2.3.) and both TCA-precipitable and specifically precipitable material isolated (Methods 1.2.2.), from 10μl (cell lysate) or 100μl (cell superantant) aliquots. Radioactivity incorporated was measured by liquid scintillation counting (Methods 8.1.2.).

FIG. 23: PAGE analysis of reticulocyte lysate and M.D.L. endogenous radioactively labelled products; analysis of radioactively labelled products synthesized in M.D.L. in response to the addition of polysomes or poly(A) RNA from myeloma cells.

Standard 50µl M.D.L. assays containing 5µCi L-(^{3}H) leucine were incubated as described in Methods 7.2., with the additions noted below. After incubation, 5µl aliquots were prepared for electrophoresis as described in Methods 9.2.5., and PAGE was carried out at 15mA/gel for 8 hours. The gel was processed and fluorography performed as described in Methods 8.3.1., except that the X-ray film was briefly exposed to light to improve sensitivity (Bonner and Laskey, 1974). Exposure was for four days. Numbers of counts/min loaded per gel well were measured in duplicate 5µl aliquots as described in Methods 8.2.1.

Key	Well	Description	(countd/min loaded)
	1	-	
	2	Reticulocyte lysate	(34,000)
	3	M.D.L.	(330)
	4	M.D.L. + 0.2µg reticulocyte poly(A) RNA	(10,000)
	5	M.D.L.	(367)
	6	-	(-)
	7	M.D.L. + 1µg Pl.17 I polysomes	(7,235)
	8	M.D.L. + µg Pl.17 I poly(A) RNA	(6,430)
	9	-	(-)
	10	M.D.L. + µg Pl.17 II polysomes	(14,100)
	11	M.D.L. + µg Pl.17 II poly(A) RNA	(6,300)
	12	-	(-)
	13	M.D.L. + µg 5563 I polysomes	(17,000)
	14	M.D.L. + µg 5563 I poly(A) RNA	(10,650)
	15	-	(-)
	16	M.D.L. + µg 5563 II polysomes	(5,660)
	17	M.D.L. + µg 5563 II poly(A) RNA	(2,980)
	18	-	(-)

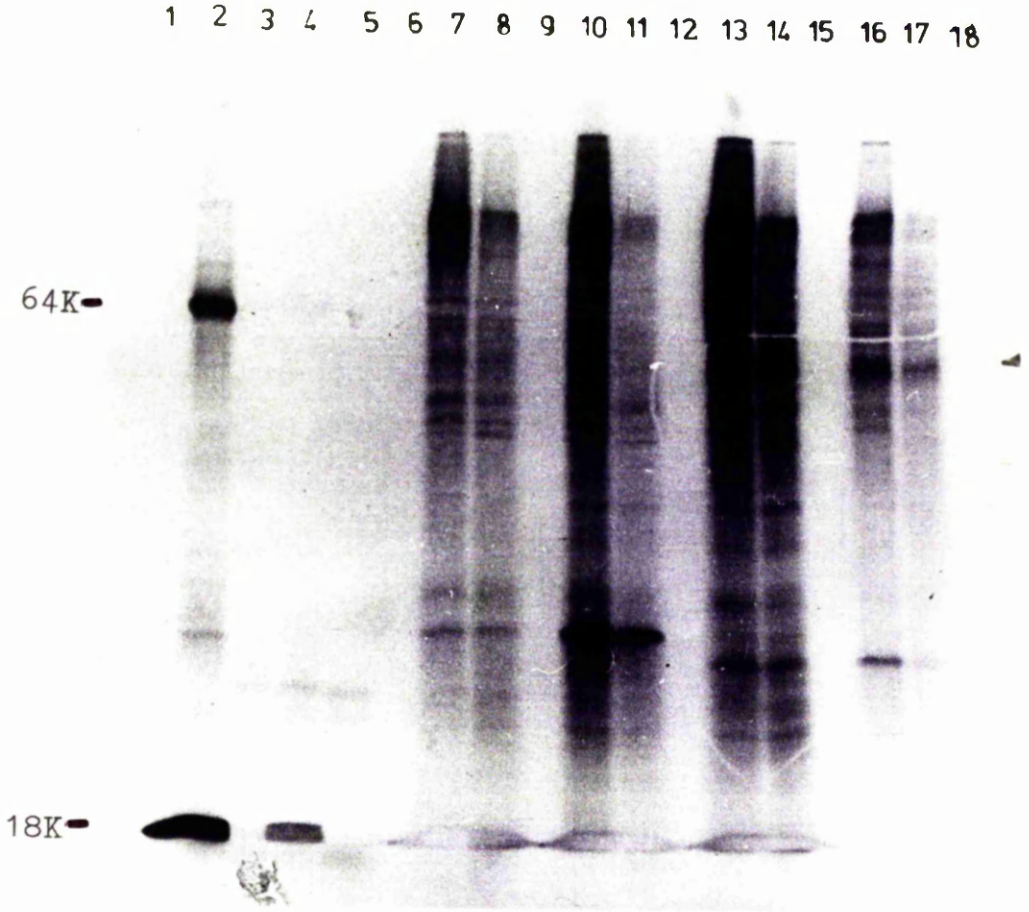
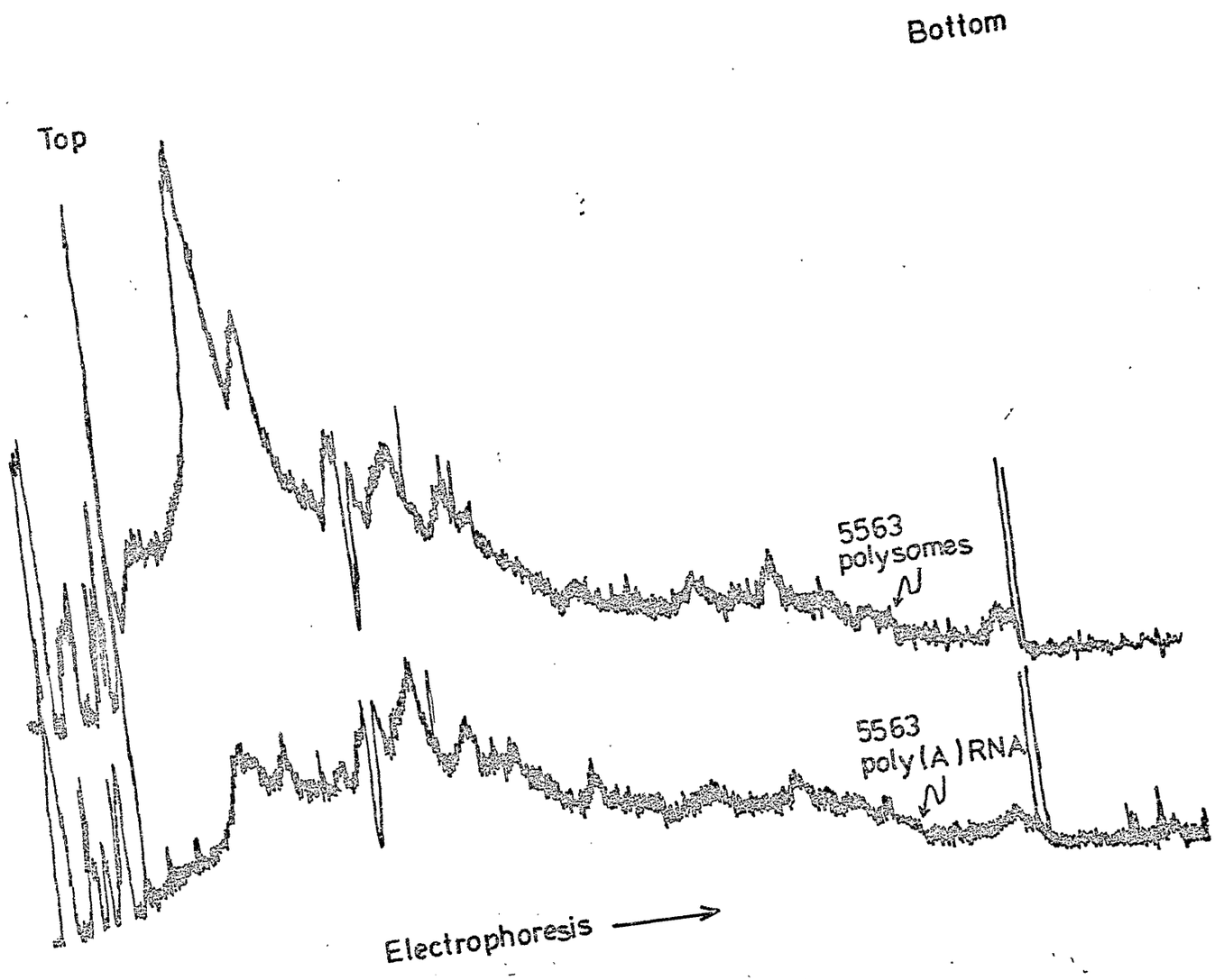

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
64K
18K

FIG. 23a: Densitometric analysis of Wells 13 and 14.

Wells 13 and 14 were scanned by a Joyce-Loebel densitometer to determine quantitatively the distribution of radioactivity. Comparisons between polysomal and poly(A) RNA-containing wells were made.

A. 5563 II polysomes and poly(A) RNA.


Bottom
Top
5563
polysomes
5563
poly(A)RNA
Electrophoresis

18,000, although many minor bands were detectable to the naked eye. Lodish and Small, 1976, have reported the presence of a prominent 64,000 MW protein in rabbit reticulocytes. Treatment with micrococcal nuclease (Wells 3,5) resulted in elimination of all reticulocyte proteins and the appearance of two fast-migrating species (mobilities 0.79 and 0.83). Addition to the system of reticulocyte poly(A) RNA restored the prominent globin band (Well 4). Treatment with micrococcal nuclease therefore removed most, but not all, endogenous protein synthetic activity. The remaining species described above were at variance with the results of Pelham and Jackson, 1976, who described proteins of approximate MW 22,000 and 42,000 in control M.D.L. assays, one of which appeared to be labelled by a non-ribosomal process. The mobilities of the labelled species observed were such that they did not interfere with interpretation of myeloma products.

7.2. Comparison of translation of myeloma polysomes and myeloma poly(A) RNA.

During the extraction of RNA from polysomes and the subsequent purification of poly(A)-containing RNA on oligo dT cellulose, there were several steps at which selective loss of messenger species was conceivable. For example, during phenol extraction, trapping of messenger species with long poly(A) tails at the phenol-buffer interface has been reported (Lee et al., 1971). In addition, loss of non-poly(A) containing mRNA species could occur on passage over oligo dT cellulose. This possible translational capacity difference between polysomes/

polysomes and poly(A) RNA was investigated by translation in the M.D.L. Four polysome preparations, consisting of the first and second polysome extractions of Pl.17 and 5563 tissue culture cells, i.e. Pl.17I, Pl.17II and 5563I, 5563II polysomes were each divided into two portions. One of each was treated by phenol extraction and oligo dT cellulose purification to yield poly(A)-containing RNA. Aliquots of each polysome sample and the appropriate poly(A) RNA fraction were assayed in the M.D.L. The resulting products were analysed by PAGE. As Fig. 23 demonstrates, the products of each polysome sample and the appropriate poly(A) RNA fraction showed apparently complete qualitative homology. When analysed densitometrically the poly(A) RNA products showed an apparent selective loss of very high MW species, which may have been artefactual. (see Fig. 23a). The loss appeared to be confined to the very high MW species, and did not appear to include products with MW < H chain. Differences between the products of Pl polysomes/poly(A) RNA and 5563 polysomes/poly(A) RNA at lower MW's were mainly seen in the faster-migrating products of both cell lines, where a prominent band in 5563 products was replaced in Pl products by another prominent band with lower mobility. These were later identified as the precursors to L chains produced by these cell-lines.

7.3. Comparison of poly(A) RNA products with cellular products.

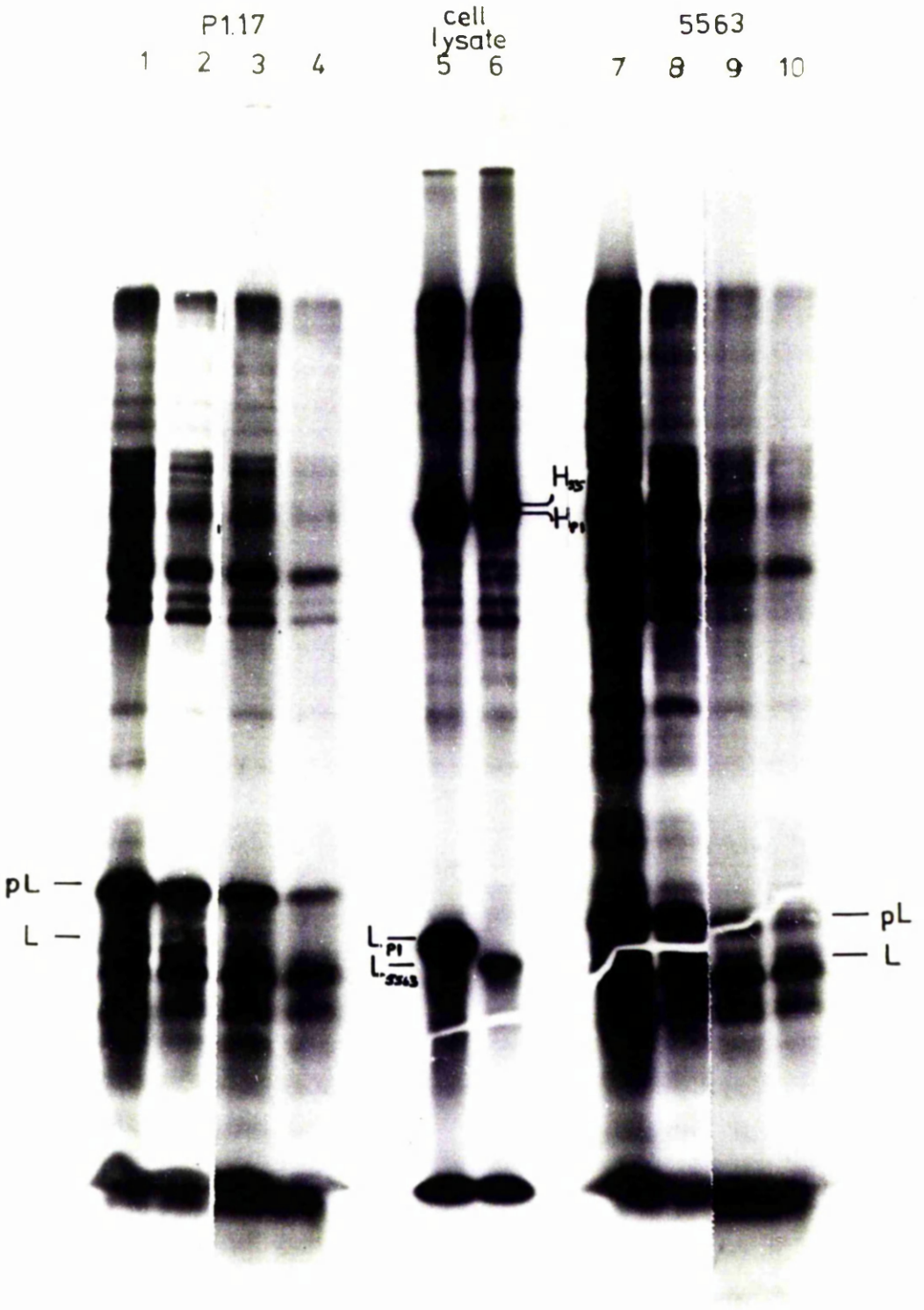
A comparison of the cellular products of myeloma cells and the M.D.L. cell-free products of myeloma poly(A) RNA was carried out by PAGE (see Fig. 24). Several observations were made:-

a/

FIG. 24: PAGE analysis of Pl.17 and 5563 poly(A) RNA-directed products from M.D.L., labelled with L-(^{35}S) methionine and comparison with L-(^{35}S) methionine labelled cellular products.

Serial dilutions of Pl.17 and 5563 poly(A) RNA were incubated with M.D.L. containing 5μCi L-(^{35}S) methionine as described in Methods 7.2. 5μl aliquots of the assays were prepared for electrophoresis as described in Methods 9.2.5. PAGE was performed at 15mA for 16 hours and the gel processed for fluorography as described in Methods 9.2.6. The processed gel was exposed to R.P. Royal X-Omat X-ray film.

Key	Well	Description	(counts/min).
	1	M.D.L. + 0.5μg Pl.17 poly(A) RNA	(29,100)
	2	" + 0.25μg " " "	(19,200)
	3	" + 0.125μg " " "	(9,000)
	4	" + 0.06μg " " "	(4,900)
	5	Pl.17 total cellular products	
	6	5563 " " "	
	7	M.D.L. + 1μg 5563 poly(A) RNA	(50,500)
	8	" + 0.5μg " " "	(24,160)
	9	" + 0.25μg" " "	(8,560)
	10	" + 0.125μg " " "	(5,725)


P1.17
cell lysate
5563
1 2 3 4 5 6 7 8 9 10
pL
L
L P1
L 5563
pL
L

a) In cellular products of both P1 and 5563, two prominent bands occur in PAGE, identified by immunoprecipitation elsewhere as the H and L chains of the respective Ig. Despite having the same approximate MW's, both the H and L chains of the two cell lines show distinct differences in mobility as shown below:-

	Mobility	
Cell-line	H	L
P1.17	0.34	0.76
5563	0.33	0.78

(see also Fig. 24).

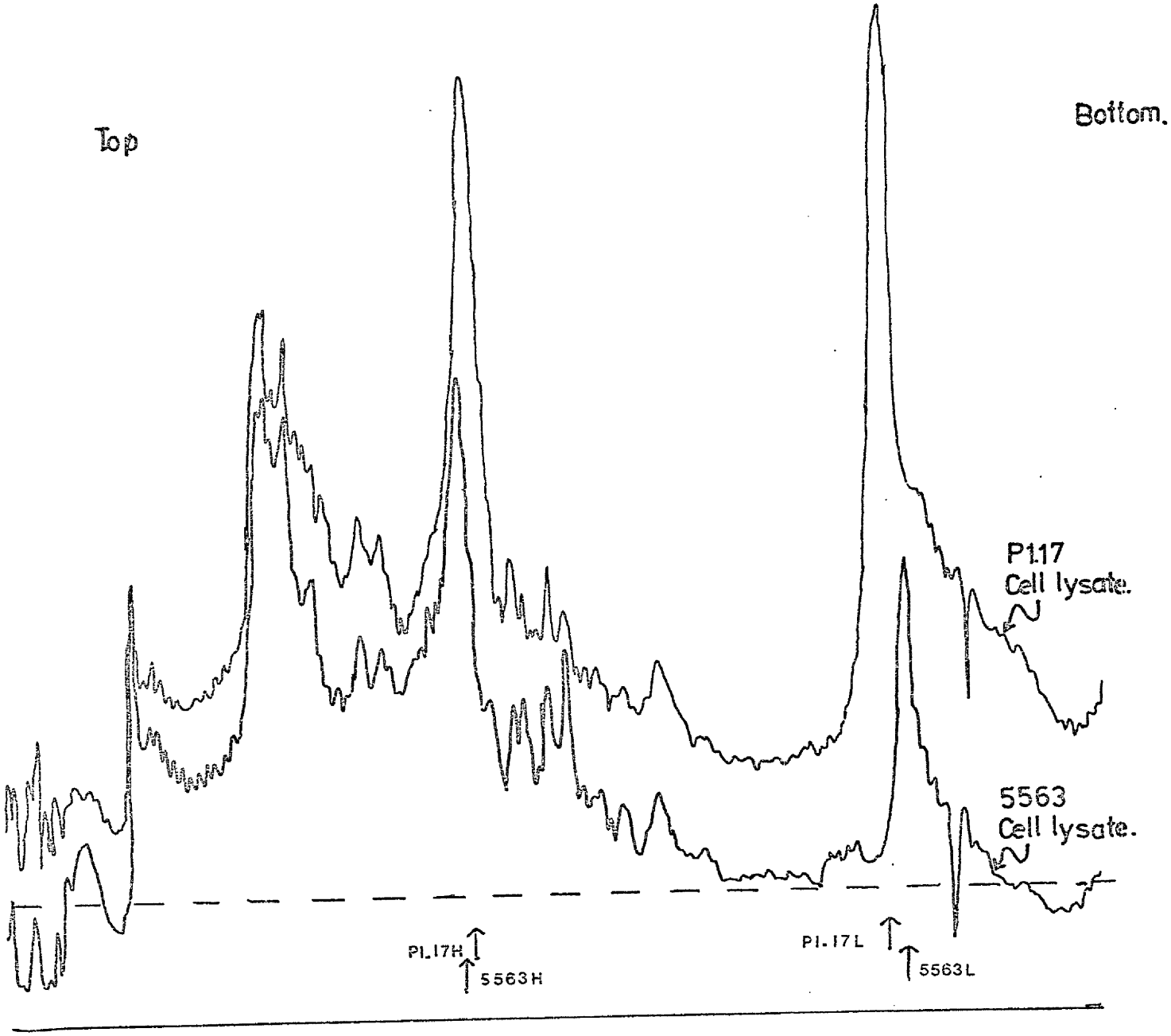
This called into question the use of molecular weight markers as a means of distinguishing the molecular weight of precursors. Instead, it was decided to use mobility as the identifying characteristic of H and L chains and their precursors. This allowed comparison of bands within the same gel but was less accurate for comparison between gels.

b) Apart from H and L chains, the proteins of the two cell lysates showed great qualitative homology, although not quantitative homology (see Fig. 24a for densitometric analysis).

c) Homologies were observed between the cellular products and the appropriate cell-free products, although there were also many differences. The most striking variations concerned the positions of the Ig chains. With both cell lines, comparison of cell lysate material and MDL-synthesized material showed the absence in M.D.L. products of the prominent band identified previously as L chain in cell lysates (see Figs. 18 and 19). In its place, a prominent band with lower mobility was observed (see Fig. 24a). This concurs with the findings of other groups with other myeloma cell types (Milstein/

FIG. 24a: Densitometric comparison of radioactively labelled cell lysates from Pl.17 and 5563 cells.

Densitometric analyses of wells 5 and 6 from Fig. 24, performed on a Joyce Loebel scanning densitometer. Positions of the H and L chains from each cell line are indicated. The trace of Pl.17 cellular products has been displaced vertically for clarity.


Top
Bottom.
P1.17
Cell lysate.
5563
Cell lysate.
P1.17H
5563H
P1.17L
5563L

Milstein et al., 1972; Swan, Aviv and Leder, 1972) and with each cell line the lowered mobility band was tentatively identified as the precursor to L chain (pre L chain). Comparison of these bands' mobilities with those of the cellular L chains are shown below.

	Mobility		
Cell line	L	pre L	%age decrease in mobility
P1.17	0.76	0.70	7.12
5563	0.78	0.74	4.64

d) In the H chain size region of the products, many bands were found but only in 5563 cell-free products was there found a band as similarly prominent as cellular H chain. P1.17 cell-free products seemed to show no band of appropriate prominence which could be tentatively identified as H chain or its precursor. The relative mobilities of cellular 5563 H chain and its tentatively identified cell-free product were 0.33 and 0.34 respectively, as accurately as could be determined. The increase in mobility of the putative 5563 cell-free H chain could result from absence of carbohydrate sidechains in the cell-free product which could increase mobility and might mask the effect of a precursor sequence in H chain. This will be discussed more fully in Section 11.

7.4. Methods for identification of Ig precursors.

Several methods were used to identify unambiguously, the cell-free synthesized H and L chain products from the M.D.L.

1. Inference from the prominence of the bands separated by PAGE.

2./

2. Inference of position from known properties of other precursors, e.g. lower mobility of pre L chains.
3. Inference of position from known effects of cell-free translation, e.g. absence of carbohydrate addition, for H chain.
4. Direct immune precipitation.
5. Indirect immune precipitation.
6. Comparison between producing and non-producing cell lines.
7. Tryptic mapping.

Some of these criteria have been discussed already (1 and 2). Of the others, immuneprecipitation and tryptic mapping offered the possibility of the most unambiguous identification of cell-free H and L chains.

8. Immunoprecipitation.

8.1. Optimisation of immunoprecipitation.

Immunoprecipitation is a powerful tool for identification of specific proteins or polypeptides. It offered one method of unambiguously identifying Ig-like products of cell-free systems which had been stimulated by myeloma cell polysomes or poly(A) RNA.

The conditions of immunoprecipitation had, however, to be optimised to ensure that the maximum amount of antigenic material was precipitated. It could not be assumed that the cell-free product would have identical antigenic properties to the intracellular or secreted/

secreted products. Reticulocyte lysate cell-free systems lack the mechanisms for translocation of synthesized H and L chains into the intra-cisternal space. They also lack mechanisms contained therein for assembly and processing of Ig molecules. This could result in the synthesis of Ig-like proteins in the cell-free system with affinities for antisera different to those of Ig synthesized in vivo. It was considered nevertheless that cell lysates of myeloma cells provided a useful model system for optimisation of immune precipitation, whilst recognising that conditions might well require adjusting for use with the cell-free systems.

Two methods of immunoprecipitation were considered. The first, direct precipitation, involved simple interaction between a specific antiserum and antigenic material. This resulted in the formation of small insoluble immune complexes which could be pelleted from the solution by centrifugation. The second method, indirect immune precipitation, initially involved interaction of a specific antiserum with the antigen as before. This was followed, however, by the addition of a more general antiserum whose specificity was directed against the Ig present in the first antiserum. This included the specific antibodies bound to the original antigen. This had the effect of producing larger, more easily isolated immune complexes and thereby improved the recovery of the antigenic material under study, as will be demonstrated (Section 8.2.3.).

8.1.1. Optimisation of indirect immunoprecipitation.

Immunoprecipitation was normally carried out in buffered saline solution to maintain protein integrity and to allow antibody - antigen/

antigen interaction. Detergents (Triton X-100, sodium deoxycholate) were present to attempt to minimise non-specific aggregation. These detergents did not appear to prevent antigen - antibody interaction and the formation of specific immune complexes (Askonas & Williamson, 1967; Parkhouse & Crumpton, 1972).

Two antisera were used. The first was raised in rabbits using purified 5563 Ig and showed a specificity of interaction both with 5563 Ig and also with P1 Ig. The second antiserum was raised in goats against rabbit Ig and hence interacted with all the rabbit Ig present in the rabbit antiserum.

Several variables required to be optimised in order to ensure maximum precipitation of antigen from the sample.

The first variable investigated was the equivalence of goat antiserum with rabbit antiserum. This was determined indirectly by titrating the goat antiserum against a fixed volume of radioactively labelled 5563 cell lysate (210,000 cpm/5µl TCA pptble) containing a fixed volume of rabbit antiserum. Increasing volumes of goat antiserum will precipitate increasing amounts of rabbit Ig-5563 complexes (and hence of radioactivity) until all such complexes have been removed from solution. Thereafter, larger volumes of goat antiserum will lead only to larger immune complexes being precipitated.

This did not require that the rabbit antiserum and 5563 Ig were at equivalence. As the data in Fig. 25 suggests, equivalence was reached at 20 volumes goat antiserum versus 1 volume rabbit antiserum. Below equivalence, the amount of radioactive material precipitated fell off sharply, indicating that not all rabbit Ig - 5563 Ig complexes were being precipitated. Above equivalence, radioactivity precipitated/

FIG. 25: Titration curve:- Goat anti-rabbit Ig antiserum vs. Rabbit anti- 5563 protein/5563 complexes in cell lysate.

5µl aliquots (∿ 180,000 counts/min) 5563 cell lysate, radioactively labelled with L-(^{35}S) methionine were incubated in 250µl PBSA containing 1% (v/v) Triton X-100, 1% (w/v) NaDOC, with 5µl rabbit anti-5563 antiserum. Incubation was for 30 min at room temperature. Subsequently, varying volumes (25µl-250µl) of goat anti-rabbit Ig antiserum was added (volumes equalised c PBSA) and the incubation was continued overnight at 4°C. The precipitates were pelleted by centrifugation, resuspended in 1 ml PBSA and this procedure repeated 2 more times. The washed precipitates were redissolved in 1 ml 0.1N NaOH, and optical density at 280nm measured by a Unicam SP1800 Ultraviolet Spectrophotometer. 0.5 ml of the solution was dissolved in 6 ml Triton/toluene/PPO scintillation fluid (Methods 8.1.2.) and radioactivity measured by scintillation counting. Each assay point was performed in duplicate. In some assays, normal rabbit serum was used instead of antiserum to measure non-specific precipitation.

FIG. 26: Titration curve:- Goat anti-rabbit Ig antiserum vs. rabbit anti-5563 antiserum/Ig-like product complexes from M.D.L. containing Pl.17 poly(A) RNA.

5µl of M.D.L. assay, incubated with Pl.17 poly(A) RNA under standard conditions (TCA precipitable material - 186,000 counts/min/5µl, background 18,770 counts/min/5µl), was incubated in 250µl PBSA containing 1% (v/v) Triton X-100, 1% (w/v) NaDOC, with 5µl rabbit anti-5563 antiserum. Incubation was for 30 min at room temperature. After this, various volumes (25µl-250µl) of goat anti-rabbit antiserum were added and the incubation continued overnight at 4^{o}C. Volumes were adjusted with PBSA. The precipitates were washed, and optical density and radioactivity measurements were carried out as described in Fig. 25. Each assay point was performed in duplicate. For some points normal rabbit serum control precipitations for background precipitation were performed.

Fig. 25

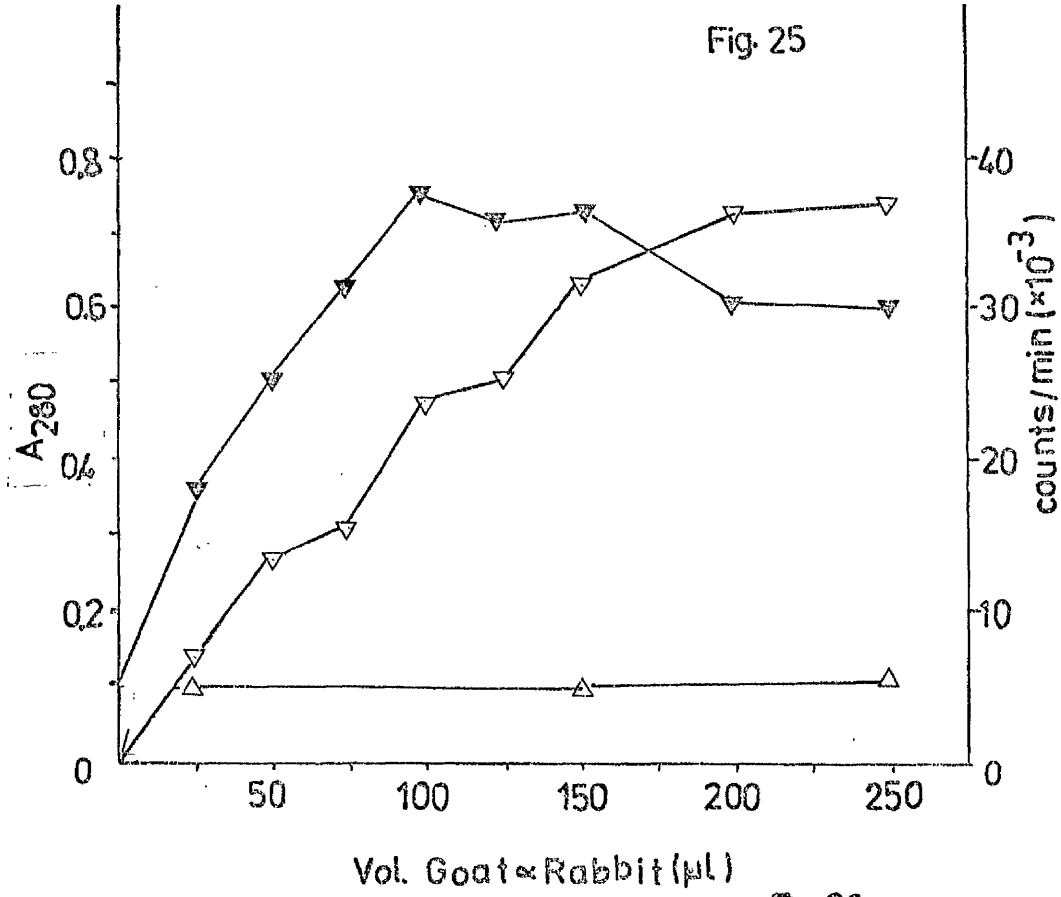
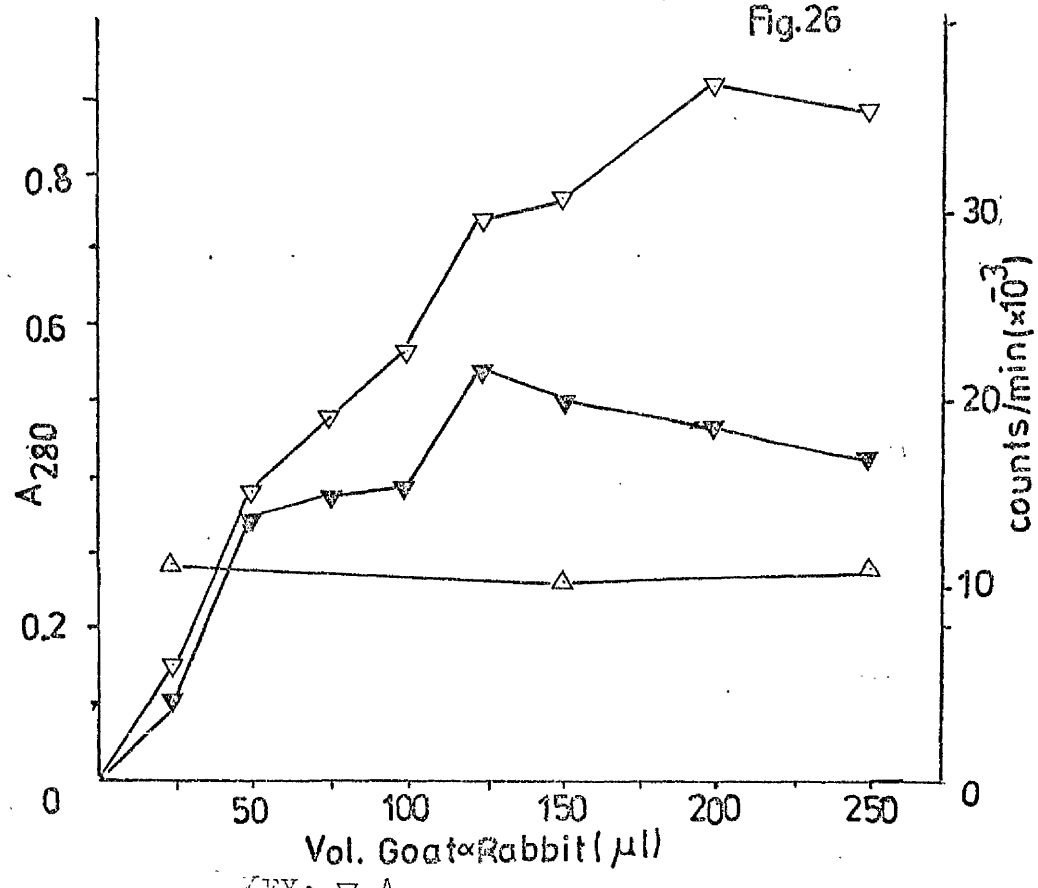


Fig.26



KEY: -▽- A_{280}

-▽- Specific ppn.(cpm)

-△- Non-sp. ppn.(cpm)

precipitated remained level over a range of goat antiserum:rabbit antiserum ratios whilst the size of precipitate formed continued to increase. To ensure maximum precipitation of radioactivity from cell lysates, rabbit and goat antisera were subsequently used in a ratio of 1:20.

This experiment was also carried out using normal rabbit serum in place of the rabbit antiserum to test for non-specific precipitation of radioactivity. The non -specific precipitation level was ∿2% of total TCA precipitable material and ∿10% of the maximum specifically precipitable levels. Later experiments suggested that this non-specific precipitation resulted from non-specific trapping of radioactively labelled proteins and/or amino acids in the immune complexes as they formed.

This method was then used to check equivalence with a sample of radioactively labelled M.D.L. Conditions were similar to that used with the cell lysate. Fig. 26 demonstrates that equivalence of goat and rabbit antiserum occurred in a ratio of 25:1 (by volume). This was slightly higher than was found using cell lysate, but not greatly so. The major difference noted was in the higher level of non-specific precipitation of radioactivity observed in control precipitations involving normal rabbit serum. This constituted 6.3% of total TCA precipitable radioactivity and approximately 50% of maximum specifically precipitable radioactivity. Such a level of non-specific precipitation was considered unacceptable. Several methods of reducing this level were investigated.

First to be investigated was the effect of repeated resuspension and pelleting of the precipitate in ice cold PBSA containing excess unlabelled methionine. As the data in Figure 27 shows, after 3

washes/

FIG. 27: Effects of washing immunoprecipitates on the amount of radioactivity detected.

Quintuplicate samples were set up, each containing 5µl P1.17 M.D.L. assay (186,000 counts/min) and 5µl rabbit anti-5563 antiserum in PBSA containing 1% (v/v) Triton X-100 and 1% (w/v) NaDOC. After 45 min incubation at room temperature, 100µl Goat anti-rabbit Ig antiserum was added and incubation was continued at $4^{\circ}C$ for 2 hours. The precipitates were pelleted by centrifugation and washed from 1 - 5 times with PBSA. Each washed, pelleted precipitate was dissolved 400µl 0.1N NaOH and duplicate 100µl aliquots were measured for radioactivity in Triton/toluene/PPO (Methods 8.1.2.) by scintillation counting.

Identical manipulations were carried out on samples containing normal rabbit serum in place of specific antiserum.

FIG. 29: Relationship between immunoprecipitate size and amount of radioactivity precipitated.

Serial dilutions of rabbit anti-5563 antiserum were added to 5µl P1.17 M.D.L. (186,000 counts/min) in precipitation medium (Methods 11.2.2.). After the standard incubation period, goat anti-rabbit Ig antiserum in the ratio 30:1 (by volume) over rabbit antiserum was added and incubated at 4°C overnight. Precipitates were isolated by standard procedures (Methods 11.2.2.) and radioactivity measured by scintillation counting (Methods 8.1.2.). With some assay points, normal rabbit serum control precipitations were included.

Fig. 27

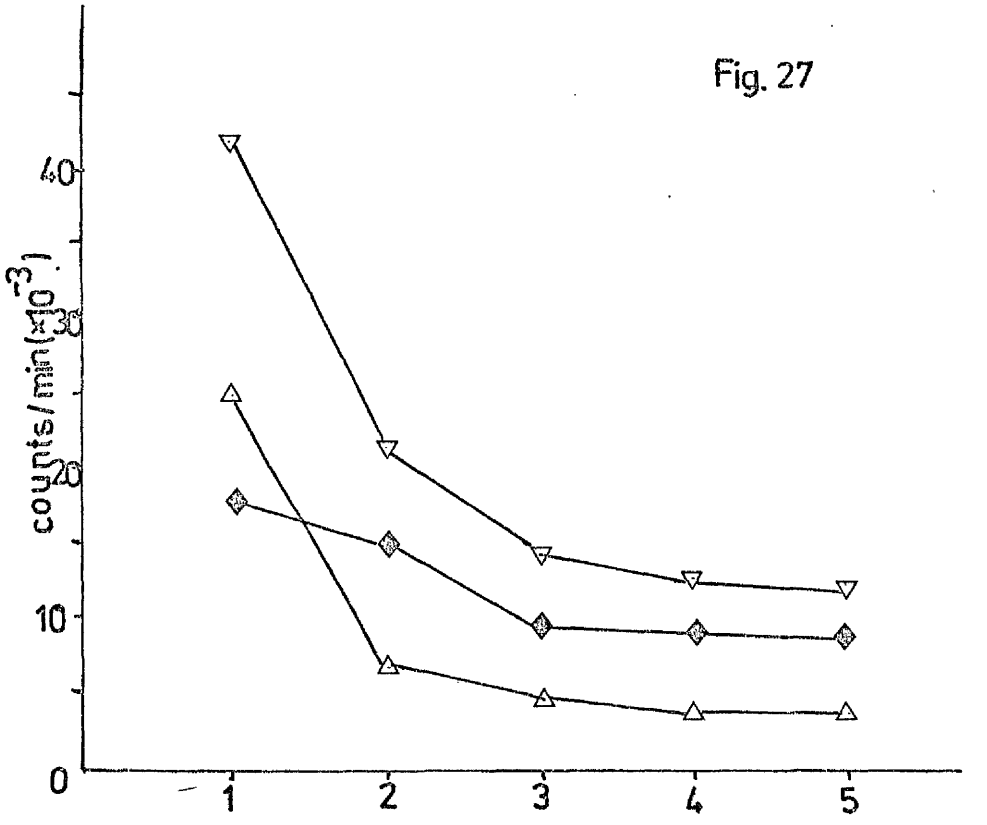
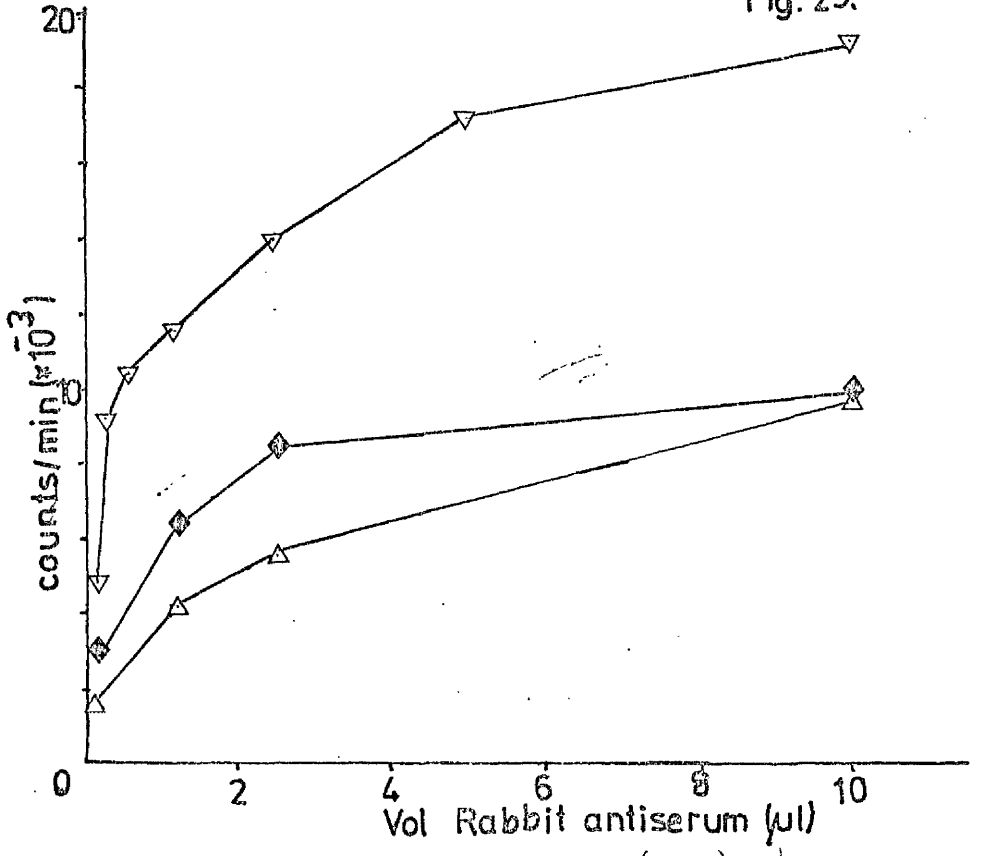


Fig. 29.



KEY: -▽- Specific ppn.(cpm)
-△- Non-sp. ppn. (cpm)
-◇- Sp.- non-sp. (cpm)

washes with PBSA, the level of radioactivity detected in immune precipitates from a sample of M.D.L., either specific or non-specific, remained constant. After this treatment, non specific precipitates contain 31% of the amount of radioactivity contained in specific precipitates. Thus washing of precipitates reduced but did not eliminate non-specific precipitation. Two further possible methods were investigated:-

i) ribonuclease was used to destroy RNA present in the M.D.L. sample. This was used to prevent possible contamination of precipitates by non-specific adsorption of tRNA molecules charged with radio-actively labelled amino acids,

ii) normal rabbit serum, followed by goat antiserum were used for the purpose of clearing non-specifically adhering material from the sample. After transfer of the resulting supernatants to fresh tubes, specific precipitation was carried out, theoretically in the absence of non-specifically adhering species. As demonstrated by Table 16, addition of pancreatic ribonuclease to M.D.L. samples before immunoprecipitation increased rather than eliminated non-specific precipitation. This problem has been found by other workers (Sussman et al., 1976). Thus, nuclease treatment was concluded to be ineffective in reducing non-specific precipitation. As shown by Fig. 28, the use of a clearing precipitate was also ineffective with regard to this problem. After the clearing precipitate, non-specific precipitation was reduced to approximately 50% of the level found without a clearing precipitate. Specifically precipitable counts, however, fell to 40% of their previous level. An indication that interference with antibody-antigen interactions was occurring was found in the shape of the titration curve obtained after using the clearing/

TABLE 16: Effect of ribonuclease on non-specific trapping of radioactivity in immune precipitates.

Legend:

Duplicate 50µl aliquots of M.D.L., stimulated by Pl.17 poly(A) RNA, containing 1.8 x 10^6 cpm/aliquot (as determined on paper discs) were treated as follows:-

Aliquot a) no additions, incubated @ 37°C for 30 minutes.

Aliquot b) made 15mM in EDTA pH 7.3, 25µg/ml pancreatic ribonuclease: incubated @ 37°C for 30 minutes.

After this, 5µl samples were added to 250µl PBSA containing 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate. To these were added either 5µl of specific rabbit antiserum or 5µl of normal rabbit serum. This solution was incubated at room temperature for 30 minutes. Thereafter, various volumes of goat antiserum were added and sufficient PBSA to make the volumes of all assays equal. These solutions were incubated at room temperature for 30 minutes, then overnight at 4°C. The precipitates were pelleted by centrifugation, then washed, dissolved and counted as described in Methods 11.2.2. and 8.1.2.

TABLE 16: Effect of ribonuclease on non-specific trapping of radioactivity in immune precipitates.

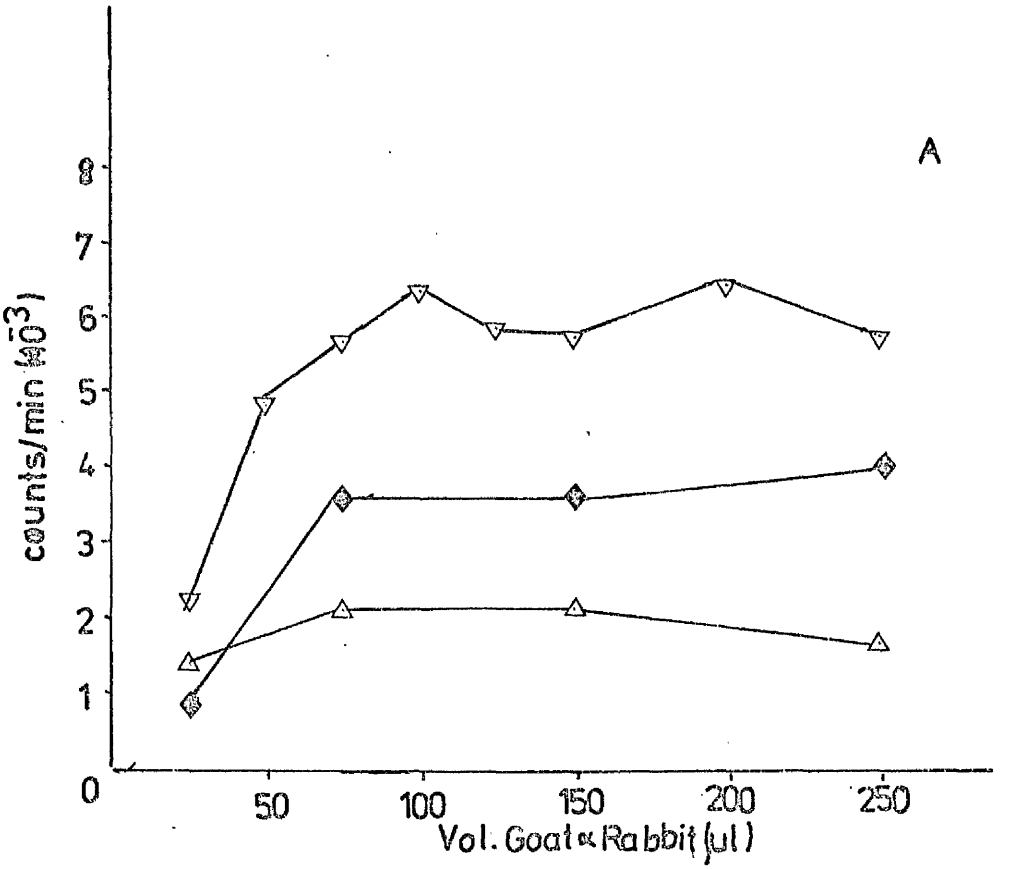
Treatment	Volume of rabbit antiserum (µl)	Vol.of normal rabbit serum (µl)	Volume of of goat antiserum (µl)	c.p.m./ precipitate	cpm -NRS ppte
Control	5	-	20	1716	384
"	5	-	150	12326	10210
"	5	-	300	8886	6300
"	-	5	20	1232	-
"	-	5	150	2116	-
"	-	5	300	2586	-
Pancreatic ribo-nuclease	5	-	20	2356	not valid
"	5	-	150	16622	7570
"	5	-	300	9570	not valid
"	-	5	20	3984	-
"	-	5	150	9052	-
"	-	5	300	15618	-

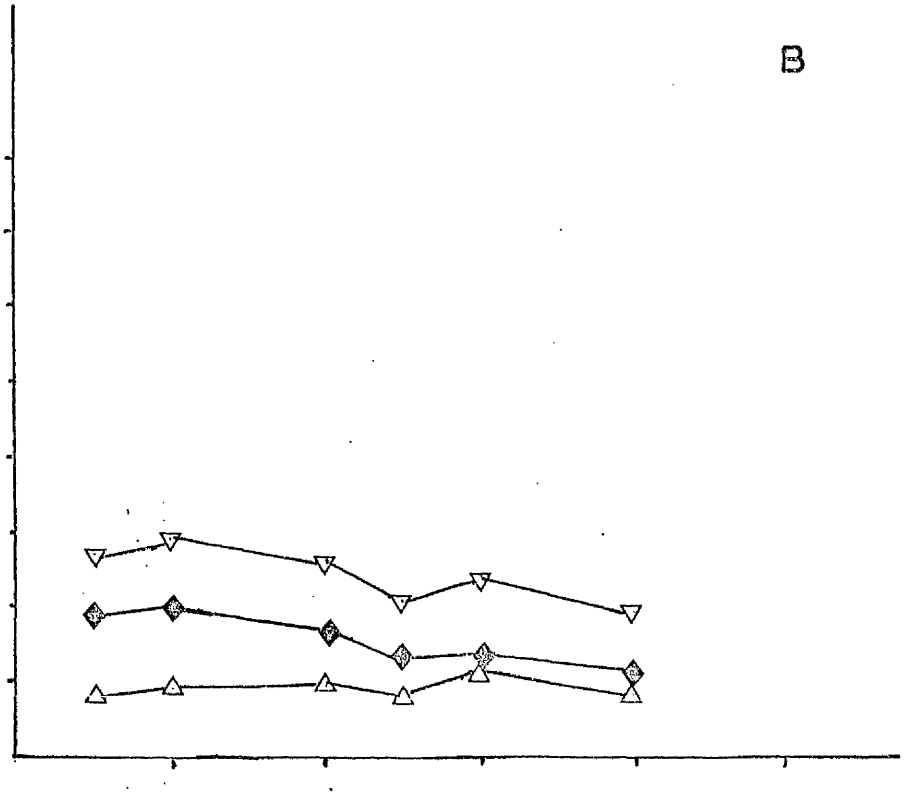
FIG. 28: Comparison of the effect of a pre-precipitation step on titration curves and M.D.L.

Duplicate series of immunoprecipitation assays were performed using varying rabbit antiserum:goat antiserum ratios (1:5 to 1:50) by standard methods (see Fig. 25) and using Pl.17 M.D.L. products as radioactive label. In some cases, normal rabbit serum replaced the specific rabbit antiserum. In one of the duplicate series, the assays had previously been subjected to a normal rabbit serum:goat anti-rabbit Ig antiserum precipitate (as a step to remove non-specifically binding material). The other duplicates had not been treated this way.

A. Titration curve without pre-precipitation with NRS/goat antiserum.

B. Titration curve after pre-precipitation with NRS/goat antiserum.





KEY: ▽ Specific ppn. (cpm).
△ Non-sp. ppn. (cpm).
◇ Sp.-nonsp. (cpm).

clearing precipitate.

Neither of these two methods yielded appreciable improvements in the level of non-specific precipitation. Later work showed that the inclusion in the precipitation assays of 0.5% (w/v) SDS reduced the level of non-specific precipitation significantly (T.R. Mosmann, personal communication).

One disadvantage of indirect immuncprecipitation was that large quantities of unlabelled IgG were present in the immune precipitate. When immuneprecipitate samples were separated by PAGE, even quite small quantities of immuneprecipitate caused distortion of the gel tracks, and of the radioactively labelled products separated on them. This was because local concentrations of unlabelled IgG H and L chains caused local overloading of the gels. This was not very important for cell-free synthesized L chains which, being synthesized as larger precursors, had mobilities lower than the area of L chain distortion. Nor was it a serious problem with α- and μ-type H chains which migrate more slowly than the γ H chain of IgG and hence avoid distortion. In the cases of 5563 and Pl.17 cells, both of which produce γ-type H chains, gel distortion in the region of gel at which the cell-free product migrated was a serious problem, resulting in "bullet-shaped" fuzzy bands (see Fig.31) rather than sharp straight bands. One way of circumventing this problem was to investigate whether less antiserum could precipitate the same amount of radioactivity. Consequently, the equivalence of cell-free system-produced antigenic material with rabbit antiserum was determined. The ratio of rabbit antiserum to goat antiserum was kept constant while varying the volume of rabbit antiserum added to a system containing a constant volume of M.D.L., containing 5563 cell-free products/

products. Figure 29 showed that, allowing for non-specific precipitation of radioactivity > 5µl of rabbit antiserum would precipitate a constant amount of radioactivity from 5µl of M.D.L. containing 5563 antigenic material,but that below 5µl of rabbit antiserum, the amount of radioactivity dropped sharply, indicating that equivalence had not been reached. Consequently, the size of immunoprecipitate could not be reduced to any advantage. The alternative precipitation method, direct precipitation, offered some advantages in terms of amount of protein precipitated and was therefore investigated.

8.1.2. Optimisation of direct precipitation.

Direct immune precipitation, relying on the primary antibody-antigen reaction to produce insoluble immune complexes large enough to be pelleted by centrifugation was investigated as an alternative to indirect immunoprecipitation, with its disadvantages.

Direct precipitates were performed as described in Methods 11.2.1. In view of the likely size of the immunoprecipitate, the sucrose layer method of isolating precipitates was used, followed by dissolving the precipitate in 9M deionised urea.

The relative effectiveness of direct precipitation and indirect precipitation was studied, by comparing the amount of radioactivity recovered by each method from a sample of cell lysate. Cell lysates and secreted material from two cell lines, 5563 and P1.17, were used. As the data in Table 17 demonstrate,in general, directly immunoprecipitable radioactive material amounted to only

∿/

TABLE 17: Comparison of direct and indirect immuneprecipitation from Pl.17 and 5563 cell lysate.

Legend:

Immunoprecipitations were carried out using conditions described in Methods 11.2.1. (direct) or Methods 11.2.2. (indirect). 10µl L(^{3}H) leucine-labelled 5563 cell lysate (140,000 counts/min), 10µl L(^{3}H) leucine-labelled Pl.17 cell lysate (175,000 counts/min) or 50µl L(^{3}H) leucine-labelled Pl.17 cell supernatant (26,600 counts/min) were used. Immunoprecipitates were washed as described in Methods 11.2.1. (direct) or Methods 11.2.2.(b) (indirect) and dissolved in either 100µl 9M urea (direct) or 500µl 9M urea (indirect). Radioactivity was measured by scintillation counting of samples dissolved in triton/toluene/PPO (Methods 8.1.2.)

Key:

I, indir.= indirect immunoprecipitation

D, dir. = direct immunoprecipitation

C = indicates normal rabbit serum control precipitation

NRS = normal rabbit serum

ppte = precipitate

Sp = specific

n.a. = not applicable

TABLE 17: Comparison of Direct and Indirect immunoprecipitation from P1.17 and 5563 cell lysates.

	Vol. rabbit antiserum (µl)	Vol. normal rabbit serum (µl)	Vol.5563 protein added (µl)	Vol. goat antiserum (µl)	cpm/½ppte (ave)	Sp. cpm/ppte -cpm/NRS ppte	cpm dir. cpm indir.(%)	cpm NRS cpm Sp.(%)	Type of precipitation
a) P1.17 cell lysate	5	-	-	100	30389	28813	100	n.a.	I
	-	5	-	100	1576	-	n.a.	5.20	IC
	5	-	5	-	8347	8139	28.9	n.a.	D
	-	5	5	-	208	-	n.a.	2.49	DC
	5	-	2.5	-	9707	9324	32.4	n.a.	D
	-	5	2.5	-	383	-	n.a.	3.90	DC
	5	-	-	-	11405	11165	38.8	n.a.	D
	-	5	-	-	240	-	n.a.	2.10	D
b) P1.17 secreted	5	-	-	100	7358	7000	100	n.a.	I
	-	5	-	100	357	-	n.a.	4.85	IC
	5	-	-	-	7832	7654	"109"	n.a.	D
	-	5	-	-	186	-	n.a.	2.37	DC
c) 5563 cell lysate	5	-	-	100	15017	12729	100	n.a.	I
	-	5	-	100	2288	-	n.a.	15.23	IC
	5	-	5	-	4470	4401	34.6	n.a.	D
	-	5	5	-	69	-	n.a.	1.54	DC
	5	-	2.5	-	4642	4230	33.2	n.a.	D
	-	5	2.5	-	412	-	n.a.	8.86	DC
	5	-	-	-	4745	4225	33.2	n.a.	D
	-	5	-	-	520	-	n.a.	10.96	DC

∿ 33% of the radioactive material precipitable by indirect immunoprecipitation. The possibility that the immune complexes being formed were too small to allow precipitation from solution was investigated. Varying amounts of purified antigen (in this case, pure 5563 protein) were added to the solution before the antiserum. This was intended to provide more antigen material to allow formation of larger immune complexes.

The amount of radioactivity precipitated from 5563 cell lysate remained unchanged in the presence or absence of added 5563 Ig. This indicated the possibility that limiting amounts of antiserum were not being used. It also suggested that there was no significant pool of soluble immune complexes containing radioactively labelled material. Alternatively, the data could be interpreted as suggesting that any pool existing contained only poorly interacting species.

In the presence of 5563 Ig, the amount of radioactivity detected in direct precipitates from Pl.17 cell lysates showed a marked decrease. This may have been due to competition between the radioactively labelled Pl Ig and the unlabelled 5563 Ig. The antiserum was raised against 5563 Ig and whilst showing cross-reactivity with Pl Ig might be expected to contain some antibodies having greater affinity for 5563 Ig.

Immunoprecipitations were carried out in the medium retained after labelling of Pl.17 cells. This medium contained radioactively labelled secreted Pl Ig. Both direct and indirect immunoprecipitation techniques yielded the same amount of radioactivity precipitated. A hypothesis consistent with this and the above data was that strongest affinity was shown to L chains by the antiserum.

Thus/

Thus the larger immune complexes formed in direct immunoprecipitation contained a higher proportion of free L chain than that found in the cell. This was later confirmed by gel analysis of direct and indirect immunoprecipitates (see Fig. 19).

With direct immune precipitation the effect of varying the amount of antiserum used was investigated. The optimum volume of antiserum was found to be 5µl. At this volume, the amount of radioactivity precipitated levelled off.

When direct precipitation was used on samples of M.D.L. assays, the results obtained were variable. Direct precipitation did not always yield more radioactivity than was precipitated in normal rabbit serum control precipitations. When, however, specific direct precipitation did yield levels of radioactivity higher than background, PAGE analysis showed bands corresponding to H and L chain-like material (see Fig. 30a).

8.2.1. Direct immunoprecipitation of Pl.17 and 5563 myeloma products in the M.D.L.

Direct immunoprecipitation of samples of M.D.L. assays containing Pl.17 or 5563 poly(A) RNA was carried out as described in Materials and Methods, and the direct precipitates and total M.D.L. synthesized material were compared by PAGE (Fig. 30). Densitometric analysis of the direct precipitate and comparison with both the total M.D.L. products and intracellular products indicated the presence of a small amount of material electrophoresing with a mobility slightly greater than the mobility of intracellular H chain. A band with a mobility

lower/

FIG. 30: PAGE analysis of direct and indirect immunoprecipitation from M.D.L. containing 5563 poly(A) RNA.

Standard M.D.L. assays, either containing or omitting 2µg 5563 poly(A) RNA,were carried out as described in Methods 7.2 , with 2.4µCi L-(^{35}S) methionine as radioactive label. Direct and indirect immunoprecipitations were carried out on 5µl aliquots of the assays as described in Methods 11.2.1. and 11.2.2(b) except that the precipitate size was 1/4 the standard amount in the indirect precipitation. Samples of cell lysate were run as markers. The gel showed abnormal running characteristics but this did not appear to affect separation.

Key	Well	Description	(counts/min)
	1	M.D.L., no added mRNA - total products	(12,000)
	2	M.D.L., no added mRNA - direct precipitate	(3,380)
	3	M.D.L., no added mRNA - indirect precipitate	(2,200)
	4	M.D.L., 2µg 5563 poly(A) RNA - total products	(183,000)
	5	M.D.L., 2µg 5563 poly(A) RNA - direct precipitate	(5,400)
	6	M.D.L., 2µg 5563 poly(A) RNA - indirect precipitate	(6,060)
	7	5563 cell lysate - total products	

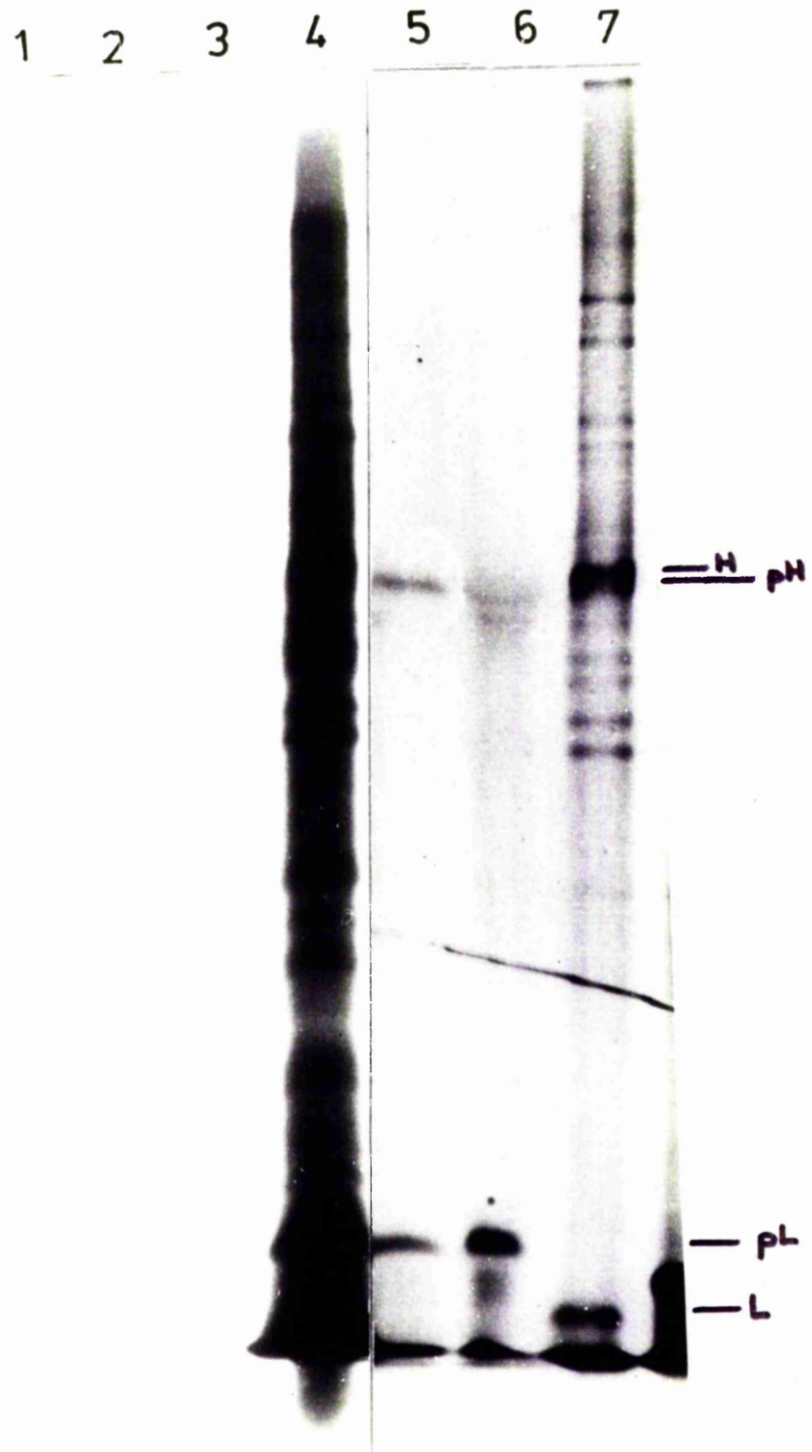
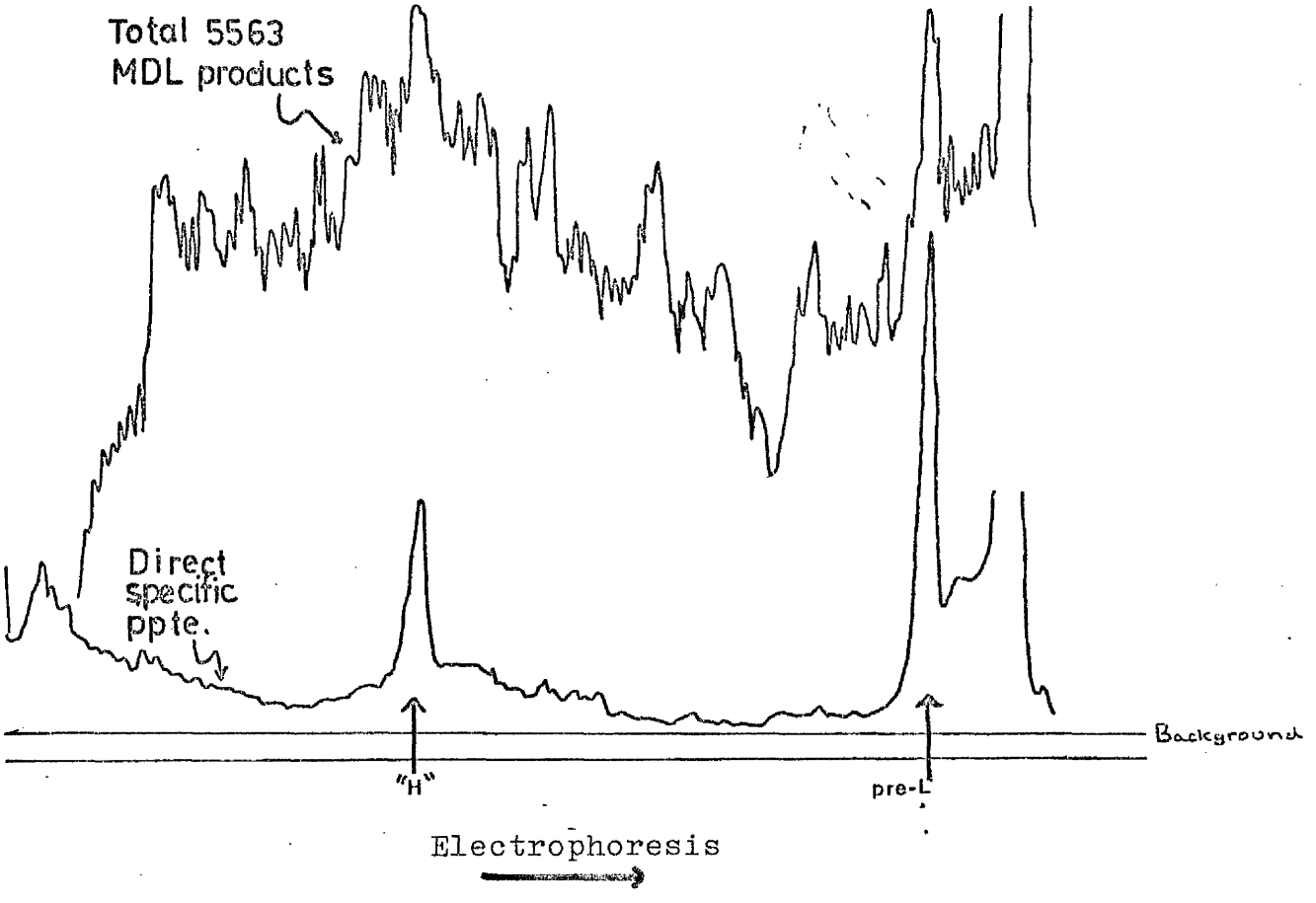

1
2
3
4
5
6
7
H
pH
pL
L

FIG. 30a: Densitometric analysis of total products and direct precipitates from M.D.L. containing 5563 poly(A) RNA, as shown in Fig. 30.

Densitometric analysis was performed on a Joyce-Loebel Scanning Densitometer, (Methods 8.3.3.). A wedge giving OD range 0 - 0.15 was used to scan the direct immunoprecipitate. A wedge giving OD range 0 - 1.5 was used to scan the total products. The total products trace has been reduced in height by approximately 50% for greater ease of comparison with the direct precipitate.


Total 5563
MDL products
Direct
specific
ppte.
Background
"H"
pre-L
Electrophoresis

lower than that of intracellular L chain was also observed. These separations were reproducible on a further gel suggesting that they were not merely artefactual. Direct precipitation of a Pl.17 M.D.L. aliquot revealed no clearly defined material with mobility close to that of intracellular H chain although prominent bands of material were observed at positions of lower and equal mobility with intracellular L chain. As with direct precipitation from cell lysates, the precipitation was not quantitative but was indicative of the presence of H and L-chain-like material in M.D.L. assays containing myeloma poly(A) RNA. Quantitative precipitation of material was investigated using indirect immunoprecipitation.

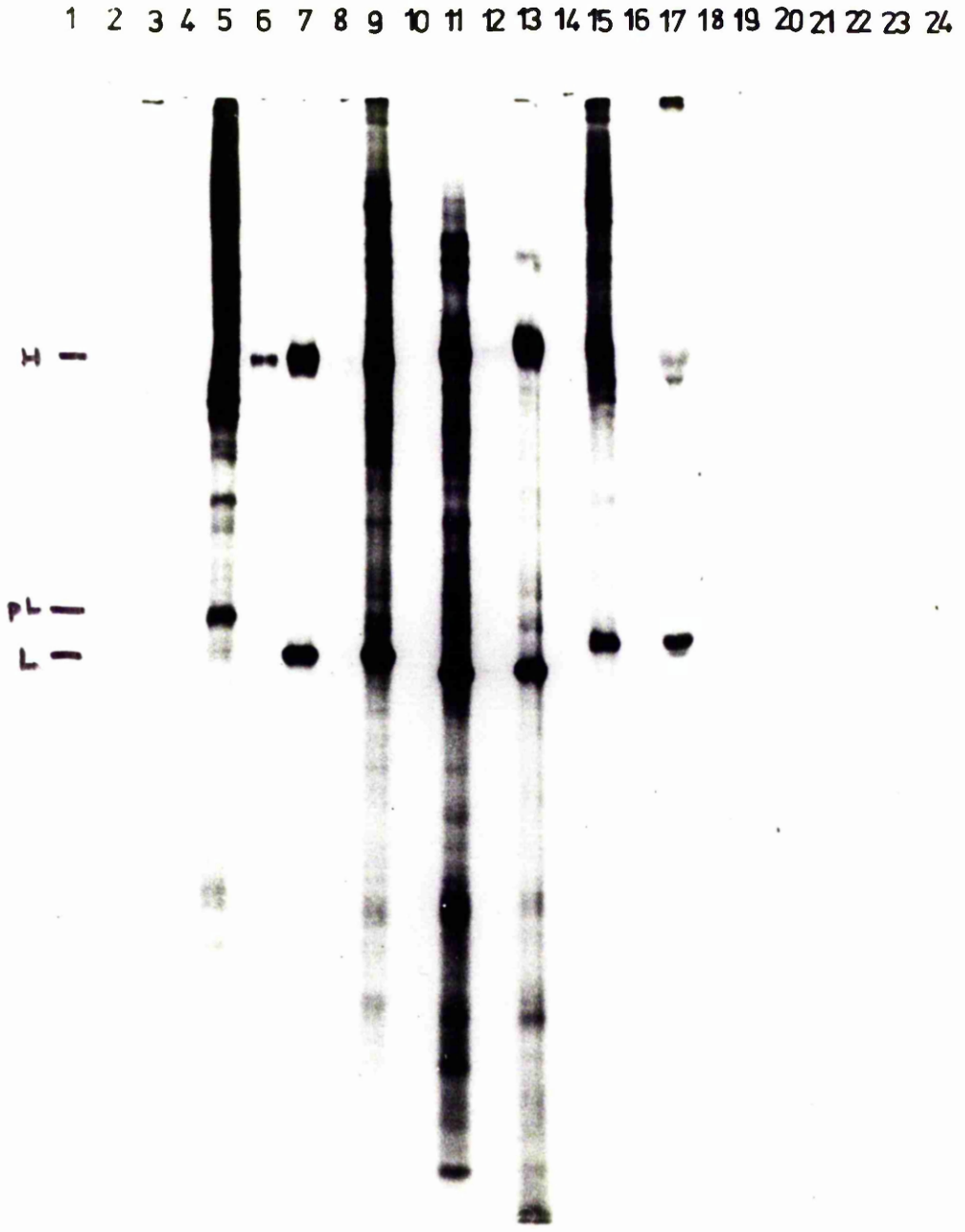
8.2.2. Indirect immunoprecipitation of Pl.17 and 5563 myeloma products in the M.D.L.

Indirect precipitation of Ig-like species synthesized in the M.D.L. containing 5563 and Pl.17 myeloma polysomes or poly(A) RNA was carried out as described in Methods 11.2.2(a). NRS controls for non-specific precipitation were used. The immunoprecipitates were redissolved in deionised 9M urea after washing and aliquots of these solutions were prepared for PAGE. These were electrophoresed with samples of cell lysate and total M.D.L. products. Fig. 31 shows the radioactive banding pattern obtained after fluorography. A prominent band corresponding to the position of putative pre-L chain was observed in precipitates from M.D.L. containing either 5563 or Pl.17 polysomes. In the case of 5563, precipitation of this material appeared quantitative by densitometry. Pl.17 indirectly/

FIG. 31: PAGE analysis of M.D.L. products from P1.17 and 5563 polysomes.

Aliquots from (b) assays shown in Table 18, were treated as described in Methods 9.2.5. and electrophoresed overnight for 16 hrs. at 15mA/gel. Gel processing and exposure to X-ray film was as described in Methods 8.3.1. Films were developed by standard techniques. Approximately 1000cpm were readily detectable.

Key	Well	Description	(counts/min)
	1	M.D.L./P1.17 polysomes, direct immuneprecipitation	(1,000)
	2	" " " NRS control	(60)
	3	" indirect immuneprecipitation	(1,000)
	4	" " " NRS control	(300)
	5	" total cell-free products	(10,000)
	6	P1.17 secreted, direct immuneprecipitate	(2,500)
	7	" " total secreted material	(3,300)
	8	" cell lysate, direct immuneprecipitate	(200)
	9	" " " total cell lysate material	(15,400)
	10	Blank	(-)
	11	5563 cell lysate, total cell lysate material	(1,800)
	12	" " " direct immuneprecipitate	(500)
	13	" secreted, total secreted material	(6,900)
	14	" " direct immuneprecipitate	(2,000)
	15	M.D.L. /5563 polysomes, total cell-free products	(17,000)
	16	" " indirect immuneprecipitable, NRS control	(450)
	17	" " indirect immuneprecipitate, NRS control	(1,700)
	18	" " direct immuneprecipitate, NRS control	(700)
	19	" " direct immuneprecipitate	(3,000)
	20	M.D.L. total cell-free products	( )
	21	" indirect immuneprecipitate	(120)
	22	" " " NRS control	(240)
	23	" direct immuneprecipitate	(50)
	24	" " " NRS control	(1,600)


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
H
PL
L

indirectly immunoprecipitated material was not quantitative for pre L chain material. In both cases, the area corresponding to the expected mobility of putative pre-H chain was distorted, implying overloading of the gel with protein at this point on the gel. This was probably due to a high concentration of unlabelled γ chains present from the indirect immunoprecipitate. This heterogenous mixture of H chains from Goat and Rabbit presented severe problems. Its distorting effect prevented accurate measurement of the mobility of putative pre H chain. It also hampered attempts to quantify production of putative pre-H chains,by immunoprecipitation. These problems have not been entirely resolved in this project nor have they been by other workers. Indirect immunoprecipitation of M.D.L. containing 5563 polysomes nevertheless yielded material which had a mobility approximately the same, or slightly greater than cellularly produced H chain. This agreed with the results of direct immuno-precipitation. Comparison with the gel profiles of immunoprecipitates with NRS showed that this radioactivity was specifically precipitated and not non-specifically "trapped" in the precipitate. Reducing the fraction of the immuno-precipitate added to the gel from 1/2 to 1/5 to 1/10 in later experiments did not significantly improve the distortion by unlabelled H chain.

8.2.3. Comparison of direct and indirect immunoprecipitation of M.D.L.-synthesized Ig-like material.

It was shown that 5μl of antiserum removed 90% of precipitable material from a similar volume of M.D.L. containing myeloma mRNA-directed/

directed products by the indirect immunoprecipitation method. This volume of antiserum was therefore used to compare direct and indirect immunoprecipitation of Ig-like products from M.D.L. stimulated by myeloma polysomes. Polysomes from two myeloma cell lines were used, Pl.17 and 5563, and Fractions I and II of polysomes from these lines were tested separately. As shown in Table 18, direct immune-precipitation was erratic in the amount of radioactively labelled material it precipitated, varying by up to 10 fold between two precipitations with the same polysome fraction. Consequently analysis by PAGE was not pursued in this experiment. Indirect immunoprecipitation, although experiencing high background as shown in NRS precipitates, nevertheless gave more consistent figures for radioactive material precipitated. In both Pl.17 and 5563 experiments, a higher percentage of total products seemed to be specifically precipitable using the Fraction II polysomes.

8.2.4. Comparison of levels of specifically precipitable material from M.D.L. assays containing myeloma polysomes and from myeloma cells labelled in culture.

The cell labellings described in Sections 5.1.2 & 5.2.2 were carried out using small samples of the cells used in the preparation of the polysomes assayed in Section 8.2.3 above. The cells were removed for labelling prior to the first step of the polysome isolation procedure (i.e. addition of cycloheximide to 100µg/ml; see Methods Section 3.) Consequently, they allowed a direct comparison between the levels of production of Ig intra-cellularly and the levels of production of immunoreactive material in/

TABLE 18: Comparison of direct and indirect immunoprecipitation from M.D.L. assays of various polysome fractions.

Legend:

Two separate series of assays were run using respectively a) 60 min incubation, 6μCi (L-(^{35}S) methionine and 50μl M.D.L. and b) 120 min incubation, 30μCi L(^{35}S) methionine and 100μl M.D.L. 5μl polysomes were added in assay a), 10μl in assay b). Otherwise, standard M.D.L. conditions were used. Direct and indirect immunoprecipitation reactions, including normal rabbit serum controls, were carried out as described in Methods 11.2.1. and 11.2.2(b) respectively. Radioactivity was detected by dissolving precipitates in 9M urea and measuring radioactivity by liquid scintillation counting, using triton/toluene/PPO as solvent (Methods 8.1.2.).

TABLE 18: Comparison of direct and indirect immuneprecipitation from M.D.L. assays of various polysome fractions.

polysome fraction		Total cpm/5µl	Total cpm -bgd cpm	Indirect: Sp pptble cpm/5µl	Indirect: NRS cpm/5µl	Indirect: Sp pptble -NRS cpm/5µl	Indirect: % Sp pptble	Direct: Sp pptble cpm/5µl	Direct: NRS cpm/5µl	Direct: Sp-Normal cpm/5µl	Direct: % Sp pptble
O	a	2556	O	125	125	O	O	168	200	O	O
	b	9350	O	589	406	O	O	693	1125	O	O
Pl I	a	72634	70078	8386	3857	4529	6.46	3030	2726	304	0.43
	b	174752	165402	26996	9460	17536	10.6	472	6736	4727	1.2
Pl II	a	37596	35040	5084	2049	3035	8.66	1834	614	1220	3.48
	b	112148	102798	15552	5342	10210	9.93	6188	1138	5050	4.9
5563 I	a	14885	12329	1590	623	967	7.84	732	282	550	4.46
	b	64258	54908	6030	3000	3030	5.52	1353	1608	O	O
5563 II	a	46410	43854	9503	2678	6825	15.56	1712	900	812	1.85
	b	146850	137500	21397	6237	15160	11.02	4349	4080	269	0.19

a,b refer to the experiment in which the results were obtained (see Legend).

in the M.D.L. cell-free system. Comparison of Tables 11 and 18 showed that in the cell line Pl.17, incorporation of radioactivity into Ig-like material (as measured by immunoprecipitation) was a lower percentage of total incorporation in the M.D.L. than it was intracellularly. With the cell line 5563, the relative specifically precipitable percentage of total incorporation in the M.D.L. and intracellularly was approximately equal. These results suggested that relative production of Ig or Ig-like material compared to total protein production was not substantially different in M.D.L. assays and in cell lysates. This argued that the M.D.L. system did not selectively enhance or repress translation of the major myeloma cell mRNA species relative to other species, although, as seen in Fig. 24, variations in the relative synthesis of some species occurred.

9. Effect of initiation inhibitors on the relative synthesis of H and L chains in the M.D.L.*

According to the hypothesis of Lodish, (1974), inhibitors of initiation of protein synthesis having a direct effect on the formation of the ^{40S}Met-tRNA$_f$-mRNA complex should have a preferential inhibitory effect on mRNA's which are poorer initiators. On the other hand, inhibitors acting after this point should show no preferential effect.

Consequently, with myeloma mRNA, if either H or L chain mRNA is a poorer initiator, the effect of inhibition of initiation of translation/

*These results require further repetition to establish their reproducibility.

translation should be more pronounced with that mRNA than with the other. Inhibitors of initiation whose site of action is temporally after the ^{40S}met-tRNA$_f$-mRNA complex formation event should have no effect, i.e. inhibition should not significantly affect the ratio in which H and L chains are synthesized.

Two inhibitors were studied for their effects on H and L chain production. One of these inhibitors, aurintricarboxylic acid (ATA) acts to prevent the formation of the ^{40S}met-tRNA$_f$-mRNA complex. The other inhibitor used (pactamycin) acts at a point after this complex has formed.

M.D.L. assays containing P1.17 poly(A) RNA were incubated with L-(^{3}H)-leucine in the presence of varying concentrations of aurintricarboxylic acid (ATA), or of pactamycin. Aliquots of these assays were analysed by PAGE, followed by fluorography. Fluorographs produced were densitometrically analysed and the relevant peaks traced on thick plastic film. The film was cut around the tracing and weighed. From these weighings the peak areas could be inferred and measurements of the H:L chain ratios made. 18 1cm square samples of the plastic film gave an average weight of 23.45 ± 1.25mg. Hence accuracy was approximately ± 5%.

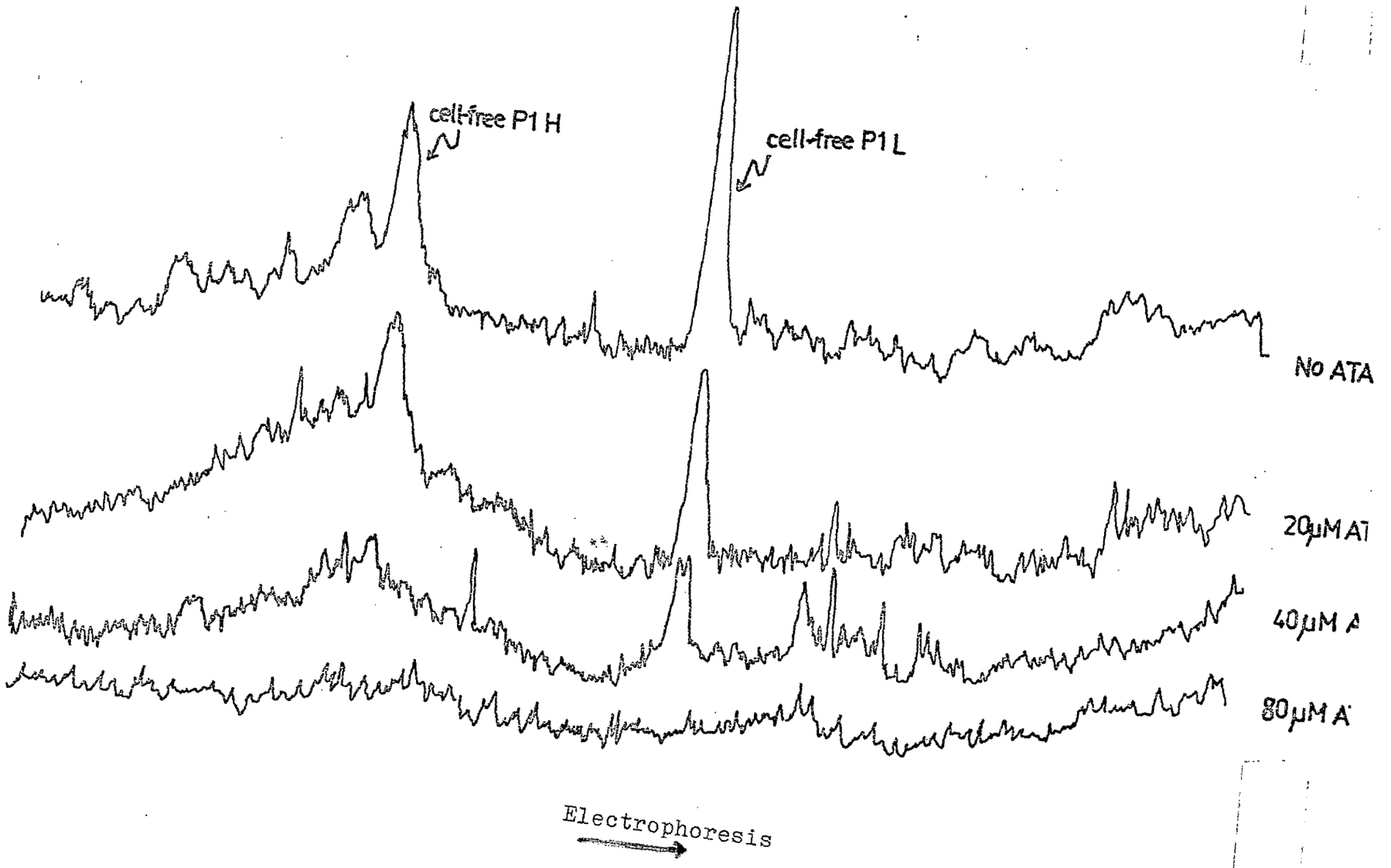
9.1. Effect of ATA on the H:L chain ratio.

Aliquots of M.D.L. assays with P1 poly(A) RNA, and containing ATA at final concentrations of 0, 20, 40, and 80μM, were analysed, after incubation, by PAGE and fluorography. Densitometric traces of the appropriate gel slots are shown in Fig. 32. The

total/

FIG. 32: Effect of aurintricarboxylic acid (ATA) on translation of Pl.17 poly(A) RNA in M.D.L.

Standard M.D.L. assays, containing 1µg Pl.17 poly(A) RNA, 2.5µCi L-(^{3}H) leucine and 50 pmoles unlabelled leucine, were incubated at 30^{o}C for 40 min in the presence of serial dilutions of ATA (0,20,40,80µM). 5µl aliquots were analysed by PAGE as described in Methods 9.2.5. and 9.2.6. Gels were processed and exposed to X-ray film (R.P. Royal X-omat) (Methods 8.3.1.). The developed fluorographs were analysed densitometrically by a Joyce-Loebel Scanning Densitometer (Methods 8.3.3.). The traces obtained were displaced vertically from each other for clarity.


cell-free P1 H
cell-free P1 L
No ATA
20μM AT
40μM A
80μM A
Electrophoresis

total incorporation and the H:L chain ratios, measured as described above are shown in Table 19.

TABLE 19. Effect of ATA on total incorporation and on the H:L ratio in M.D.L. assays. (for legend see Fig. 32).

{ATA} (μM	counts/min incorporated - background (3600 cpm)	% of control	H chain (mg)	% of control	L chain (mg)	% of control	H:L	H:L *
0	44600	100	58.2	100	68.3	100	0.85	0.42
20	11450	25.7	60.9	104.6	31.3	45.8	1.94	0.97
40	2000	4.5	27.8	47.8	24.1	35.3	1.35	0.67
80	300	0.7	n.d.	n.d.	n.d.	n.d.	n.d.	

* Adjusted to take account of relative numbers of methionines (i.e. H chain ÷ 2).

From the Lodish hypothesis, this suggests that H chain mRNA is the better initiator of the two species of mRNA, since inhibition of its translation is less affected by low levels (20μM) of ATA, whilst translation of L chain mRNA is markedly inhibited. At higher concentrations of ATA, elongation steps are also inhibited, resulting in a partial recovery of L chain synthesis relative to H chain synthesis. These results contradict those of Sonenshein & Brawerman (1976b). ATA inhibited total protein synthesis relatively more than Ig synthesis at all levels of inhibition tested. This agreed with results of Sonenshein and Brawerman (1976b), using a wheat germ cell-free protein synthesizing system to test the effect of high salt concentrations in inhibition of initiation of myeloma cell mRNA. The results are also consistent with work on the effect of initiation inhibitors on myeloma cell cultures by Sonenshein and Brawerman, (1976a), and by Nuss/

Nuss and Koch, 1976.

9.2. Effect of pactamycin on the H:L chain ratio,

Aliquots of M.D.L. assays containing Pl.17 poly(A) RNA and pactamycin at final concentrations of 0, 60, 120 and 250 nM were analysed, after incubation, by PAGE and fluorography. Densitometric traces of the appropriate gel slots are shown in Fig. 33. The total incorporation and the H:L chain ratios, measured as described above are shown in Table 20

TABLE 20: Effect of pactamycin on total incorporation and on the H:L chain ratio in M.D.L. assays. (For legend see Fig. 33).

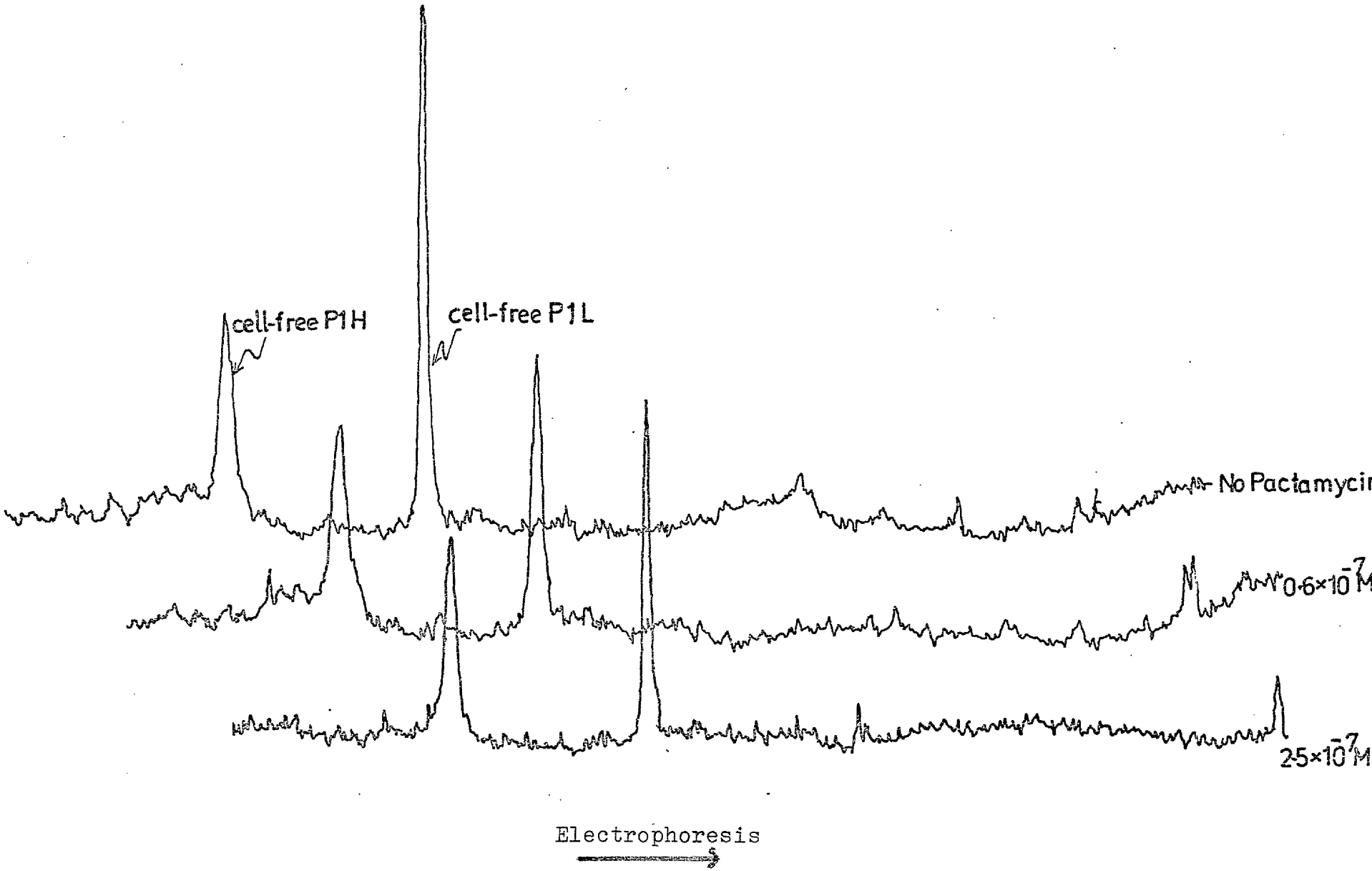
{pactamycin} (nM)	counts/min incorporated - background (3600cpm)	% of control	H chain (mg)	% of control	L chain (mg)	% of control	H:L	H:L *
0	47300	100	33.5	100	49.1	100	0.68	0.34
60	10275	21.72	40.6	121.2	34.8	70.9	1.16	0.58
120	9740	20.6	n.d.	n.d.	n.d.	n.d.	n.d.	-
250	6550	13.8	34.92	104.2	37.8	77.0	0.922	0.46

* Adjusted to take account of relative numbers of methionines (i.e. H chain ÷ 2).

Lodish predicted and showed with α and β haemoglobin synthesis that pactamycin would not greatly affect the relative translation of two species of mRNA since its site of action is after the formation of the met-$tRNA_f$-mRNA complex. These results showed a significant relative inhibition of L chain in the presence of pactamycin. As was found for ATA , total non-Ig protein synthesis was inhibited more severely/

FIG. 33: Effect of pactamycin on translation of Pl.17 poly(A) RNA in M.D.L.

Standard M.D.L. assays, containing 1µg Pl.17 poly(A) RNA, 2.5µCi L-(^{3}H) leucine and 50pmoles unlabelled leucine were incubated at 30^{o}C for 40 min in the presence of several concentrations of pactamycin (0, 0.6 x 10^{-7}M, 2.5 x 10^{-7}M). 5µl aliquots of these assays were analysed by PAGE (Methods 9.2.5 and 9.2.6.). The gels were processed and exposed to X-ray film (R.P. Royal X-omat) (Methods 8.3.1.). The developed fluorograph was analysed by a Joyce-Loebel Scanning Densitometer, (Methods 8.3.3.). The traces obtained were displaced slightly both horizontally and vertically for clarity.


cell-free P1H
cell-free P1L
No Pactamycin
0.6×10⁻⁷M
2.5×10⁻⁷M
Electrophoresis

severely by pactamycin than was Ig synthesis. Ig H chain synthesis was relatively unaffected by pactamycin whilst L chain synthesis dropped by a maximum of 30%. Total protein synthesis however, fell by up to 88% suggesting that non-Ig protein synthesis was relatively even more inhibited. These results appear to contradict the hypothesis of Lodish (1974). They will be discussed later.

10. Comparison of cell-free products of polysomes from Pl.17.1 and Pl.17.4 cells.

Pl.17.1 was a recloned cell line derived from Pl.17 selected for high levels of Ig production, whereas Pl.17.4 was a similarly derived cell line selected on the basis of an absence of detectable H chain synthesis. They therefore provided a means for locating the position of the H chain precursor synthesized by the M.D.L. in response to myeloma polysomes. The band corresponding to this species would be absent in fluorographs of PAG's of cell-free products from Pl.17.4 but present in cell-free products of Pl.17.1. As shown in Figs 34 and 34a, both visual and densitometric inspection revealed the presence of a major band with a mobility slightly greater than that of secreted H chain or cellular H chain, which appeared in densitometric traces of Pl.17.1 total cell-free products but not of Pl.17.4 total cell-free products. The relative mobilities of the secreted and cellular H chain and of the putative H chain precursor were 0.26, 0.26 and 0.27 respectively.

11./

FIG. 34: PAGE analysis of total M.D.L. synthesized products of Pl.17.1 and Pl.17.4 polysomes.

Aliquots of standard M.D.L. assays, containing either Pl.17.1 or Pl.17.4 polysomes were analysed by PAGE (Methods 9.2.5. and 9.2.6.). Gels were processed and exposed to X-ray film (R.P. Royal X-omat) (Methods 8.3.1.).

Key	Well	Description
	1	Pl.17 secreted total products
	2	Pl.17 cell lysate total products
	3	M.D.L./Pl.17 polysomes - total products
	4	M.D.L./Pl.17.1 polysomes - total products
	5	M.D.L./Pl.17.1 polysomes - total products
	6	M.D.L./Pl.17.4 polysomes - total products
	7	M.D.L./Pl.17.4 polysomes - total products
	8	Pl.17 cell lysate total products

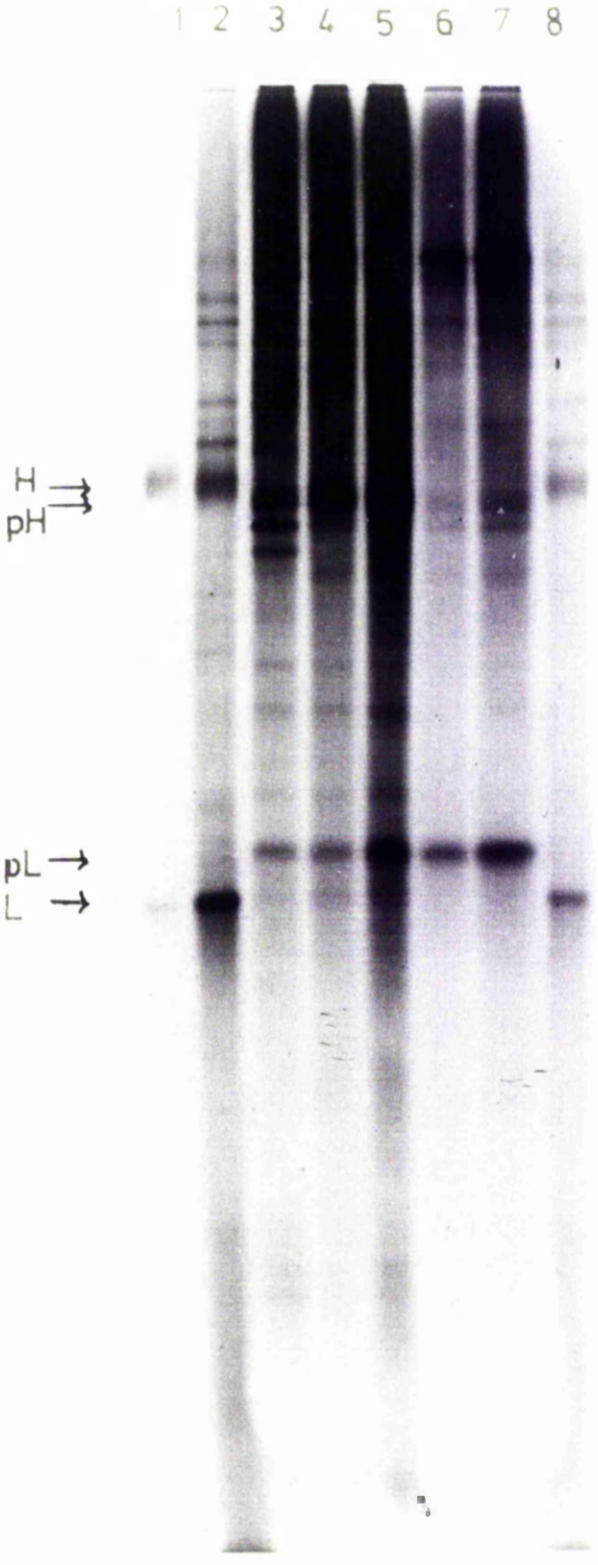
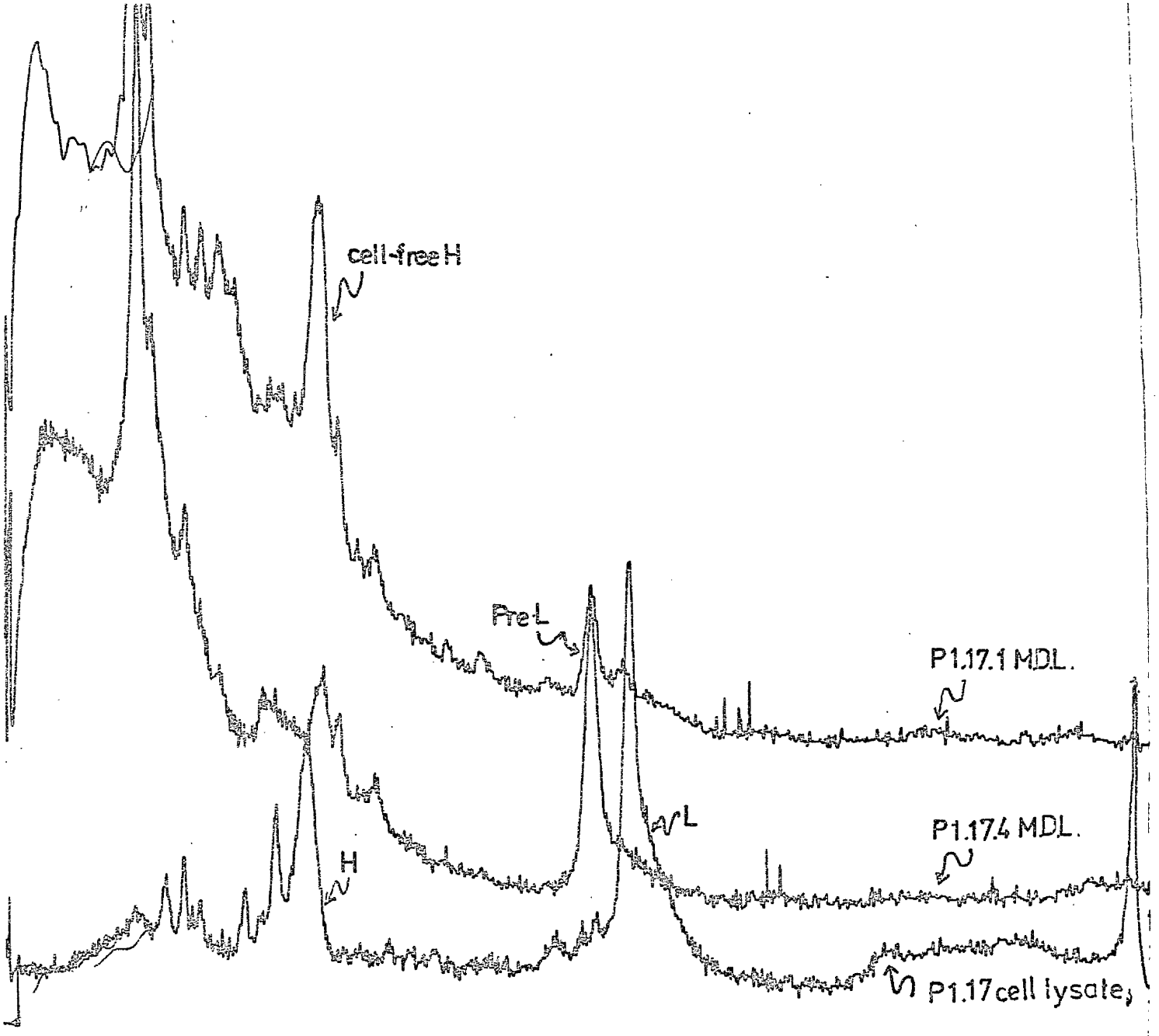

1 2 3 4 5 6 7 8
H
pH
pL
L

FIG. 34a: Densitometric analysis of Wells 2,4,7 of Fig. 34.

Wells 2,4,7 of Fig. 34 were analysed densitometrically by a Joyce-Loebel Scanning Densitometer (Methods 8.2.3.). The traces obtained have been displaced vertically from each other for clarity.


cell-free H
Pre-L
P1.17.1 MDL.
L
P1.17.4 MDL.
H
P1.17 cell lysate

11. Effect of 2-deoxyglucose on myeloma Ig products.

2-deoxyglucose, an analogue of glucose,has been shown to block the addition of carbohydrate to Ig H chains (Melchers, 1973) and to Ig L chains (Eagon and Heath, 1977). In the cell lines used in this study, only H chains contain carbohydrate residues. It also acts as a glucose antagonist, causing general inhibition of metabolism by blocking energy-supplying reactions. It has, however, other effects on cellular metabolism whose mode of action is not clear. M.D.L. synthesized H chain appeared to be higher in mobility than either secreted or intracellular H chain. It is known that the presence of carbohydrate side chains decreases the mobility of proteins on polyacrylamide gels by a greater amount than can be accounted for merely by the actual increase in MW which they cause. Murine γ_{2a} chains generally have relatively low amounts of carbohydrate attached (Fougereau et al., 1976). Nevertheless, the increase in mobility displayed by the putative pre-H chain could have been due to a lack of carbohydrate residues, missing because the necessary enzymes for addition of carbohydrate were missing or inactive in the M.D.L. It was demonstrated (Table 21) that M.D.L. containing myeloma polysomes or poly(A) RNA failed to incorporate significant amounts of D (6-^{3}H)-glucosamine into TCA precipitable material.

2-deoxyglucose was used to attempt to produce H chains lacking in carbohydrate residues in cultured cells. These would provide markers for comparison with in vitro synthesized putative H chain.

To determine the optimum concentration of 2-deoxyglucose, 5563 cell cultures were labelled with L-(^{35}S) methionine in the presence of/

TABLE 21: Incorporation of D-(6-^{3}H) glucosamine in M.D.L. containing myeloma polysomes or poly(A) RNA.

M.D.L. containing:-	(^{3}H) * counts/min/assay	(^{35}S)* counts/min/assay
0	17800	135000
P1 polysomes	18800	424800
5563 polysomes	15400	547700
5563 poly(A) RNA	19000	659300

* Separate assays

Legend:

Standard M.D.L. assays containing excess unlabelled methionine were incubated with various polysome fractions in the presence of 5μCi D-(6-^{3}H) glucosamine as described in Methods 7.2. Duplicate assays, containing L(^{35}S) methionine were also incubated as above. Radioactivity incorporated into TCA-precipitable material was measured as described in Methods 8.2.1.

of different concentrations of 2-deoxyglucose, as described earlier in this section. The results in Table 12 showed that, in the presence of increasing concentrations of 2-deoxyglucose, incorporation of radioactivity into TCA precipitable material fell rapidly to an approximately constant level of 20% of the control cultures at concentrations of > 20mM 2-deoxyglucose. Incorporation of radioactivity into specificially immunoprecipitable material, however, proved more sensitive to the presence of 2-deoxyglucose. Radioactivity specifically precipitated continued to decrease with rising concentrations of 2-deoxyglucose, although its rate of decline decreased at higher concentrations of 2-deoxyglucose. 20mM 2-deoxyglucose was used as the optimum concentration, giving 80% inhibition of specifically precipitable material. Analysis of aliquots of several of the cell lysates by PAGE suggested that the presence of 2-deoxyglucose produced a small but visible increase in the mobility of the cellular H chain of 5563. (see Fig. 35).

12. Translation of polysomes from SAMM 368 in M.D.L.

SAMM 368 is a "double-producing" cell line which synthesizes and secretes both α and γ_{2b}-type H chains (Morse et al., 1977), and two κ-type L chains (T.R. Mosmann, personal communication). As such, it offered the possibility of comparing in vitro the relative synthesis of two Ig H chains normally synthesized together in vivo. (The two κ chains are generally not well resolved by PAGE, except with 4M urea present, and hence unsuitable for quantitative study). The α-type H chain of Ig molecules, moreover, have generally more attached carbohydrate/

FIG. 35: PAGE analysis of 5563 cell lysate products, labelled with L-(^{35}S) methionine in the presence of various concentrations of 2-deoxyglucose.

5563 cells were labelled, as described in Methods 2.4. 10µl aliquots were analysed by PAGE as described in Methods 9.2.5. and 9.2.6. Gels were processed and exposed to X-ray film (R.P. Royal X-omat) (Methods 8.3.1.).

Well

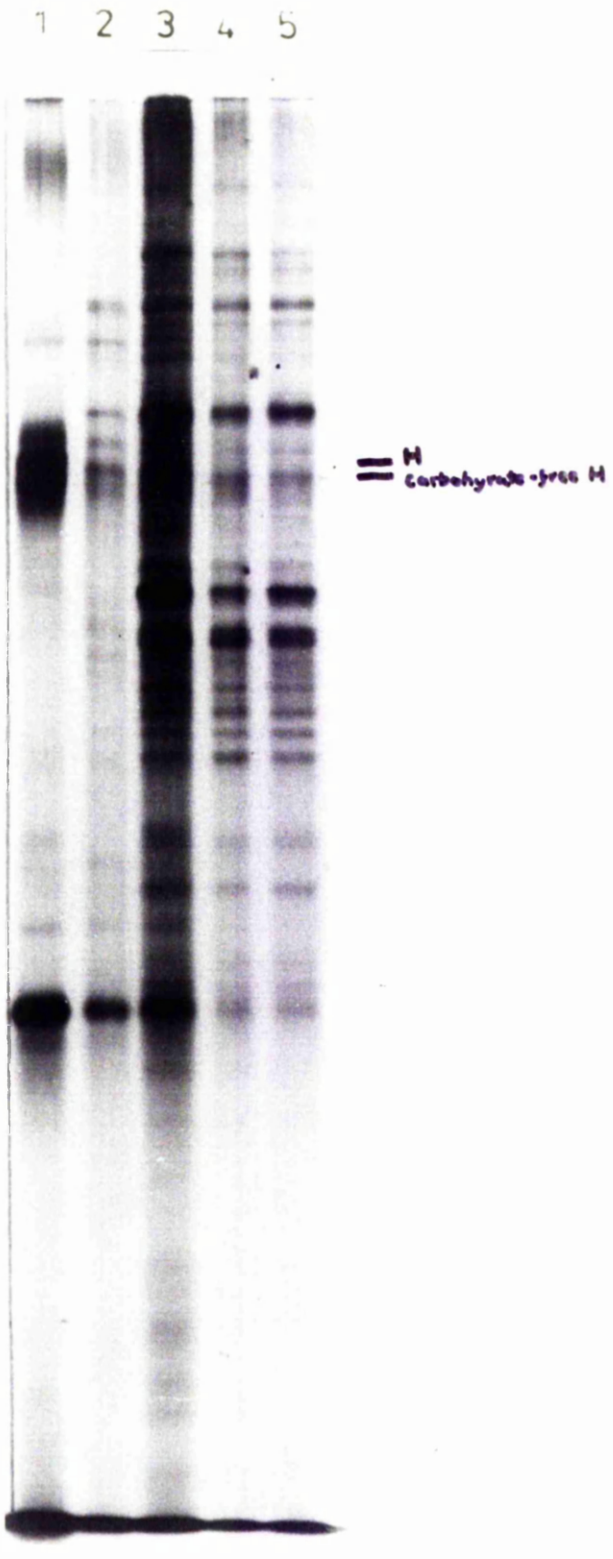
1 Secreted material from 5563 cells

2 5563 cell lysate (No 2-deoxyglucose).

3 " " " " "

4 " " " (20mM 2-deoxyglucose).

5 " " " (100mM 2-deoxyglucose).


1
2
3
4
5
= H
carbohydrate-free H

carbohydrate than γ-type H chains. Hence the relative shift in mobility caused by synthesis of the H chains in vitro should be different for α-and γ-type H chains.

Polysomes from SAMM 368 cells were translated in the M.D.L. The cell-free products were compared by PAGE with the cellularly synthesized products (Fig. 36) and the banding patterns compared. The bands indicated in the cell lysate as α, γ and κ chains had previously been identified by immunoprecipitation (Fig. 20). Cell-free products showed several differences from cellular products.

1. A band appeared in cell-free products with a mobility lower than that of SAMM 368 cellular L chains. This was tentatively identified as the precursor to L chain on the basis of its mobility and of its prominence.

2. Two prominent bands with mobilities higher than cellular α and γ chains were observed in the cell-free products. On the basis of their prominence, and also on the predicted effect of the absence of carbohydrate on mobility, they were tentatively identified as the cell-free translation products of α and γ chain mRNA. Which band corresponded to α chain and which to γ chain required clarification by immunoprecipitation.

Densitometric analysis of the banding patterns obtained revealed no bias of synthesis towards one or the other H chain in the cell-free system (data not shown).

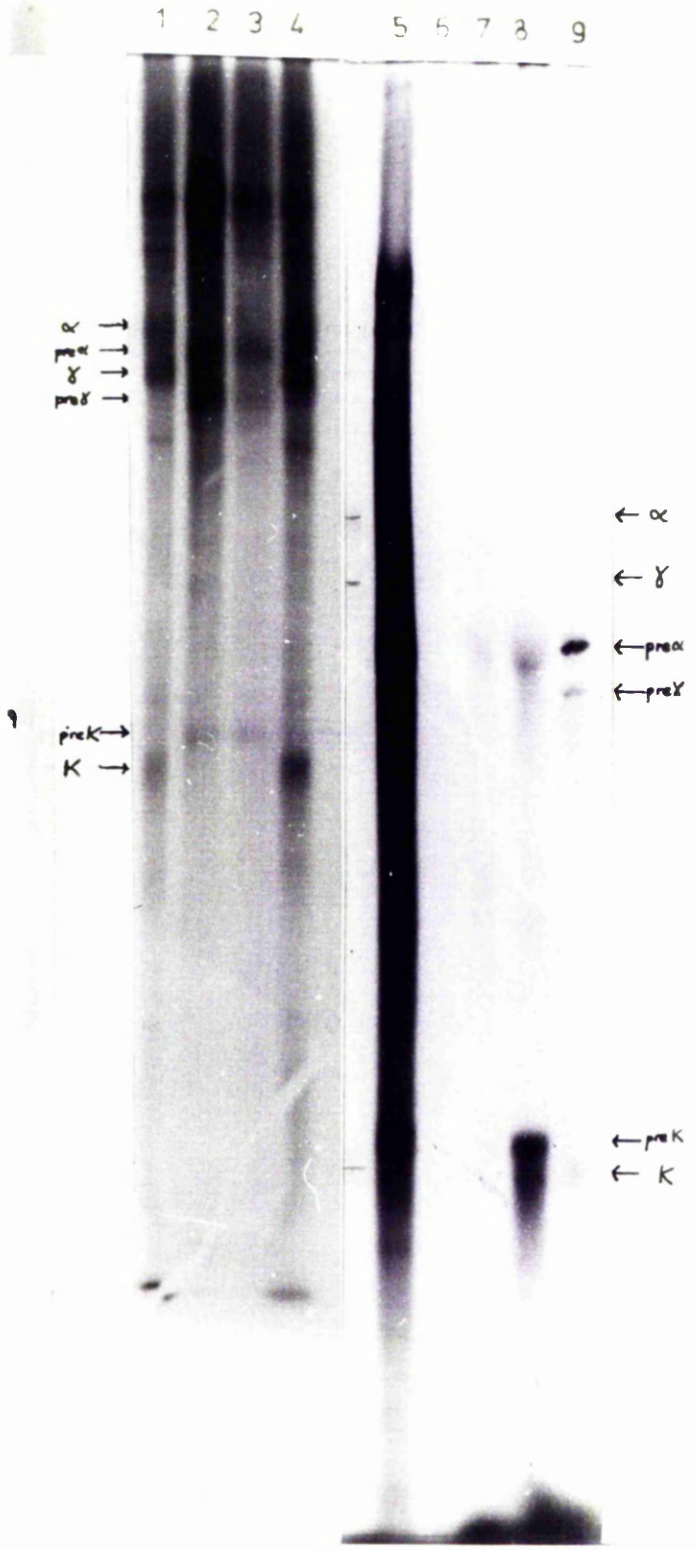
Immunoprecipitation, using antisera directed against κ, γ and α chains was investigated. PAGE analysis of the immunoprecipitates (Fig. 36) demonstrated that, as expected, the lower mobility band in the L chain region of the gel corresponded to the pre L chain.

Using/

FIG. 36: Comparison of SAMM 368 polysome-directed products in M.D.L. with SAMM 368 cellular products.

SAMM 368 polysomes, prepared as described in Methods Section 3, were incubated in the M.D.L. under conditions described in Methods 7.2. Aliquots of the assay were analysed by PAGE (Methods 9.2.6.) and the gel processed for fluorography (Methods 8.3.1.). Aliquots of cell lysate were analysed similarly. Immunoprecipitates were carried out as in Methods 11.2.2. Positions of marker cellular α, γ and κ chains are indicated.

Well	Description
1	SAMM 368 total cell lysate products
2	SAMM 368 total M.D.L. products
3	" " " "
4	" " cell lysate products
5	SAMM 368 total M.D.L. products
6	SAMM 368 normal rabbit serum precipitate
7	" anti γ chain precipitate
8	" " κ chain precipitate
9	" " α " "


1 2 3 4 5 6 7 8 9
preK
K
← α
← γ
←preα
←preγ
←preK
← K

Using anti-α antiserum, two species were precipitated, corresponding in mobility to the putative cell-free α and γ chains. The anti-α antiserum had previously been demonstrated to have cross-reactivity with γ chains, but of a weaker nature. Consequently, the more prominent precipitated species was identified as cell-free α chain, whilst the other species was identified as γ chain. From mobility considerations, the α chain mobility seemed relatively more affected by translation in the M.D.L., as would be expected due to the difference in carbohydrate composition.

Hence it has been demonstrated that translation of two types of H chain mRNA (one for α chain, one for γ chain) in a cell-free system leads to the synthesis of H chains with lower apparent MW's. That this is in part due to the lack of carbohydrate addition is shown by the relatively greater change in the mobility of that H chain which in vivo contains more carbohydrate.

13. N-terminal amino acid determination of 5563 pre L chain.

The precursors of L chain from other myeloma cell lines have been found, by amino acid sequencing,procedures, to contain methionine as N-terminal amino acid (See Fig. 1), although in some cases, the yield of methionine is very low (e.g. Burstein et al., 1976). Dansylation was used to examine the N-terminal amino acid of 5563 pre L chain. Cellular 5563 L chain contains a blocked N-terminus (Awdeh et al., 1969). The dansylated protein was hydrolysed and the amino acids separated by two-dimensional chromatography on polyamide sheets, as described in Methods (Sections

12/

12.1 and 12.2).

13.1 Separation of dansylated amino acid markers on polyamide sheets.

Mixtures of dansylated amino acids for use as markers (kindly donated by Drs. J. Lumsden and J.R. Coggins) were separated on polyamide sheets as described in Methods 12.2. Under ultra violet light the reproducible spot patterns shown in Figs. 37 A and B were obtained. The various dansylated amino acids are identified in the key. In subsequent experiments, one side of the polyamide sheet was loaded with a dansylated amino acid mixture whilst the other side was loaded with the sample under investigation.

13.2 Dansylation of L(^{35}S) methionine.

5μCi of L(^{35}S) methionine was treated in a similar fashion to the dansylation procedure for proteins described in Methods 12.1. After hydrolysis and separation of products on polyamide sheets, the sheet was examined under U-V light and by autoradiography (data not shown). Ultraviolet examination detected a weak spot in the position of DNS-methionine and a stronger spot in the position of DNS-leucine as well as several other weaker spots. Autoradiography suggested that apart from free methionine and Dansyl-OH, the major species formed were DNS-methionine and DNS-methionine sulfoxide. The spot seen at the DNS-leucine position with ultraviolet light was not radioactive.

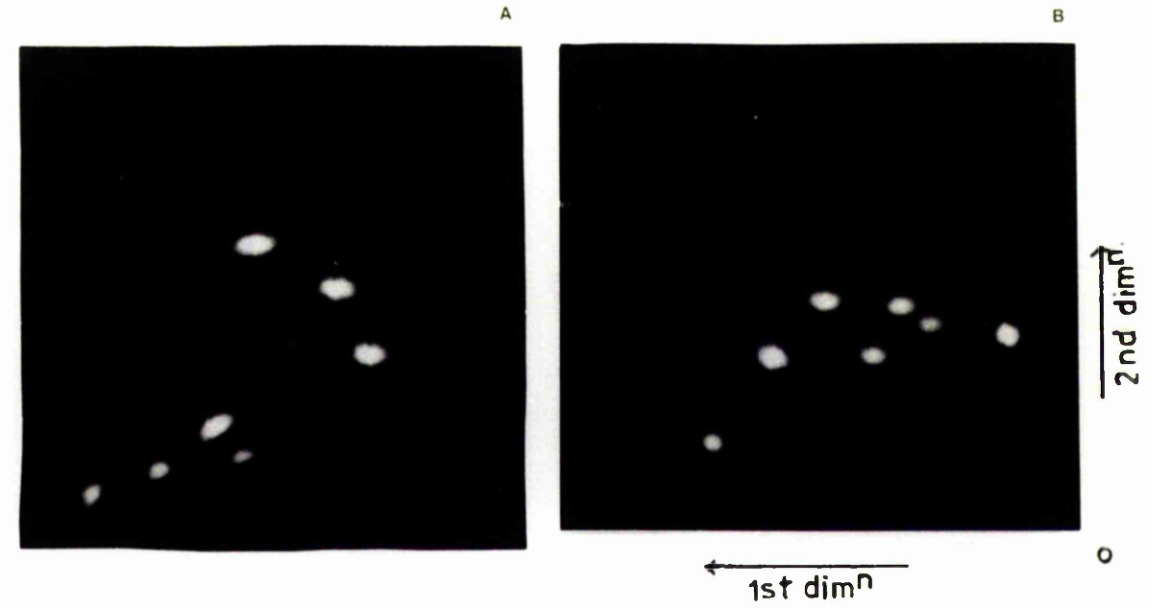
13.3/

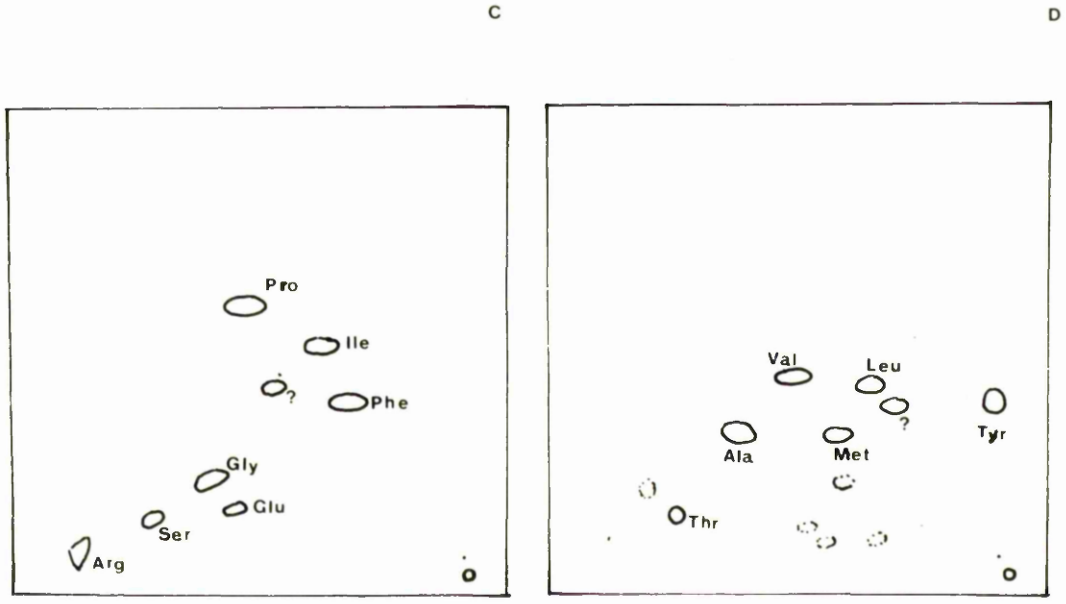
FIG. 37: Dansylation studies I: Analysis of dansylated amino acids on polyamide sheets.

A: Dansylated amino acid mixture I: DNS-Arg, DNS-Glu, DNS-Gly, DNS-Ile, DNS-Phe, DNS-Pro. Fig. C is a representation of Fig. A, with the dansylated amino acids corresponding to each spot represented by the appropriate three-letter amino acid designation. Unknown or minor spots represented by question marks and dotted lines, respectively.

B: Dansylated amino acid mixture II: DNS-Ala, DNS-Leu, DNS-Met, DNS-Thr, DNS-Tyr, DNS-Val. Fig. C is a representation of Fig. B, with the dansylated amino acids corresponding to each spot represented by the appropriate three-letter amino acid designation. Unknown or minor spots represented by question marks and dotted lines, respectively.

Dansylated amino acids were applied to polyamide sheets and chromatographed as described in Methods 12.2. Polyamide sheets containing dansylated residues were photographed under short-wave ultraviolet light.


A
B
2nd dimn
1st dimn


C
D
Pro
Ile
?
Phe
Gly
Glu
Ser
Arg
Val
Leu
?
Tyr
Ala
Met
Thr

13.3. Behaviour of L(^{35}S) methionine on polyamide sheets.

Free L(^{35}S) methionine moved with the solvent front in the first dimension buffer and had no mobility in the second dimension (data not shown).

13.4. Dansylation of micrococcal nuclease.

The N-terminal amino acid of micrococcal nuclease (E.C.3.1.4.7) is alanine and is unblocked (Croft, 1973). After dansylation, digestion and polyamide sheet analysis, inspection under ultraviolet light revealed a dansylated amino acid spot in the position of DNS-alanine (Fig. 38).

13.5. Dansylation of 5563 H and L chains.

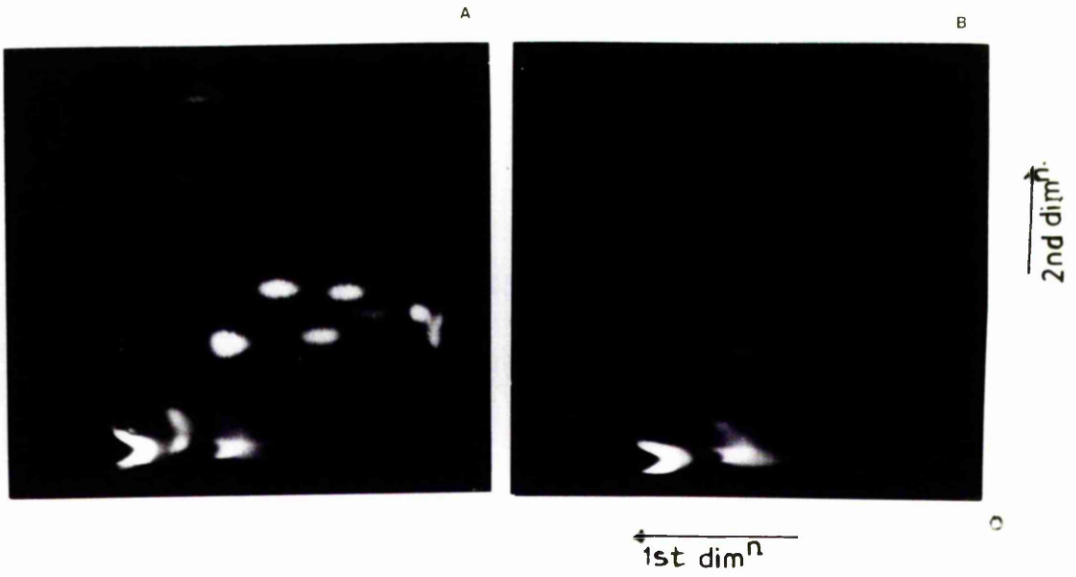
5563 H and L chains synthesized *in vivo* are known to have "blocked" N-terminal amino groups (Awdeh *et al.*, 1969) and hence should be unreactive with Dansyl chloride (except for internal tyrosine and lysine residues). 5563 H and L chains, separated by G-100 Sephadex in a urea-formate buffering system, were taken separately through the dansylation, hydrolysis and analysis procedure as described in Methods 12.1. (Fig. 39). Ultraviolet light inspection revealed fluorescent spots only in the positions associated with O-Tyrosine and ε- or α-lysine. Hence, neither 5563 H or L chains had reactive (i.e. unblocked) N-terminal amino acids. (Lysine is ruled out as N-terminal amino acid since such a residue would be dansylated both in the α- and the ε- positions, thus altering its mobility on polyamide sheets. An alteration such as this was not found/

FIG. 38: Dansylation studies II: Dansylation of a protein of known N-terminus.

Dansylation and hydrolysis were carried out as described in Methods 12.1. The hydrolysed protein was separated by chromatography on polyamide sheets as described in Methods 12.2 and the dansylated species identified under ultra-violet light.

A: Dansylated amino acid mixture II + dansylated and hydrolysed micrococcal nuclease (S. aureus). Hatched spots indicate spots not present in the marker mixture alone.

B: Dansylated and hydrolysed micrococcal nuclease (S. aureus). The dansylated N-terminal residue is marked (DNS-ala).


A
B
2nd dim.
1st dim.

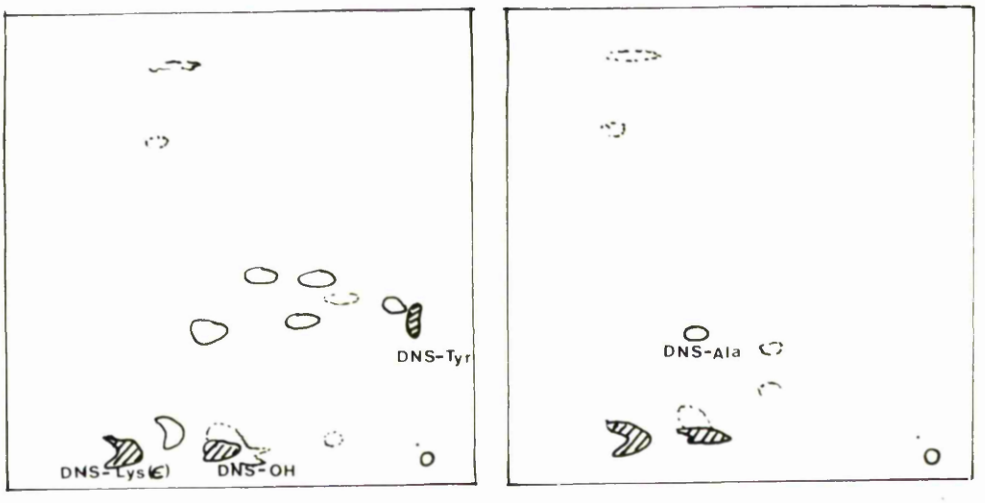

DNS-Tyr
DNS-Lys(ε)
DNS-OH
DNS-Ala

FIG. 39: Dansylation studies III: Dansylation of cellular 5563 H and L chain and of L-(^{35}S) methionine-labelled cell-free synthesized L chain-like material.

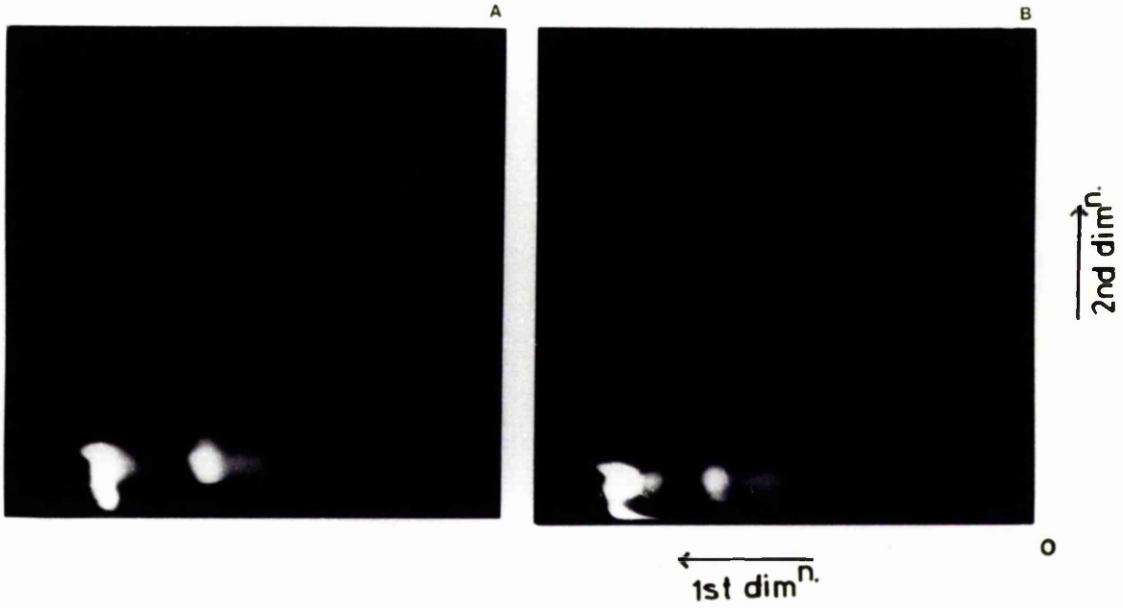
A: Dansylated products of 5563 H chain. 5563 H chain, separated from 5563 L chain by column chromatography (Methods 10.3.), was dansylated and hydrolysed (Methods 12.1.) before separation of dansylated residues on polyamide sheets (Methods 12.2.). The dansylated derivatives were examined under ultra-violet light.

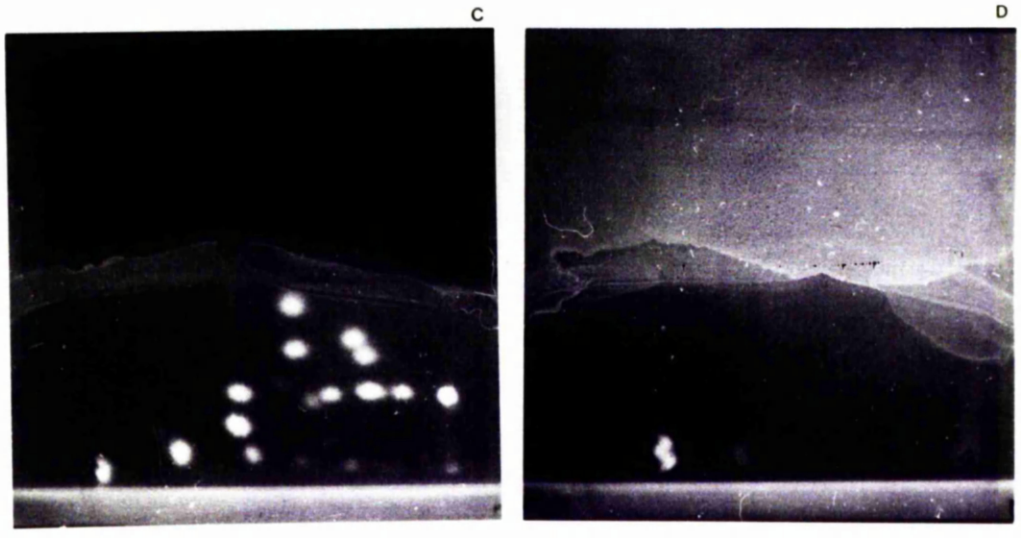
B: As for A with 5563 L chain.

C*: Dansylated amino acid mixtures I and II.

D*: Dansylated products of pre-L chain of 5563. Pre L chain, radioactively labelled with L-(^{35}S) methionine in an M.D.L. assay was separated from other radioactive cell-free products by PAGE. The pre L chain-containing gel region was excised and the pre-L chain material extracted (Methods 9.2.7.). After lyophilisation of the extract, the radioactive material was dansylated, then hydrolysed, in the presence of carrier unlabelled 5563 L chain (Methods 12.1.). Separation of dansylated species was carried out on polyamide sheets (Methods 12.2.).

* The ultra-violet photographs of these sheets appear lighter than normal, due to the presence of PPO, which was introduced into the sheets to improve the detection of radioactivity by fluorography. Removal of the PPO by washing with ether did not reveal any more ultra-violet fluorescent spots.


A
B
2nd dimn.
O
1st dimn.


C
D

found.

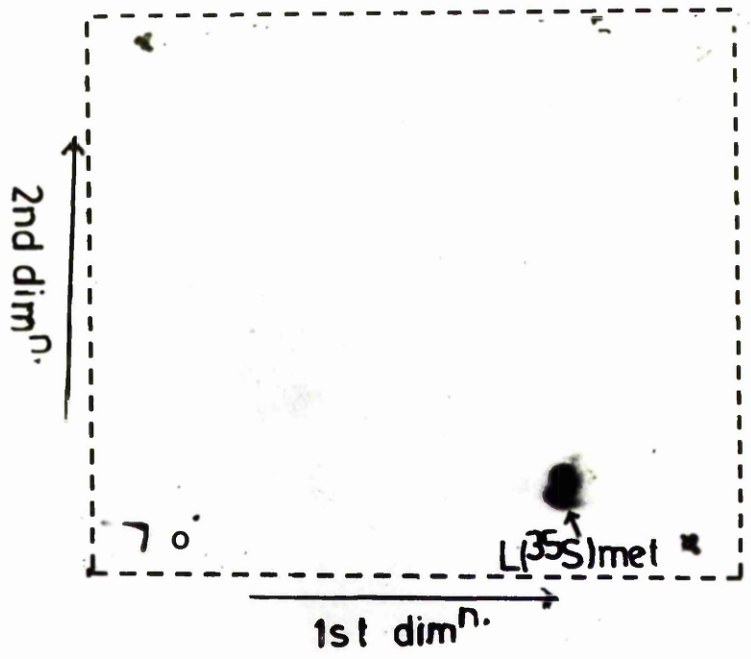
13.6. Dansylation of 5563 pre-L chain.

Bands corresponding to 5563 pre-L chain as localised by comparison with fluorographs, were excised from a polyacrylamide slab gel and the protein extracted as described in Materials and Methods (Section 9.2.7.). (4 times as much radioactivity was released by the first extraction than was released by a second extraction). The extracted radioactively labelled material was lyophilised and re-dissolved in dansylation medium, dansylated, hydrolysed and analysed as described in Methods 12.1 and 12.2. Ultraviolet inspection (Fig. 39) revealed the presence of ε-DNS-lysine and O-DNS-Tyrosine as well as a few minor spots. Fluorography, however, (Fig. 40) showed the presence of radioactivity only at the solvent front in the first dimension indicating the presence of free methionine. This suggested either that in 5563 pre-L chain synthesized in the M.D.L. system, methionine is not the original N-terminal residue or (more probably) that the N-terminal amino acid is cleaved off rapidly by the system.

14./

FIG. 40: Dansylation studies IV. Fluorograph of hydrolysed products of dansylated 5563 pre L chain, radioactively labelled with L-(^{35}S) methionine.

Pre L chain was treated as described in Fig. 39 D. After separation of hydrolysed products polyamide sheets, the sheet was chromatographed in the second dimension with 7% (w/v) PPO in diethyl ether as solvent (Methods 8.3.2.). Following chromatography, the polyamide sheet was dried and exposed to R.P. Royal X-omat film for 3 weeks. The position of L-(^{35}S) methionine is indicated.


2nd dim^n.
7 o
L(35S)met
1st dim^n.

14. Tryptic mapping.

14.1. General.

Proteolytic cleavage by trypsin followed by separation of the peptides generated by electrophoresis and chromatography was investigated as a method of comparing cellularly produced H and L chains with H and L chain-like products of the cell-free system. Extra peptides, present in the cell-free system products but not in the cellular products, were looked for. The technique was also used to try to establish the identity of the cell-free products related to that of the cellular products.

14.2. Peptide mapping of cellular 5563 H and L chains.

Immunoprecipitates from 5563 cells, radioactively labelled with L(^{35}S) methionine in the presence of 20mM 2-deoxyglucose, were reduced, alkylated and acetone precipitated. The precipitate (4.6 A_{280} units:36000 cpm) was dissolved in 6M urea/20% formate buffer and separated on a 50ml G-100 Sephadex column equilibrated with 6M urea/20% formate buffer (Methods 10.3). 2.745 A_{280} units of protein were detected in the eluant in one peak with a shoulder to the lighter side. This peak was separated into two fractions, labelled "H" and "L". The eluant fractions were acetone precipitated, and the precipitates redissolved in ammonium bicarbonate buffer. Trypsin digestion was followed by electrophoresis and chromatography. The silica gel plates containing the peptides were then chromatographed in 7% (w/v) PPO in ether. Radioactivity was detected by fluorography. Figs. 41 and 42 also show H and L chain L(^{35}S) methionine labelled peptide maps rexpectively. In Fig. 41A, for H chain, 8 prominent

and/

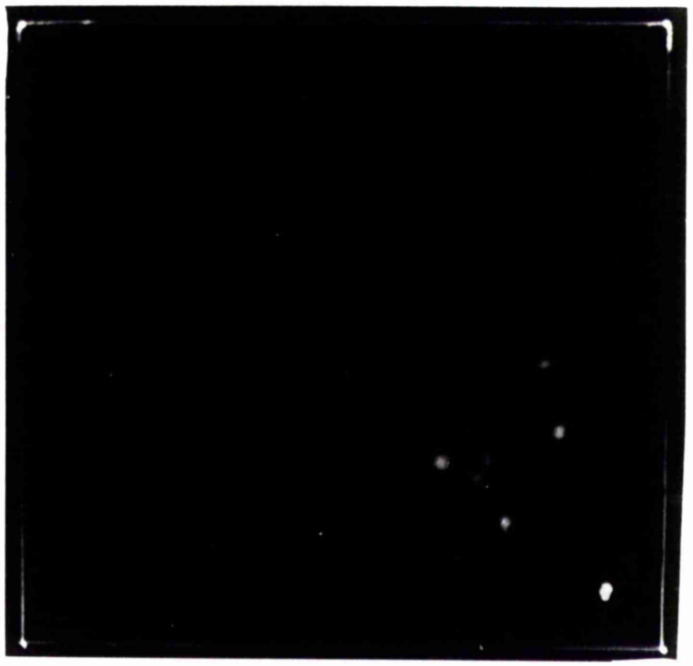
FIG. 41: Peptide maps of cellular and cell-free 5563 H chain.

A: 5563 cells were incubated with 20mM 2-deoxyglucose and L-(^{35}S) methionine as described in Methods 2.4. Cell lysates were prepared (Methods 2.3.) and 5563 Ig was isolated by indirect immunoprecipitation (Methods 11.2.2.). After reduction and alkylation the immunoprecipitable 5563 Ig H and L chains were separated by G-100 Sephadex chromatography (Methods 10.3.). 5563 H chain (+ unlabelled carrier H chain from the immunoprecipitate) was digested with Trypsin-TPCK (Methods 13.1.) and the digestion products separated by electrophoresis and chromatography on cellulose sheets (Methods 13.2.). Electrophoresis was carried out at pH 3.5 for 5 hours at 20mA/sheet. Ascending chromatography was carried out using B.A.W.P. (Butanol: acetic acid: water: pyridine 15:3:12:10: by volume) as solvent. Cellulose sheets were then subjected to ascending chromatography, using 7% (w/v) PPO in diethyl ether as solvent. After drying, the plates were placed in contact with R.P. Royal X-omat X-ray film for 3 weeks. The film was developed by standard methods. Visualisation of peptides by ninhydrin was not carried out due to the presence of excess heterogenous peptides from the carrier Ig H chain.

FIG. 41: Peptide maps of cellular and cell-free 5563 H chain.

B: L-(^{35}S) methionine labelled products of M.D.L. containing 5563 polysomes were separated by PAGE and the positions of the various species determined by fluorography. The putative cell-free H chain band was excised from the gel and the radioactive material extracted (Methods 9.2.7.). Tryptic digestion (in the presence of excess unlabelled 5563 Ig)was carried out as described in A.

A



B

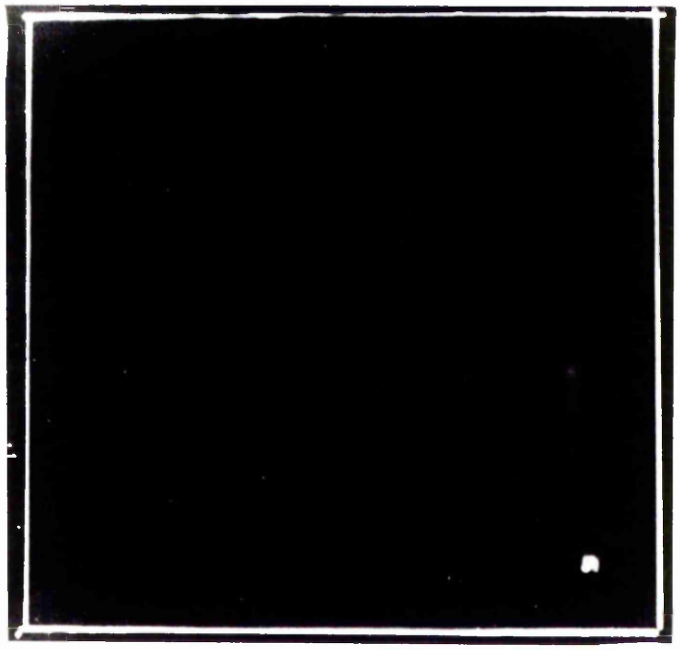
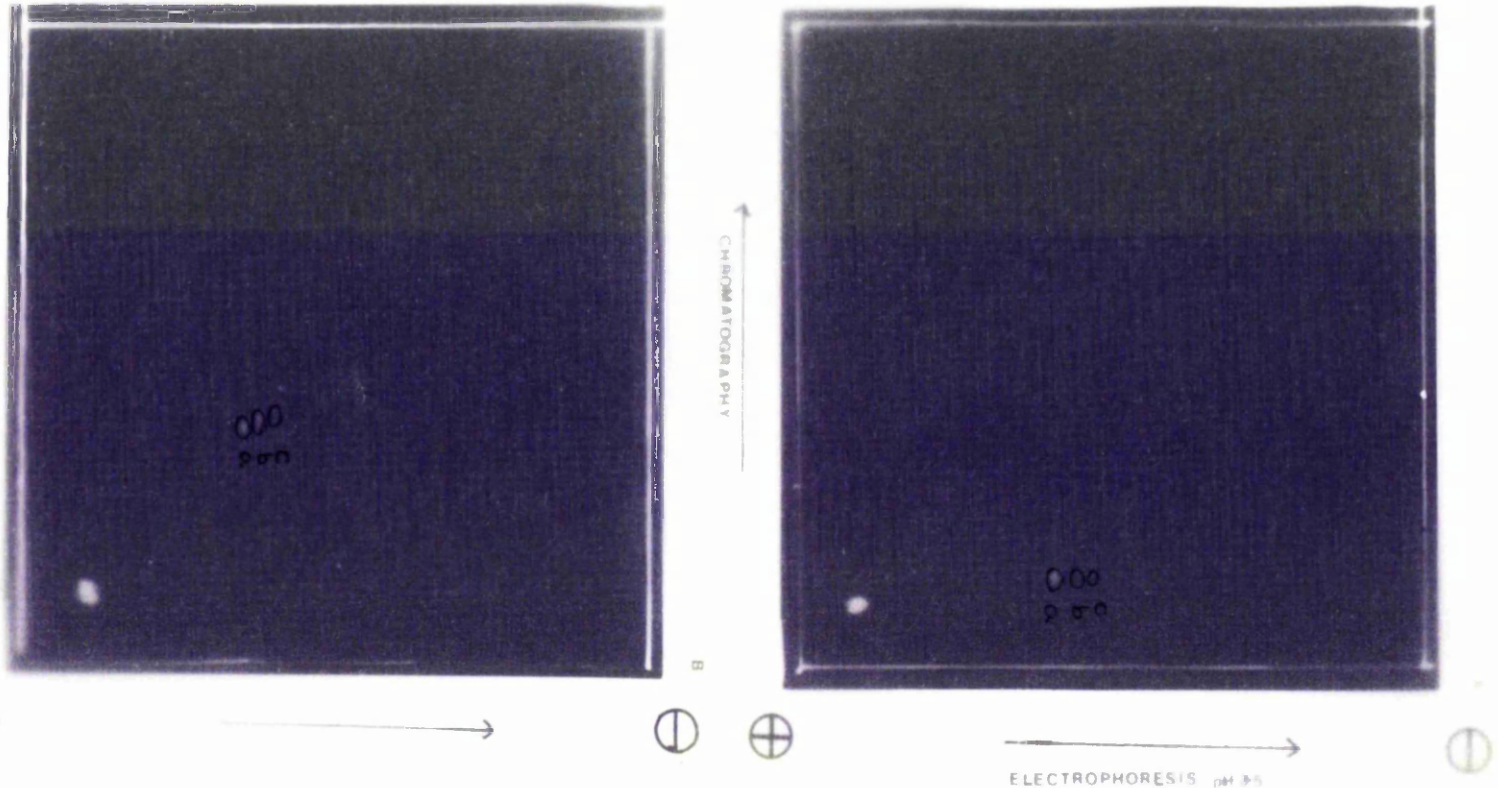


FIG. 42: Peptide maps of cellular and cell-free 5563 L chain.

A. 5563 L chain isolated and digested as described in Fig. 41 A.

B. 5563 pre-L chain, isolated and digested as described in Fig. 41 B.


CHROMATOGRAPHY
ELECTROPHORESIS

and several minor spots can be seen. 5563 H chain contains 10 methionine residues (A.R. Williamson, unpublished results). In Fig. 42A for L chain, four prominent spots and several less prominent spots can be seen.[a,b,c] Three of the prominent spots have similar chromatographic mobilities and vary only in electrophoretic mobility. These may be due to charge heterogeneity of a single species. The minor spots may be due to a contamination by H chain peptides caused by insufficient resolution of H and L chains by the gel filtration step. 5563 L chain contains 3 methionine residues (A.R. Williamson, unpublished results).

14.3. Peptide mapping of putative 5563 H and L chains produced in M.D.L.

Bands corresponding to putative 5563 H and L chains on PAG's were located by comparison with fluorographs. The bands were excised and extracted as described in Section 9.2.7 (Materials and Methods). Radioactive material (containing 9600 counts/min) extracted from the pre L chain region of the gel and material (containing 21000 counts/min) was extracted from putative pre H chain region. The extracts were lyophilised and redissolved in Tris HCl buffer pH 7.4, containing either 125µg 5563 L chain or 250µg 5563 H chain (as appropriate). The sample was then precipitated with acetone, re-dissolved in ammonium bicarbonate and digestion, electrophoresis chromatography and fluorography were performed as described in Methods 13 and 8.3.2. The fluorographs are shown in Figs. 41 and 42. 5563 pre L chain (Fig. 42B) shows three major spots similar in grouping to,/

to, but with different mobilities from those found with cellular L chain. Such differences may have occurred due to differences in the silica gel plates used. The spot pattern obtained with digests of putative 5563 H chain did not closely resemble that of 5563 H chain cells treated with 2-deoxyglucose (Fig. 41B).

From this data, it can be stated that the spot patterns for cellular L chain and cell-free synthesized putative pre-L chain are similar and suggest that the cell-free species identified by mobility and immunoprecipitation contains similar methionine peptides to 5563 L chain.

The area removed from polyacrylamide gels and thought to contain putative pre H chain did not give a spot pattern similar to that of cellularly produced H chain. The region containing putative H chain (identified by immunoprecipitation) contains many other protein species. This may have obscured the spot pattern of putative H chain.

15. Attempted improvements of the M.D.L. cell-free system.

15.1. Rationale.

Earlier work (Results 4.3) had shown that the M.D.L. system contained large endogenous pools of amino acids. Even the relatively small pools of leucine and methionine, which were primarily used for radioactive incorporation experiments, were at least an order of magnitude larger than the amount of radioactively labelled amino acid added. For example, the specific activity of added L(^{35}S) methionine was generally decreased by ∿70 fold due to dilution/

dilution in assays with unlabelled methionine, either free or bound in met-tRNA complexes. Nevertheless, the high fidelity of translation of all sizes of mRNA and low background incorporation made the M.D.L. the cell-free system of choice for translation studies. The cell-free protein synthesizing system derived from wheat germ, by contrast, has much lower efficiency in translating long mRNA's (Green et al., 1976). It gives, however, much greater incorporation of radioactivity since endogenous amino acids are removed by a gel filtration step (Roberts and Paterson, 1971). Methods of preparation of several other cell-free protein synthesizing systems have included a gel filtration step (McDowell et al., 1972; Hartley et al., 1975). Gel filtration was considered in the first instance as a method of removing endogenous amino acids and thereby improve incorporation of radioactively labelled amino acids. The use of dialysis to remove endogenous free amino acids was also investigated. The development of a cell-free system with low levels of endogenous amino acids was seen as a means whereby polypeptides, radioactively labelled with equal specific activity in most residues, could be obtained. These would be suitable for micro techniques of amino acid sequencing.

15.2. Preparation and running of gel filtration columns.

Sephadex beads were swollen as described in Methods 10.2 and packed on a 25 x 1.5cm glass column. Final settled bed volume was ∿120ml. The column was equilibrated with column buffer (20mM HEPES pH 7.6, 120mM KCl, 5mM MgOAc, 6mM 2-mercaptoethanol (Roberts

&/

& Paterson, 1971). After equilibration, 3-4ml reticulocyte lysate (containing 25μM haemin) was loaded carefully on the column. After entering the column, the sample was washed through under a 50cm pressure head by column buffer. The void volume, identified by the colour, was collected in 2ml fractions, pooled, then dispensed in 0.8ml aliquots and stored frozen at -70°C. Passing over the column approximately doubled the volume of the 'lysate fraction'.

15.3. Preparation of M.D.L. from column eluant (cM.D.L.).

The eluant from gel columns differed in several ways from the original reticulocyte lysate, apart from an absence of amino acids:-

1. The eluted lysate was diluted approximately twofold compared with the original lysate.
2. The following low MW molecules were expected to be absent.
   a) ATP/GTP
   b) haemin
   c) possibly tRNA, because of the closeness of its MW to the exclusion MW of the gel (∿25000)
   d) unspecified other small MW components.
3. The levels of salt, etc., should approximate those of the column buffer, i.e. K^{+}=120mM, Mg^{++}=5mM, HEPES 20mM.

Compensation for the losses described above were made as follows/

follows:-

a). ATP/GTP. To each 0.8ml aliquot of column eluant was added an ATP/GTP micture sufficient for a final concentration of 100μM ATP, 20μM GTP.

b). Haemin. As in M.D.L. preparations, haemin was added as part of a haemin/Tris/K^+ mixture to a concentration of 25μM.

c). tRNA and other components. These will be discussed where appropriate.

Preparation of cM.D.L. was as described in Methods 5.1.2. for M.D.L. except that no solution A was added. Instead, 50μl of a ATP/GTP solution was added. Nuclease digestion conditions were as described in Methods 5.1.2.

15.4. Effect of G-25 Sephadex gel filtration on reticulocyte lysate.

Two 0.8ml aliquots of G-25 column eluant and two 0.8ml aliquots of reticulocyte lysate were supplemented, as described above and in Methods 5.1.2. respectively. In one of each pair, however, addition of unlabelled amino acids was omitted. Volumes were equalised with deionised water. 50μl samples from each were incubated with 5μCi (^{3}H)-amino acid mixture (15 amino acids) for 40 minutes at 30^{o}C and aliquots taken for estimation of total incorporation of radioactivity.

TABLE 21/

TABLE 21a. Comparison of incorporation of radioactivity by reticulocyte lysate and G-25 column eluant

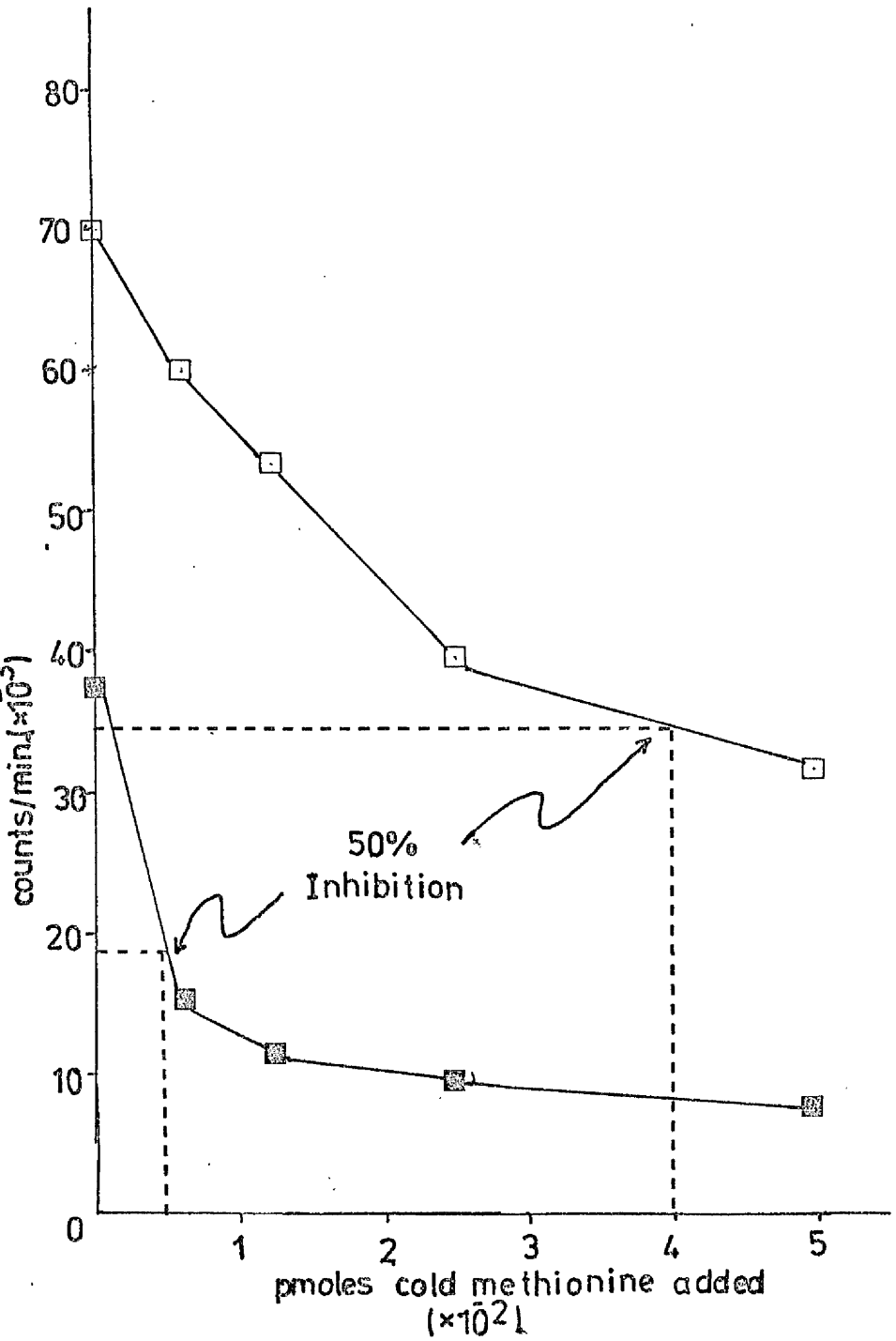
Description	40μM unlabelled amino acids	Total incorporation of radioactivity (cpm/assay)
Reticulocyte lysate	+	157200
"	-	240000
G-25 column eluant	+	49200
" " "	-	918000

As Table 21a above showed, passage through a G-25 Sephadex column resulted in decreased incorporation of radioactivity compared with an equal volume of lysate in the presence of excess added unlabelled amino acids. This was probably due to a decrease in mRNA concentration, caused by the diluting effect of column treatment. In the absence of these unlabelled amino acids, however, the more dilute column eluant gave almost fourfold higher incorporation of radioactivity/unit volume. This suggested that treatment of lysate with G-25 Sephadex removed a substantial proportion of endogenous amino acids. This interpretation was corroborated by comparing the effect of addition of serial dilutions of a stock unlabelled methionine solution to assays of G-25 column eluant and of reticulocyte lysate. These assays contained all other amino acids except methionine in excess and L-(^{35}S) methionine as label, (see Figure 43). This experiment suggests that reticulocyte lysate contained 400 pmoles unlabelled endogenous methionine/50μl assay (compare with Results 4.3.). Column eluant however, contained <55 pmoles unlabelled endogenous methionine/50μl assay/

FIG. 43: Estimation of endogenous methionine pool sizes in normal and gel-filtered reticulocyte lysate.

Reticulocyte lysate and G-25 Sephadex treated reticulocyte lysate were supplemented as described in Methods 7.1.2. and Results 15.3., respectively. Assays of each with L-(^{35}S) methionine as radioactive amino acid were performed in the presence of serial dilutions of unlabelled methionine. Radioactivity incorporated into TCA-precipitable material was measured as described in Methods 8.1.1. No second radioactive amino acid was included to measure overall protein synthesis independently.

KEY: -□- Reticulocyte lysate.
-▩- G-25 Sephadex-treated reticulocyte lysate.


80
70
60
50
40
30
20
10
0
counts/min(×10⁻³)
50%
Inhibition
1
2
3
4
5
pmoles cold methionine added
(×10⁻²)

assay (the measured 55 pmoles included ∿25 pmoles radioactively labelled methionine). Allowing for dilution (1:2) during gel filtration this indicated a removal of >80% of endogenous methionine. That level detected as not removed would include methionine present in association with tRNA. Amino-acid analysis of samples of G-25 eluant also confirmed the removal of almost all free endogenous amino acids (Fig. 14).

15.5. Incorporation of radioactivity in column eluant M.D.L. (cM.D.L.).

The next experiment was devised to investigate whether an M.D.L. made from column eluant (cM.D.L.) would translate exogenous polysomes or poly(A) RNA. Four different polysome fractions, viz. PlI, PlII, 5563I and 5563II, and poly(A) RNA derived from each of these fractions, were added to M.D.L. assays. These were prepared either from reticulocyte lysate (diluted 1:2 with column buffer - dil M.D.L.) or from column eluant (cM.D.L.), and either contained or omitted normal levels of unlabelled amino acids. Translation was measured by the incorporation of a mixture of 15 (^{3}H)-labelled amino acids into TCA precipitable material. Results are shown in Tables 22 and 23. In all cases addition of polysomes to cM.D.L. caused a stimulation of incorporation over background. This stimulation varied with polysome fractions but was between 50X and 70X background incorporation, when no added unlabelled amino acids were present, but was only about 2X to 3X background incorporation in the presence of 40μM unlabelled amino acids. In comparison, assays of diluted M.D.L. showed a stimulation of between 12X and 17X over background in the absence/

TABLE 22: Comparison of incorporation of (^{3}H) amino acids by M.D.L.'s or cM.D.L.'s containing P1.17 polysomes or P1.17 poly(A) RNA.

Legend:

Dilute M.D.L. (dil M.D.L.) and gel filtered M.D.L. (cM.D.L.) were prepared as described in Results 15.3. Assays with P1.17 polysomes or P1.17 poly(A) RNA were performed as described in Methods 7.2. with 5μCi (^{3}H) amino acid mixture as the radioactive label. Radioactivity incorporated into TCA-precipitable material was measured as described in Methods 8.1.1.

Key:

U+ = Normal M.D.L. containing unlabelled amino acid mixture (M.D.L.).

U- = Normal M.D.L. lacking unlabelled amino acid mixture (M.D.L.).

C+ = M.D.L. from G-25 treated lysate containing unlabelled amino acid mixture (cM.D.L.).

C- = M.D.L. from G-25 treated lysate lacking unlabelled amino acid mixture (cM.D.L.).

TABLE 22: Comparison of incorporation of (^{3}H) amino acids by M.D.L.'s or cM.D.L.'s containing P1.17 polysomes or P1.17 poly(A) RNA.

Polysome fraction	Poly (A) RNA fn	Treatment of M.D.L.	Radioactivity incorporated counts/min/assay ($X10^{-3}$)	Stimulation over appropriate:- background assay	non-treated assay	amino acid containing assay
-	-	U+	8.88	-	-	-
-	-	U-	9.85	-	-	-
-	-	C+	12.4	-	-	-
-	-	C-	7.94	-	-	-
PlI	-	U+	23.14	2.6	-	-
"	-	U-	148.8	15.1	-	6.43
"	-	C+	34.2	2.76	1.48	-
"	-	C-	485.64	61.16	3.26	14.2
-	PlI	U+	10.3	1.16	-	-
-	"	U-	12.1	1.23	-	1.17
-	"	C+	11.46	-	1.11	-
-	"	C-	20.62	2.6	1.70	1.8
PlII	-	U+	16.75	1.89	-	-
"	-	U-	133.13	13.52	-	7.95
"	-	C+	26.57	2.14	1.59	-
"	-	C-	380.04	48.0	2.85	14.3
-	PlII	U+	8.78	-	-	-
-	"	U-	8.93	-	-	1.01
-	"	C+	10.33	-	1.17	-
-	"	C-	20.63	2.6	2.31	2.0

TABLE 23: Comparison of incorporation of (^{3}H) amino acids by M.D.L.'s or cM.D.L.'s containing 5563 polysomes or 5563 poly(A) RNA.

Legend:

Dilute M.D.L. (dil M.D.L.) and gel filtered M.D.L. (cM.D.L.) were prepared as described in Results 15.3. Assays with Pl.17 polysomes or Pl.17 poly(A) RNA were performed as described in Methods 7.2. with 5μCi (^{3}H) amino acid mixture as the radioactive label. Radioactivity incorporated into TCA-precipitable material was measured as described in Methods 8.2.1.

Key:

U+ = Normal M.D.L. containing unlabelled amino acid mixture (M.D.L.).

U- = Normal M.D.L. lacking unlabelled amino acid mixture (M.D.L.).

C+ = M.D.L. from G-25 treated lysate containing unlabelled amino acid mixture (cM.D.L.).

C- = M.D.L. from G-25 treated lysate lacking unlabelled amino acid mixture (cM.D.L.).

TABLE 23: Comparison of incorporation of (^{3}H) amino acids by M.D.L.'s or cM.D.L.'s containing 5563 polysomes or 5563 poly(A) RNA.

Polysome fraction	Poly (A) RNA fn	Treatment of M.D.L.	Radioactivity incorporated counts/min/assay ($X10^{-3}$)	Stimulation over appropriate:- background assay	non-treated assay	amino acid containing assay
-	-	U+	8.88	-	-	-
-	-	U-	9.85	-	-	-
-	-	C+	12.4	-	-	-
-	-	C-	7.94	-	-	-
5563I	-	U+	23.7	2.69	-	-
"	-	U-	174.0	17.66	-	7.34
"	-	C+	42.75	3.45	1.80	-
"	-	C-	625.40	78.77	3.59	14.63
-	5563I	U+	10.28	1.15	-	-
-	"	U-	10.24	1.04	-	-
-	"	C+	10.1	-	-	-
-	"	C-	19.0	2.4	1.87	1.87
5563II	-	U+	18.6	2.1	-	-
"	-	U-	127.4	12.93	-	6.85
"	-	C+	32.9	2.65	1.77	-
"	-	C-	452.5	57.0	3.55	13.75
-	5563II	U+	9.57	1.07	-	-
-	"	U-	9.76	-	-	1.02
-	"	C+	11.13	-	1.16	-
-	"	C-	19.14	2.41	1.96	1.72

absence of added unlabelled amino acids and between 1.9X and 2.6X over background in the presence of unlabelled amino acids. Hence, column treatment of lysate had improved incorporation in cM.D.L. about 3 fold over an M.D.L. prepared from diluted reticulocyte lysate.

Incorporation with poly(A) RNA was generally much lower. Only cM.D.L. with no added unlabelled amino acids gave incorporation of radioactivity at much above the background levels. This was not due to degradation of the poly(A) RNA. In subsequent experiments with normal M.D.L., using L-(^{35}S) methionine as label, samples of the same poly(A) RNA gave stimulations of incorporation of between 4X and 9X background (data not shown). Lack of stimulation of incorporation by poly(A) RNA suggested that perhaps initiation of translation was unable to take place in the cM.D.L. It also suggested that incorporation observed with polysomes was due merely to "run-off" of existing ribosomes from mRNA, resulting in completion of existing polypeptides but with no reinitiation of translation. Other explanations for this lack of incorporation were as follows:-

1. A limiting amino acid. This seemed unlikely in view of the incorporation observed with polysomes. Nevertheless in the cM.D.L. without added unlabelled amino acids (C-assays), 5 of the twenty amino acids, viz., those not present in the (^{3}H) amino acid mixture - methionine, cysteine, glutamine, asparagine and tryptophan, were present in very low amounts (in the case of methionine < 55pmoles). Consequently, subsequent assays were supplemented with unlabelled amino acids to contain at least 100 pmoles of each added amino acid (including the contribution of the radioactively labelled amino acids) per 50µl assay. This decreased the possible effect of limitation (see/

(see Table 24).

2. Limiting tRNA. With 25,000 MW as the exclusion limit of the gel beads, tRNA species might be retarded by the gel to produce a lack (either general or specific) of tRNA in the column eluant. This possibility was investigated.

3. Limitation of some other low MW species. Possible candidates were:-

a) Low MW initiation factors,

b) haemin loss during the column separation,

c) Unidentified low MW cofactors.

4. Presence of some species which prevented initiation. Possible candidates were excess reducing power (6mM mercaptoethanol) or excess divalent cations (Mg^{++} = 5mM).

5. Effect of column treatment. The Sephadex beads might have released an inhibitory substance which blocked initiation but not elongation reactions. Alternatively, the time required to run sample through the column might have allowed the inactivation of some labile component.

6. Effect of dilution. Dilution of the lysate might have lowered the concentration of some substrate below a crucial level for protein synthesis. The low incorporation with poly(A) RNA observed in an M.D.L. derived from diluted lysate supported this view.

7. Some combination of the above.

These possibilities were systematically investigated.

15.6. Effect of an inhibitor of initiation on radioactive incorporation/

TABLE 24: Amino acid supplement for cM.D.L.

Amino Acid	pmoles/5µl(^{3}H) amino acid mixture	pmoles amino acid added to give 100pmoles/50µl assay
Ala	12	88
Arg	21	79
Asp	2416	-+
Asn	-*	100
Cys	-*	100
Gln	-*	100
Glu	221	-+
Gly	71	29
His	5	95
Ile	15	85
Leu	10	90
Lys	9	91
Met	-*	100
Phe	28	72
Pro	7	93
Ser	14	86
Thr	853	-+
Trp	-*	100
Tyr	5	95
Val	9	91

* Not present in (^{3}H) amino acid mixture

+ >100 pmoles (^{3}H) amino acid/50µl assay

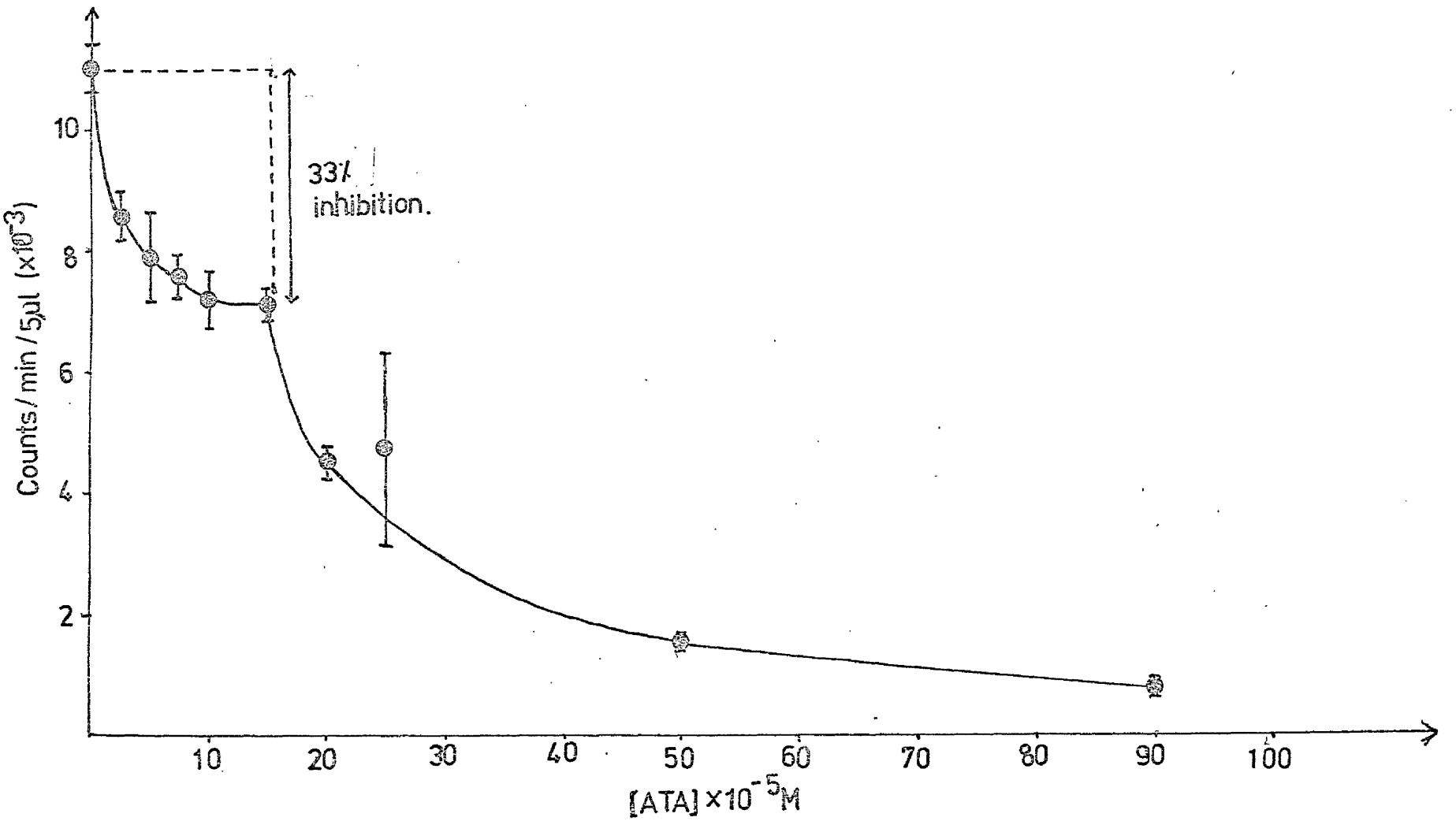
15.6. Effect of an inhibitor of initiation on radioactive incorporation by cM.D.L. with exogenous polysomes.

Aurintricarboxylic acid (ATA) is an inhibitor of initiation of protein synthesis at low concentrations, and an inhibitor also of elongation reactions at higher concentrations. Consequently, if re-initiation occurs in the cM.D.L. with added polysomes, a biphasic inhibition curve of incorporation of radioactivity should be observed over a range of ATA concentrations. Serial dilutions of a stock ATA solution were made and each added to duplicate 60µl aliquots of cM.D.L. containing Pl.17 polysomes and (^{3}H) amino acid mixture. After incubation, total TCA-precipitable incorporation was measured for each assay. As Fig. 44 demonstrates, a biphasic inhibition curve was observed in the range 0 - 1mM ATA. In the concentration range 0 - 150µM, incorporation fell off sharply then levelled off at approximately 66% of the original level. In the concentration range 150µM - 1mM, incorporation again fell off sharply before beginning to level off. At 1mM ATA, incorporation was 10% of that observed with no ATA present. This suggested the operation of two inhibitable processes, one being labile to low ATA concentrations and the other to higher ATA concentrations. If these two processes are initiation and elongation, initiation of protein synthesis in the cM.D.L. therefore accounted for approximately 33% of the total incorporation.

By comparison, studies on the effect of ATA on initiation in the M.D.L. with poly(A) RNA (Results 9.1.) showed that in the M.D.L. incorporation of radioactivity was much more sensitive to inhibition (all incorporation of radioactivity was effectively halted by 80µM concentrations of ATA). This reflected the much more prominent role of/

FIG. 44: Dose response of incorporation of (^{3}H) amino acids by cM.D.L. in the presence of ATA.

1 ml cM.D.L. was prepared and 100µl P1.17 polysomes and 100µCi (^{3}H) amino acids were added. 50µl aliquots of this solution were added in duplicate to assay tubes containing serial dilutions of ATA in 5µl volume (0-100 x 10^{-5}M). Assays were incubated at 30°C for 120 min. and radioactive incorporation into TCA-precipitable material was measured (Methods 8.1.1.), using duplicate 5µl aliquots from each duplicate assay.


Counts / min / 5µl (x10⁻³)
10
8
6
4
2
33%
inhibition.
10
20
30
40
50
60
70
80
90
100
[ATA] x10⁻⁵M

of initiation processes in assays containing poly(A) RNA.

As a result of this finding, subsequent experiments were carried out to attempt to identify the reasons for lack of initiation in the cM.D.L. with poly(A) RNA and to optimise translation of polysomes.

15.7. Effect of tRNA on incorporation.

Addition of calf liver tRNA (15µg/50µl assay) stimulated incorporation of radioactivity by the cM.D.L. containing either PlI polysomes or 5563 polysomes (see Table 25). The effect was more pronounced with PlI polysomes. In view of these results, calf liver tRNA (15µg/50µl) was added to subsequent assays. The effect of the calf liver tRNA may be due to substitution for tRNA species removed by gel filtration treatment. Alternatively, the stimulation of protein synthesis may be due to addition to the endogenous tRNA pool of tRNA species which supplement those present in low amounts. Addition of tRNA produced no noticeable stimulation of incorporation with poly(A) RNA suggesting that tRNA limitation was not responsible for the lack of response to poly(A) RNA by cM.D.L.

15.8. Experiments with G-10 cM.D.L.

Among the possible reasons for the lack of stimulation of cM.D.L. by poly(A) RNA was the absence of some low MW species. Removal of such species would occur during the gel filtration step, along with amino acids, ATP and GTP. Another possible cause of poor translation of poly(A) RNA was the presence of high levels of reducing/

TABLE 25: Effect of tRNA on incorporation of L(^{35}S) methionine by the cM.D.L. containing various myeloma polysome or poly(A) RNA fractions.

Legend:

G-25 cM.D.L. assays, containing various myeloma polysome or poly(A) RNA fractions were incubated with L(^{35}S) methionine in the presence or absence of 3µg calf liver tRNA under standard conditions (Methods 7.2.). Radioactivity incorporated into TCA-precipitable material was measured as described in Methods 8.2.1.

TABLE 25: Effect of tRNA on incorporation of L(^{35}S) methionine by the cM.D.L. containing various myeloma polysome or poly(A) RNA fractions.

Polysome fraction		Calf liver tRNA	Radioactivity incorporated cpm/assay	Fold stimulation
Pl.17I	Expt. 1	-	100000	-
		+	555000	5.54
	Expt. 2	-	91400	-
		+	430980	4.7
	Expt. 3	-	498700	-
		+	620950	1.25
5563 II	Expt. 2	-	394900	-
		+	451500	1.14
	Expt. 3	-	533130	-
		+	688550	1.3
0	Expt. 1	-	17120	-
		+	21270	1.24
	Expt. 2	-	8580	-
		+	11170	1.302
	Expt. 3	-	10855	-
		+	12350	1.13
Pl.17I poly A	Expt. 1	-	20244	-
		+	21270	-
	Expt. 2	-	21084	
5563 poly A	Expt. 2	-	17940	

reducing compounds (6mM 2-mercaptoethanol) as a result of the gel filtration step. Reticulocyte lysate and M.D.L. assays normally contain only endogenous levels of reducing compounds. For these reasons a smaller pore-size for the gel (G-10 Sephadex) was tested and variations in buffer composition investigated. With a lower MW exclusion limit, G-10 Sephadex might not retard small factors concerned with translation of poly(A) RNA, but would still remove amino acids. Samples of reticulocyte lysate were run on G-10 Sephadex columns, as described for G-25 Sephadex columns. In one column, 6mM 2-mercaptoethanol was omitted from the buffer. In this column, after collection of the coloured fraction of the eluant, several ml of the subsequent eluant was collected and pooled. This fraction was collected to look for factors slightly retarded by the column. The pooled fractions (∿5ml) were lyophilised and resuspended in 100µl of deionised H_2O.

M.D.L. prepared from G-10 Sephadex eluant was assayed with Pl.17 polysomes or Pl.17 poly(A) RNA. These RNA fractions were shown to give stimulations of 4 fold and 3 fold respectively over background incorporation in M.D.L. assays. The results of these assays are shown on Table 26.

Several points can be made about the results:-

1. Removal of 2-mercaptoethanol stimulates incorporation of radioactivity in assays containing polysomes, but did not significantly stimulate incorporation with poly(A) RNA.

2. Use of a smaller pore size gel did not improve the stimulation of the system by poly(A) RNA. This suggested that any retarded "factor" had a MW < 10,000.

3. Addition of a concentrate of the eluant fractions recovered from the column after the coloured fraction did not stimulate incorporation/

TABLE 26: Effect of 6mM 2-mercaptoethanol and/or 0.5M KCl wash factors on incorporation of radioactivity by cM.D.L. containing Pl.17 polysomes or poly(A) RNA.

Legend:

cM.D.L., prepared from G-25-treated reticulocyte lysate, was incubated with Pl.17 polysomes or poly(A) RNA, using L(^{35}S) methionine as radioactive label. Assays were performed in the presence and absence of 6mM 2-mercaptoethanol and/or 0.5M KCl wash factors from reticulocyte polysomes (prepared as described in Methods 6.1.5.). Assays were incubated at 30^{o}C for 120 min. Radioactive incorporation into TCA-precipitable material was measured as described in Methods 8.2.1. Control assays, using diluted reticulocyte lysate, were performed for comparison of incorporation.

TABLE 26: Effect of 6mM 2-mercaptoethanol and/or 0.5M KCl wash factors on incorporation of radioactivity by cM.D.L. containing Pl.17 polysomes or poly (A) RNA.

RNA	6mM 2-mercaptoethanol	(µl) Added "factors"	Radioactivity incorporated cpm/assay	Fold stimulation over background
0 Expt. 1	+	-	9400	-
Expt. 2	+	-	21576	-
Expt. 1	-	-	9900	-
Expt. 2	-	-	16980	-
Pl.17 polysomes	+	-	105900	11.27
	-	-	178000	18.0
Pl.17 poly(A)				
Expt. 1	+	-	10560	1.12
Expt. 2	+	-	21606	1.0
Expt. 2	+	+	23338	1.08
Expt. 1	-	-	12700	1.28
Expt. 2	-	-	20176	1.19
Expt. 2	-	+	20398	1.20

Controls

1:2 diln of retic lysate with column buffer (including 6mM 2-mercapto-ethanol).

0	+	-	6000	-
Pl.17 polysomes	+	-	27400	4.6
Pl.17 poly(A)	+	-	8300	1.38

incorporation with poly(A) RNA significantly. This suggested that any retarded "factor" had low mobility through the column.

As a result of this experiment, 6mM 2-mercaptoethanol was omitted from further gel filtration buffers. Since G-10 Sephadex appeared to yield no improvement in the cM.D.L.'s ability to translate poly(A) RNA, further studies were carried out using G-25 Sephadex.

15.9. Experiments with G-25 coarse cM.D.L.

Previous experiments using G-25 Sephadex for gel filtration had used G-25-FINE Sephadex. Roberts and Paterson, 1971 used COARSE grade G-25 Sephadex for their preparation of the wheat germ cell-free system. G-25 COARSE Sephadex was used for preparation of gel-filtered reticulocyte lysate and was found to have better flow properties and fewer 'fines' than G-25 FINE Sephadex. Thus, the time taken for the reticulocyte lysate to pass through the column was reduced by two thirds. This reduced the time available for inactivation of labile factors in the reticulocyte lysate. Several batches of gel filtered reticulocyte lysate were made, using various column buffer compositions. This investigated whether the column buffer system was optimal for producing cM.D.L. capable of translation of poly(A) RNA. From each of these reticulocyte lysate eluants was prepared cM.D.L. Conditions for preparation of cM.D.L. were as before, except that since L-(^{35}S) methionine was used as radioactive amino acid, a stock solution of 19 amino acids minus methionine was added, as for the M.D.L. system. L-(^{35}S) methionine was/

was used to allow improved fluorography of cM.D.L. samples separated by PAGE.

The following buffer systems were used:-

a) 20mM HEPES pH 7.6, 100mM KCl, 5mM $MgCl_2$

b) 20mM HEPES pH 7.6, 100mM KCl, 5mM $MgCl_2$, 25μM haemin

c) 20mM HEPES pH 7.6, 100mM KCl, 0.5mM $MgCl_2$

d) 20mM HEPES pH 7.6, 10mM KCl, 100mM KCl

e) 20mM HEPES pH 7.6

In addition, a 0.5M KCl wash of reticulocyte ribosomes was prepared as described in Methods 6.1.5 by the method of Gross, 1977. The high salt wash factors thus isolated were intended to replace protein factors essential for initiation of protein synthesis which might have been removed from reticulocyte lysate by the gel filtration step. The results of assays using the variously prepared cM.D.L.'s and the effect of added factors are shown in Table 27.

The following points can be made about the results shown in Table 27.

1. Poly(A) RNA translation in cM.D.L. was not stimulated by the presence of haemin in the column buffer during gel filtration of the reticulocyte lysate. This suggested that the formation of translational inhibitor during column treatment was not the major cause of the lack of response to poly(A) RNA.

2. Variation of K^+ and Mg^{++} concentrations in the column buffer (and hence in the cM.D.L. itself) did not significantly enhance translation of poly(A) RNA. In fact, the various buffer conditions tested prevented any incorporation above background levels. The original buffer system allowed some incorporation over background levels with poly(A) RNA.

3./

TABLE 27: Comparison of incorporation of L(^{35}S methionine by cM.D.L. prepared using several different buffer systems.

Legend:

Reticulocyte lysate was passed over G-25 Sephadex in a number of buffer systems described in Results 15.9.

Incorporation of radioactivity by M.D.L.'s prepared from the G-25 treated lysates in response to the addition of 5563 polysomes or poly(A) RNA was measured as described in Methods 8.2.1.

TABLE 27: Comparison of incorporation of L(^{35}S) methionine by cM.D.L. prepared using several different buffer systems.

Cell-free system	Buffer system	RNA	High salt (5µl) wash factors	Radioactivity incorporated/assay cpm/assay in Expt. ($\times 10^{-3}$) 1	2	3	4
M.D.L.	-	0	-	35	201	-	61
	-	5563 polysomes	-	482	708	-	-
	-	5563 poly(A) RNA	-	158	369	-	168
cM.D.L.	a	0	-	28	171	105	47
	a	5563 polysomes	-	3534	3945	-	-
	a	5563 poly (A) RNA	-	178	323	505	145
	a	0	+	-	-	140	45
	a	5563 poly (A) RNA	+	-	-	410	119
	b	0	-	-	197	-	-
	b	5563 polysomes	-	-	5041	-	-
	b	5563 poly (A) RNA	-	-	321	-	-
	c	0	-	-	-	-	68
	c	5563 poly (A) RNA	-	-	-	-	41
	c	0	+	-	-	-	63
	c	5563 poly (A) RNA	+	-	-	-	58
	d	0	-	-	-	-	57
	d	5563 poly (A) RNA	-	-	-	-	72
	d	0	+	-	-	-	72
	d	5563 poly (A) RNA	+	-	-	-	60
	e	0	-	-	-	-	64
	e	5563 poly (A) RNA	-	-	-	-	47
	e	0	+	-	-	-	54
	e	5563 Poly (A) RNA	+	-	-	-	57

3. Additions of high salt wash factors did not cause a stimulation of incorporation in response to poly(A) RNA by any cM.D.L. tested. Those cM.D.L.'s which already gave stimulation over background in response to poly(A) RNA gave less stimulation in the presence of high salt wash factors.

As a result of the experiments described above using either G-10 or G-25 Sephadex, the following conclusions were reached about the lack of stimulation of cM.D.L. by added poly(A) RNA.

a. Salt concentration did not appear to be a cause of the lack of stimulation. It could be argued that the results supporting this conclusion could be interpreted as being due to degradation in the poly(A) RNA fraction used. This seemed unlikely since this fraction gave incorporation of radioactivity over background levels in the same experiment with G-25 Sephadex cM.D.L. prepared previously. Alternatively, RNase or some other degradative enzyme may have been present in the solutions used to prepare the cM.D.L.'s in question. The same stringent nuclease precautions were used, however, as had been successfully employed at other times (Methods 10.1).

b. Lack of high salt wash factors was not responsible for the lack of stimulation. Such a cause had already seemed unlikely in view of the probable size of any missing factors (<10,000MW) as deduced from previous experiments.

c. Absence of haemin during the column separation was not responsible for the lack of stimulation.

These results suggested that some small MW factor was involved which was either:-

1. Reduced to a limiting concentration by Sephadex/

Sephadex filtration either by partial removal, or by dilution.

2. Removed completely by Sephadex filtration.

3. Inactivated during the time involved in gel filtration.

15.10. Incorporation of radioactivity by M.D.L.:cM.D.L. mixtures.

The effect of mixing M.D.L. and cM.D.L. in various proportions was investigated. Reticulocyte poly(A) RNA was used as the added mRNA. M.D.L. and cM.D.L. with exogenous poly(A) RNA were used to ensure that initiation of translation was studied. Varying proportions of M.D.L. and G-25 coarse cM.D.L. were mixed and incubated with reticulocyte poly(A) RNA. Protein synthesis was detected by incorporation of L-(^{35}S) methionine into TCA-precipitable material. The data shown in Fig. 45 suggested that with less than 40% (v/v) (∿60% w/w) M.D.L. present no significant protein synthesis occurred. Above this level, TCA-precipitable radioactivity increased rapidly to over 13 times the incorporation at lower levels. Incorporation fell slightly again as the proportion of M.D.L. in the assay reached 100%. This result suggested that the factor responsible for initiation was active only above a critical concentration. Moreover, the increase in incorporation observed when 80% (v/v) rather than 100% (v/v) M.D.L. was used was 16%. This was roughly the increase to be expected due to removal of ∿20% of the endogenous methionine. Below 80% (v/v) decreased incorporation suggested a progressive decrease in incorporation due to the "low MW" factor becoming limiting.

15.11/

Fig. 45

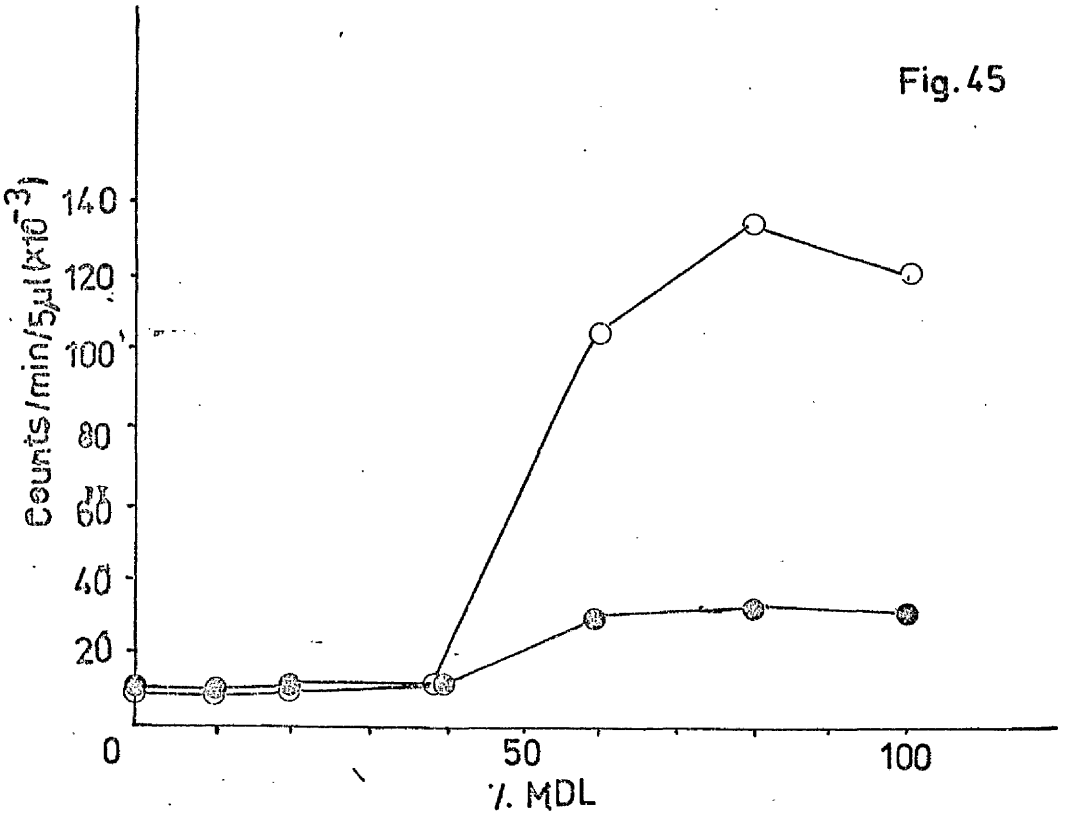

Counts/min/5µl (x10⁻³)
140
120
100
80
60
40
20
0
50
100
% MDL

Fig. 46

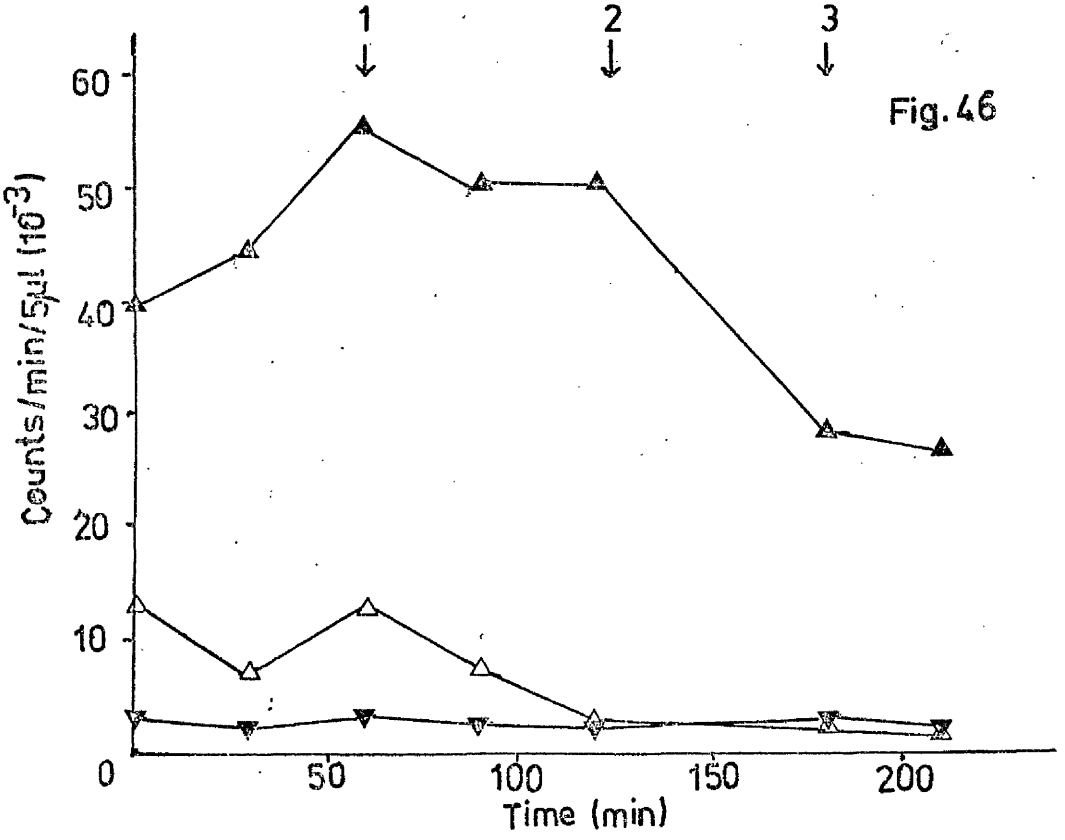

1
2
3
Counts/min/5µl (10⁻³)
60
50
40
30
20
10
0
50
100
150
200
Time (min)

FIG. 45: Incorporation of L-(^{35}S) methionine by various M.D.L.:cM.D.L. mixtures.

M.D.L. and cM.D.L. were mixed in various proportions and incubated with L-(^{35}S) methionine and 1µg reticulocyte poly(A) RNA/assay under standard conditions. 5µl aliquots were removed in duplicate and radioactivity incorporated into TCA-precipitable material was measured as described in Methods 8.2.1.

KEY: -o- Incorporation with poly(A) RNA.
-●- Incorporation with no additions.

FIG. 46: The effect of dialysis on incorporation of L-(^{35}S) methionine directed by 5563 polysomes and poly(A) RNA in the M.D.L.

5 ml of reticulocyte lysate (25µM haemin added), was dialysed against 3 changes of 1 litre of 100mM KCl, 0.5mM $MgCl_2$ solution. The top of the dialysis bag could be opened to allow removal of 500µl aliquots of lysate at various time intervals. Dialysis medium was changed hourly. Aliquots removed were treated to produce M.D.L. The incorporation of these M.D.L.'s (dM.D.L.'s) in response to the addition of 5563 polysomes or poly(A) RNA was measured in 50µl standard assays, using L-(^{35}S) methionine as label. Radioactive incorporation into TCA-precipitable material was measured as described in Methods 8.1.1.

KEY: -●- Incorporation with polysomes.
-▲- Incorporation with poly(A) RNA.
-●- Incorporation with no additions.

15.11. Dialysis of M.D.L.

Dialysis was explored as a means of investigating the progressive changes occurring in incorporation in the cM.D.L. due to removal of small MW factors. A small volume of reticulocyte lysate (+ haemin) was dialysed against 3X 1 litre of column buffer (no 2-mercaptoethanol). Aliquots of lysate were removed at intervals and dialysed M.D.L. (dM.D.L.) prepared from these as for cM.D.L. Incorporation of radioactivity was measured in these dM.D.L.'s in the presence of 5563 myeloma polysomes and 5563 poly(A) RNA. The results obtained are shown in Fig. 46.

As can be seen, incorporation of radioactivity by dM.D.L.'s prepared from samples removed after short periods of dialysis (0 - 60 min) showed an increase using polysomes and remained fairly constant using poly(A) RNA, relative to undialysed M.D.L. After the first buffer change, however, incorporation with both polysomes and poly(A) RNA declined consistently in dM.D.L. samples. After 180 minutes of dialysis, incorporation with polysomes was lower than incorporation measured with M.D.L. prepared from undialysed reticulocyte lysate. Incorporation with poly(A) RNA had fallen to background levels by 120 min.

This fall-off in incorporation both with polysomes and poly(A) RNA may be time-dependent due to inactivation of labile components. If it is due to removal of a factor concerned with initiation, it suggests that incorporation with polysomes is affected by its removal but only when it reaches much lower levels than for poly(A) RNA.

15.12/

15.12. Gel analysis of cM.D.L. products.

Products of cM.D.L. assays with myeloma polysomes and poly(A) RNA were analysed by PAGE and fluorography, sometimes followed by scanning densitometry. As shown in Fig. 47, a comparison of banding patterns produced by 5563 polysome-directed cell-free products in M.D.L. and cM.D.L. shows many similarities. This was more evident by comparison of fluorographs visually than by densitometry, where a high background tended to obscure detail. This high background may be due to premature termination in the cM.D.L. resulting in many heterogeneous peptide sizes forming a uniform background to those polypeptides synthesized as normal. The banding pattern obtained with incorporated radioactivity in response to 5563 poly(A) RNA was much simpler. Above an even background of radioactivity, only one major band, corresponding to L chain precursor, was visible. This appeared to be the only message translated in sufficient quantities to yield a recognisable band. It has been shown that cM.D.L. lacks some factor required for normal levels of initiation. Consequently, it is suggested by this result that only those mRNA's present in large numbers are translated. Alternatively, only those mRNA's with the most favourable initiation constants are translated. The second hypothesis would support the findings of Nuss and Koch (1976) and of Sonenshein and Brawerman (1976a,b) that L chain mRNA is less affected by factors tending to block initiation of protein synthesis. They do not, however, agree with results obtained in this project with Pl.17 poly(A) RNA (Results 9) which suggested that under conditions of inhibition of initiation, H chain mRNA was a better initiating message. Admittedly, however/

FIG. 47: PAGE analysis of cell-free products directed by 5563 polysomes or poly(A) RNA in cM.D.L.

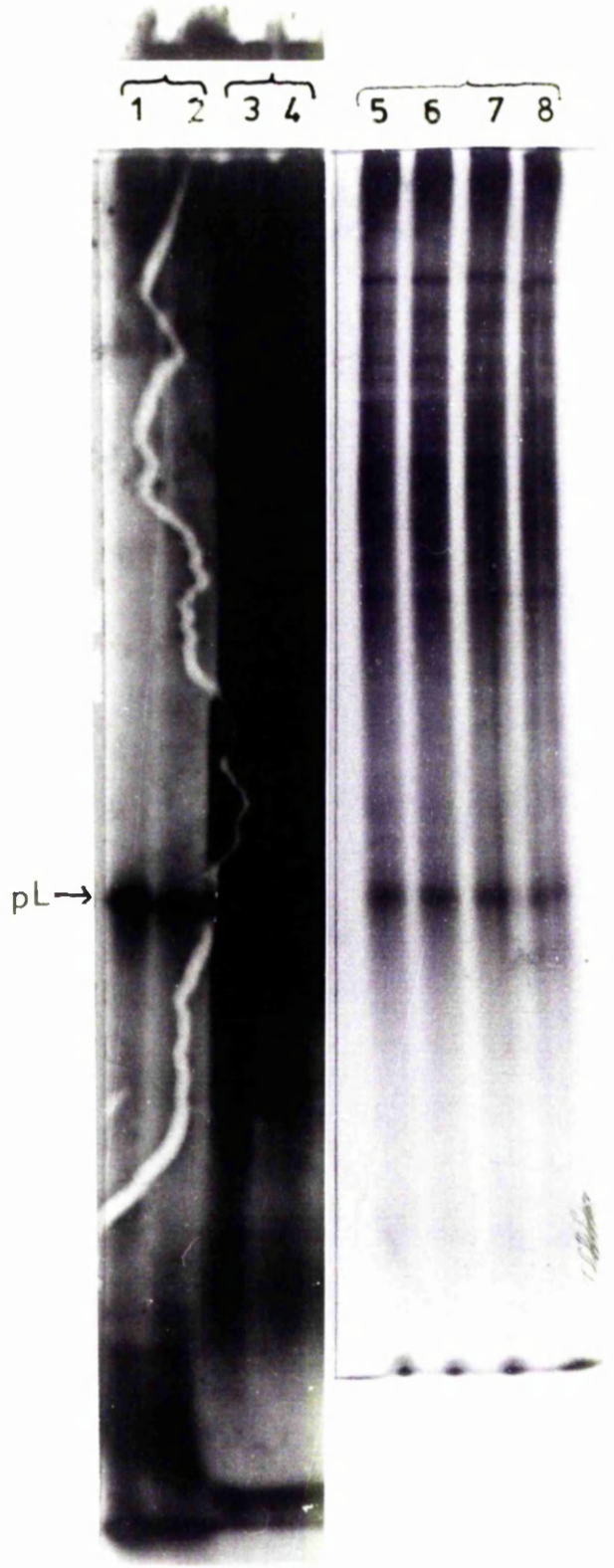
5563 polysomes or poly(A) RNA were assayed in G-25 Sephadex cM.D.L. under standard conditions (Methods 7.2.) and analysed by PAGE and fluorography (Methods 9.2.6. and 8.3.1.).

Well

1)) 2) Cell-free products of 5563 poly(A) RNA in cM.D.L. (12,000 counts/min/well).

3)) 4) Cell-free products of 5563 polysomes in cM.D.L. (120,000 counts/min/well).

5-8 Cell-free products of 5563 polysomes in cM.D.L. (second gel; lower exposure).


1 2 3 4
5 6 7 8
pL

however, the two results were obtained with mRNA from two different cell lines.

15.13. Conclusion.

The aim of the attempts described above to remove endogenous amino acids from reticulocyte lysate was to produce a cell-free system which would incorporate high levels of radioactive amino acids. Such a system could be used to label polypeptides to an equal specific activity in many amino acids, hence allowing amino acid sequencing to be readily carried out. While the system developed so far has little capacity for translation of poly(A) RNA, a measure of success was achieved in labelling cell-free products directed by polysomes to high level. Several improvements to the system to allow translation of poly(A) RNA are discussed later (see Discussion).

DISCUSSION

1. The existence of precursor sequences on secreted proteins.

Part of the intention of this project was to investigate the primary translation products of mRNA from several myeloma cell lines, using a heterologous cell-free protein synthesizing system. In particular, evidence was sought for the synthesis *in vitro* of higher MW precursors to Ig H and L chains.

1.1. Ig L chain precursors and their nature.

As was reviewed in the Introduction (5.4.2.) the evidence for the synthesis of a precursor to Ig L chains in cell-free systems is strong. Precursors to L chains (pre L chains) have been demonstrated to be synthesized in a variety of cell-free systems, using mRNA isolated from a wide range of murine myelomas (see Table 2). Their identity as precursors to mature L chain has been established by a number of techniques:-

a) Most importantly, amino acid sequence analysis has revealed, either in part or in whole, the amino acid sequence at the N-terminus of a number of cell-free synthesized Ig L chains (see Fig. 1). These studies, mainly by Schechter and his coworkers, have demonstrated the existence, at the N-terminus of cell-free synthesized L chain, of ∿20 amino acid residues not found in the corresponding mature L chain.

b) Tryptic mapping of cell-free synthesized L chains from several myeloma cell-lines (Stavnezer and Huang, 1971; Milstein *et al.*, 1972; Schechter, 1973) indicated the existence of a peptide in/

in the cell-free product with altered properties in electrophoresis and chromatography. It suggested that this peptide was located at the N-terminus.

c) Immunoprecipitation of cell-free synthesized pre L chain with antisera specific for the cellular L chain established that the cellular and cell-free products were closely related antigenically.

d) Mobility studies indicated that the L chain produced in cell-free systems was of a higher apparent MW than the mature cellular L chain. This was the first but least satisfactory piece of evidence for the synthesis of precursors to secreted proteins in cell-free systems.

Evidence that these cell-free products may be synthesized as a normal part of the in vivo process comes from a number of sources.

1. The determination of the N-terminal amino acid sequence of several cell-free precursors to L chain has indicated that the cell-free product is not synthesized following an incorrect initiation of translation; the precursors contain methionine as their N-terminus, suggesting initiation occurs at an AUG codon. The L chain amino acid sequence following the precursor sequence is identical with the sequence of the mature L chain, suggesting that translation in the cell-free system is in the correct reading frame. It could be argued that there perhaps exists a secondary initiation site on the mRNA's which is only exposed or favoured for initiation as a result either of the mRNA isolation procedure or due to the conditions prevailing in the cell-free systems. The amino acid sequence data rule out this possibility. In the precursor, the only methionine is found at the N-terminus, and the precursor and L chain sequences

are/

are consecutive. In mature L chain no methionine is found at the N-terminus. Since, in general, eukaryotic mRNA's initiate protein synthesis at methionine,the pre L chain must be the precursor to the mature L chain. Models in which mature L chain is formed by removal only of an N-terminal methionine (as occurs in the synthesis of globin) are ruled out.

2. It has been shown that membrane fractions have the ability to cause the cell-free products of L chain mRNA translation to be synthesized as apparently mature size L chain (Introduction 5.4.1.). In most cell-free systems, an absence of membrane material exists. Consequently, these systems are unlike the normal environment of the L chain mRNA. The presence of membranes, either by virtue of the cell-free systems used (Swan, Aviv and Leder, 1972) or by deliberate addition (Dobberstein and Blobel, 1977) mimics to some extent the physiological conditions. It is difficult, however, to demonstrate a precursor-product relationship between pre L chain and mature L chain material in this system. Consequently, it could be argued that the formation of an apparently mature L chain was merely the result of non-specific proteolytic cleavage of a particularly susceptible peptide bond in the cell-free product. Until amino acid sequence analyses have been performed of L chain material processed in cell-free systems by membranes, it cannot be stated that the processing events observed are physiologically significant.

3. Schmeckpeper et al., (1975) have provided some evidence, based on mobility data and serological evidence, that in a myeloma cell line incubated in the presence of a protease inhibitor, the existence of a precursor to L chain can be demonstrated. While this/

this is indicative of an *in vivo* occurrence of pre L chains, several ambiguities remain. Firstly, only one out of five cell lines examined (MOPC 41A) showed the appearance of a precursor under conditions of protease inhibition. Secondly, only one protease inhibitor was effective in demonstrating this precursor. Thirdly, only mobility data were used to compare the cell-free pre L chain and the cellular species. Until these points have been cleared up it is too early to claim positive proof of an occurrence of pre L chain as an *in vivo* precursor to L chain.

Admittedly, the demonstration of a precursor-product relationship will be difficult if synthesis and processing are closely linked events. A demonstration of this relationship might be made by showing, for example, that the membrane-mediated cleavage of pre L chain to L chain was an event dependent on the time of addition of membranes (see Rothman and Lodish, 1977).

In this study, both mRNA and polysomes from three cell lines have been studied, viz. Pl.17 (including clones Pl.17.1 and Pl.17.4), 5563 and SAMM 368. Both mRNA and polysomes have been shown to direct the synthesis of cell-free products, which are thought to be precursors to mature L chain by several criteria.

1. Their quantitative occurrence in cell-free products is similar to their quantitative occurrence intracellularly (Results 7.3.).
2. Their mobilities are lower than those of cellular L chains, in agreement with the findings of other workers (Results 7.3.).
3. They are serologically related to cellular L chain, being specifically precipitated (both directly and indirectly) by antisera directed against the appropriate mature L chain (Results 8.2.1. and 8.2.2.)/

8.2.2.).

4. When 5563 pre L chain is digested with trypsin, radioactively labelled peptides show similar electrophoretic and chromatographic properties to peptides obtained by digestion of cellular L chain (Results 14.3.).

The tryptic digestion/peptide mapping result requires comment. Although the mobilities of the spots found by fluorography were not identical for the cell-free and cellular L chain material, the pattern of spots was the same. It is possible that the varying mobilities were due to differences in silica gel plates used. This could be tested in future by separating mixtures of cell-free and cellular peptides on one plate in addition to separating the cell-free or cellular material individually. Alternatively the differing treatments for isolation of the mature L chain (Methods 10.3.) and of the cell-free pre L chain (Methods 9.2.7.) could have altered the propoerties of the peptides subsequently generated. For example, the retention of SDS on the cell-free peptides might alter their mobility both in electrophoresis and chromatography. Extra radioactive spots in the cellular L chain peptide map were thought to be contaminating H chain spots. This was because of their general similarity in distribution to the peptide map of cellular H chain and because of the known difficulties in resolving H and L chains completely on G-100 Sephadex.

Further data on the nature of 5563 pre L chain was obtained by dansylation studies of radiolabelled 5563 pre L chain (Results 13.6.). These suggested that the N-terminal amino acid of the pre-L chain isolated from M.D.L. was not methionine. This does not necessarily imply that the initially translated product did not contain an N-terminal/

N-terminal methionine. As Schechter et al.found (see Fig. 1.) using the Krebs ascites cell-free system, some myeloma pre L chains contain labile N-terminal methionines. Moreover, the reticulocyte is known to contain enzymes which rapidly remove N-terminal methionine from newly synthesized globin chains (Jackson and Hunter, 1970). Hence the absence of an N-terminal methionine may reflect post-translational processing. That the absence of N-terminal methionine was characteristic of a majority of 5563 pre L chain was suggested by the non-appearance of an additional radioactively labelled spot in tryptic mapping studies (Results 14.3).

Consequently, it was concluded in this project that myeloma L chain mRNA from all cell lines studied is translated in the M.D.L. as a higher MW precursor to cellular L chain. Moreover, in at least one case (5563), it appears to undergo removal of its N-terminal methionine, possibly as a result of processing by the cell-free system.

1.2. Ig H chain cell-free products and their nature.

The evidence for the existence of an additional precursor sequence at the N-termini of cell-free synthesized Ig H chains is at present debatable (Introduction 5.4.3.). In this respect, the effect of carbohydrate residues on cellularly synthesized or secreted H chain has sometimes been ignored (e.g. Bedard and Huang, 1977) or not considered properly (Schmeckpeper et al., 1977). Alternatively, the cell-free systems used for translation studies have been unsuitable for the drawing of rigorous conclusions (Cowan and Milstein, 1973).

In/

In this project, an attempt was made to measure the relative effect of those mobility differences between cellular and cell-free H chain caused by lack of carbohydrate and those caused by the presence of a precursor sequence. The results obtained for each cell line used will be discussed separately.

In the M.D.L. cell-free system, 5563 polysomes or poly(A) RNA consistently synthesized a polypeptide migrating on PAG's in the region in which cellular H chain migrated (e.g. Results 7. & 8.2.1.). Although precipitation was not quantitative, both direct immunoprecipitation (Results 8.2.1.) and indirect immunoprecipitation (Results 8.2.2) indicated that this polypeptide corresponded to the cell-free synthesized H chain. Measurement of mobilities suggested that it had a slightly lower apparent MW than cellular or secreted H chain. This mobility difference could be further investigated, using lower percentage PAG's to improve the relative separation involved. Alternatively, cellular and cell-free products could be mixed and co-electrophoresed to attempt to demonstrate this difference. The large number of polypeptides migrating with approximately the same mobility might, however, make interpretation difficult. The possibility that the mobility change was caused by lack of carbohydrate addition, was investigated by the use of 2-deoxyglucose to block carbohydrate addition in vivo. Again, PAGE analysis showed a small increase in the mobility of H chains synthesized by cells radioactively labelled in the presence of 2-deoxyglucose (Results 11). Such H chains, however, appeared not to be well resolved by PAGE. The possibility exists that these H chains are more susceptible to proteolysis, due to lack of carbohydrate. A direct comparison between cell-free synthesized 5563 H chain and 5563 H chain from

2/

2-deoxyglucose treated cells will be required before the question of the existence of precursors to 5563 H chain can be settled.

Peptide analysis of tryptic digests of 2-deoxyglucose-treated cellular H chain and cell-free H chain did not yield unambiguous results as to whether extra peptides (or peptides with altered mobility) occurred in cell-free H chain. This may have been due to the possible technical problems discussed earlier for L and pre L chain (Discussion 1.1), or alternatively may have reflected the fact that many other polypeptide species could contaminate cell-free H chain isolated from PAG's.

Using the cell line Pl.17, it was shown that the cell-free synthesized H chain had higher mobility (i.e. lower apparent MW) than the H synthesized in vivo. The main evidence for this came from a study of the banding patterns obtained on PAG's using cellular Pl.17 products and the cell-free synthesized products from the cell lines Pl.17.1 and Pl.17.4 (Results 10). The presence was noted in the products of Pl.17.1 of a polypeptide species with a mobility greater than that of cellular Pl.17. H chain. The absence of such a species in the products of Pl.17.4 (which synthesizes only Pl Ig L chains) strongly suggested that this species was the cell-free H chain. Immunoprecipitation studies of the M.D.L. products, however, have not so far been sufficiently free of non-specific precipitation to allow unambiguous identification of this species (data not shown). Another line of enquiry as to the nature of this species could be developed by comparing its mobility with that of Pl.17 H chain produced by cells in the presence of 2-deoxyglucose. So far, the results obtained from these studies have not proved unambiguous/

unambiguous (data not shown).

Translation of polysomes from SAMM 368 polysomes resulted in the synthesis of two prominent polypeptide species with mobilities greater than the cellular α and γ chains, previously identified by immunoprecipitation (Results 12). Specific precipitation of M.D.L. samples with anti-α antiserum identified the two species as being antigenically related to α and γ chains (anti-α antiserum had been shown previously to cross-react with the γ-type H chain.) Some non-specific precipitation appeared to occur with both anti-κ antiserum and normal rabbit serum in the H chain regions of the gels but it did not have a similar mobility to the positions of cell-free α or γ chains. The mobility of the cell-free α chain seemed to be relatively more greatly increased compared to the cellular α chain than did the mobility of the cell-free γ chain.

These results could also be extended by investigations with 2-deoxyglucose. Especially interesting in this respect would be the relative effect on α and γ chain mobility, bearing in mind the different extents to which carbohydrate addition occurs on different H chain classes (μ>α>γ, in general).

The drawback to the demonstration of the existence of H chain precursors by mobility studies has been the problem of distinguishing between the effects caused by changes in two different processes brought about by cell-free translation. Non-addition of carbohydrate would tend to decrease the apparent MW (and increase mobility) while non-removal of a putative precursor sequence would tend to increase the apparent MW (and decrease mobility).

It may prove difficult to resolve the effects of these processes sufficiently by mobility determination to allow an unambiguous/

unambiguous decision about the existence of a precursor sequence. Even tryptic mapping studies could still lead to ambiguous results. For ultimate proof, the only unambiguous data might come from amino acid sequence analyses of the N-terminus of the cell-free and cellular H chains to discover whether a hydrophobic precursor sequence is present in the cell-free H chain but not in the cellular H chain. Given the small amounts of material involved, this sequencing could be most easily carried out using radioactively labelled material. It was with this aim in mind that gel filtration experiments were carried out on M.D.L. in an attempt to reduce endogenous amino acid pools. This would then allow the labelling of cell-free products to a fairly uniform specific activity with radioactive amino acids. Such material would be suitable for sequencing studies (Results Section 15.).

1.3. Speculations on the role of the "signal" peptide.

As shown in the Introduction (Table 1), precursor sequences at the NH_2-terminus of almost all secreted proteins and polypeptides so far synthesized are a well-established phenomenon of cell-free translation studies. The original idea that all such "signal" sequences would be identical (Blobel & Sabbatini, 1971) has now been shown to be wrong, as a result of amino acid sequencing data. "Signal" sequences vary widely in amino acid composition among different secreted proteins, although their length appears to be relatively homogeneous($\sim$ 18 - 25 amino acids). Signal sequences do not appear to be strictly conserved even within the same cell type/

type. Devilliers-Thiery et al., (1975) found heterogeneity between the sequences of "signal" peptides of dog pancreas secretory proteins, whilst Schechter's group have demonstrated wide sequence variability between the "signal" sequences of various myeloma L chain precursors (see Fig. 1.). It may, however, be argued that Ig L chains form a special case (see later). The basic point of similarity between "signal" peptides so far sequenced has been the high level of hydrophobic residues found. It has been speculated that this is a feature of "signal" peptides which is recognised by membrane receptors (Blobel and Dobberstein, 1974a). Rothman and Lodish (1977) have recently demonstrated that binding of polysomes containing a viral RNA-directed membrane protein to membranes, occurs after only a small portion of the polypeptide chain has been synthesized. While, in this case, it has not been demonstrated that the protein synthesized contains a precursor sequence, the data are consistent with the presence of a hydrophobic signal peptide on the protein. Palmiter (1977) has also claimed that transfer of s secreted proteins through membranes begins before the completion of translation. Hence, the evidence is suggestive of a membrane binding role for the "signal" peptide.

It has been suggested (Blobel and Dobberstein, 1974a) that the signal peptide is removed rapidly after its appearance on the lumen side of the endoplasmic reticulum, but the characteristic of the signal peptide which allows its specific removal without protease digestion of the mature polypeptide is not clear. The balance of evidence suggests that processing of precursors to mature/

mature polypeptides is not highly sequence specific. Dobberstein and Blobel (1977), for example, have demonstrated the apparently correct processing of murine Ig pre L chain synthesized on wheat germ ribosomes in the presence of dog pancreas microsomes. The Krebs ascites system, moreover, has been shown to process murine Ig pre L chain as it is synthesized in the system (Swan, Aviv & Leder, 1972). Admittedly, the suggestion that processing is completely accurate comes only from mobility studies by PAGE which might be unable to distinguish small discrepancies in MW (e.g. $\sim$ 5 amino acids which is the difference in length between pancreatic secretory protein "signal" peptides and murine Ig L chain "signal" peptides). Nevertheless, it would seem likely that processing is a phenomenon of wide occurrence but is relatively independent of amino acid sequence. More likely, the "signal" peptide, upon appearance within the lumen,rapidly assumes a particular three-dimensional configuration as a small "domain" which is linked to the emerging mature polypeptide chain by several exposed amino acids. These may well be labile to protease attack by virtue of their exposed position. A model involving exopeptidase digestion appears to be ruled out by the discovery by Dobberstein et al., (1977) of a small peptide corresponding to the signal peptide in cell-free studies of the synthesis of a chloroplast-specific protein.

The evidence presented in the Introduction (Section 5.4.1.) suggests that precursors are found on almost all secreted proteins. While this project has not unambiguously demonstrated the existence of a precursor sequence on Ig H chains synthesized in cell-free systems, it is important to consider what form (if any) it might take/

take. The most interesting point would be whether Ig H and Ig L chains produced by the same cell have identical precursor sequences. The evidence of Devilliers-Thiery et al., (1975) suggests that in dog pancreas, secreted proteins do not necessarily have identical precursors (although this may reflect the different cell species present in the organ). Moreover, the evidence of Schechter (1977) suggests that in myeloma L chains sequence variation among precursors so far sequenced is as great as is found among the mature L chain hypervariable regions. Consequently in Ig L chains (and hence, possibly, Ig H chains) the precursor may have to be considered as an extension of the variable region. Ig H and L chains from the same cell line (e.g. MOPC 315) have been shown to have different Variable regions. Hence, one would predict different precursor regions.

In cell lines producing two classes of H chains in the same cell (e.g. SAMM 368) sequence analysis would demonstrate whether the same variable region (and so the same precursor sequence) was found in both classes of H chains.

2. Effect of inhibitors of initiation on relative H and L chain production.

Two major experiments carried out in this project pertain to studies of translational control. These experiments have involved the use of inhibitors of initiation to study the relative synthesis of H and L chains and the relative synthesis of Ig and non-Ig protein on a cell-free system under conditions in which initiation of protein/

protein synthesis is limiting.

It was first of all established by PAGE analysis that the cell-free products directed by myeloma polysomes or poly(A) RNA showed qualitative similarity to the cellular products (Results 7.3.). Consequently, translation in the M.D.L. was used as an analogue of the in vivo situation. It was also established by immuno-precipitation techniques that the percentage of protein synthesis (directed by myeloma mRNA in the M.D.L.) which was devoted to the production of Ig-like material was approximately the same as was found intracellularly (Results 8.2.4.). With 5563 the percentage was similar, although with Pl.17 it was slightly less.

As shown in Results 9.1 and 9.2, both ATA and pactamycin had similar effects on the relative synthesis of Ig H, Ig L and non-Ig products.

ATA is an inhibitor of the initial binding of mRNA to the 40S-met-$tRNA_f$ complex. When used at the same concentrations as those employed by Lodish (1974) for investigations on α and β globin synthesis, ATA inhibited the synthesis of Ig H and L chains much less severely than non-Ig species. By the Lodish hypothesis, this suggested that Ig H and L chain mRNA's were better initiators of protein synthesis than non-Ig mRNA's. This is in agreement with the results of Nuss and Koch (1976) with an in vivo system and also with the results of Sonenshein and Brawerman (1976a) also in vivo. Sonenshein and Brawerman's in vitro results (1976b) also concur with these conclusions although their interpretation is hampered by poor H chain (and high MW species) translation in their cell-free system. Such an effect suggests that, in general, proteins produced/

produced in large quantities by differentiated cells may be translated from mRNA's with higher than average initiation constants. This would ensure preferential synthesis of these proteins even under circumstances unfavourable to protein synthesis in general, e.g. starvation. It may also explain why, for example, Ig production can occur at such a high level in tumour cells, where Ig production is non-essential. Other proteins produced for maintenance of cellular metabolism and integrity may, in general, prove to be translated from mRNA's with poorer initiation constants since their synthesis may only be required at low levels in normal circumstances, and may be "turned off" in conditions of starvation.

This does not take account of situations where increases in the rate of synthesis of all cellular proteins are required naturally (as in differentiation) or pathologically (as in cancer). Under these circumstances, replacements of initiation factors may occur.

With ATA, H chain synthesis was relatively less inhibited than L chain synthesis in the M.D.L. This may imply that the H chain mRNA is intrinsically a better initiator of protein synthesis than L chain mRNA, at least in the cell line Pl.17. Nuss & Koch (1976) and Sonenshein & Brawerman (1976a) concluded that in the cell line MPC 11, L chain mRNA is a better initiator than H chain mRNA. Moreover, in this project, translation of 5563 poly(A) RNA in the cM.D.L. (a system in which conditions are unfavourable for the initiation of translation of most species of mRNA), 5563 Ig L chain mRNA was nevertheless translated (Fig.47). This suggested that in 5563, L chain mRNA has better initiating properties than H chain mRNA.

These results suggest that, while in those myeloma cell lines studied,Ig mRNA's are better initiators of translation than non-Ig mRNA's, variation in the initiation properties of H chain and L chain mRNA's may exist.

Pactamycin, the other inhibitor studied, acts to block protein synthesis subsequent to formation of the mRNA-40S-met-$tRNA_f$ complex. In fact, it has been demonstrated that the initial dipeptide coded for by mRNA's can be formed (Goldberg et al., 1973; Cheung et al., 1973) but thereafter protein synthesis ceases, due to an inhibition of translocation. According to Lodish's hypothesis, inhibition at this stage should not lead to preferential initiation by better initiating mRNA's.

The results obtained in this project (Results 9.2.) with Pl.17 poly(A) RNA in M.D.L. inhibited by pactamycin do not agree with the model proposed by Lodish. Using a concentration range, including those levels of pactamycin investigated by Lodish (1974) with globin synthesis, it was found that Ig synthesis in M.D.L. is less inhibited than non-Ig synthesis. Moreover, it was again shown that H chain synthesis was relatively less inhibited by pactamycin than L chain synthesis. The findings with Ig and non-Ig synthesis are not directly comparable with Lodish's results with globin. The resistance to inhibition of H chain synthesis relative to L chain synthesis, however, appears to contradict the results obtained by Lodish for α and β globin which he interprets in the light of his model. A possible explanation was that the levels of pactamycin used caused inhibition of elongation steps also. The concentration range used included, however, as noted above, the levels/

levels used by Lodish. Moreover, the inhibition did not seem to have the same characteristics as elongation inhibition by cycloheximide in myeloma cells, viz. approximately equal inhibition of Ig and non-Ig synthesis. This comparison was not strictly valid, however, and it could still be argued that elongation was being inhibited. This would imply, however, with mRNA's being translated in proportion to their abundance, that a large excess of poorly initiating Ig mRNA's occurred in the normally growing cell. The results obtained with ATA, however, suggested that Ig mRNA's are good initiators of translation.

It may prove necessary to extend the scope of the Lodish model. The initiation constant K_i, which governs the rate of initiation of any particular mRNA, may prove to cover all processes up to and including the first peptide bond formation, rather than the steps leading merely to the formation of the mRNA-40S-met $tRNA_f$ complex. This seems possible since all the steps up to and including the first peptide bond formation are unique events in the translation of any mRNA. Contrasted with this are subsequent elongation and termination reactions which are essentially either repetitive processes (elongation) or common processes for all mRNA's (termination).

Further study of the initiation process could be carried out with inhibitors specific for other steps in initiation.

3. Improvements in systems.

3.1. Immunoprecipitation.

One of the major problems encountered in this project, and one not wholly overcome, was concerned with immunoprecipitation techniques. Two methods of immunoprecipitation - direct and indirect, were compared. Direct immunoprecipitation was found to have variable efficiency but was generally much less efficient than indirect immunoprecipitation. Indirect immunoprecipitation, using unfractionated rabbit antiserum and goat antiserum, was found to be very efficient in precipitation of specific proteins from cell lysates and cell-free systems (less so in cell-free systems) but involved the formation of a large precipitate. Such large quantities of protein in precipitates resulted from the specificity of the second antiserum for total rabbit IgG and from the unfractionated nature of the first antiserum. The large protein precipitates formed tended to cause problems of non-specific trapping of radioactivity, especially from the cell-free system,and overloading of Laemmli gels. Improvements in immunoprecipitation would be made as follows. Preparation of purified rabbit antibodies with specificity for 5563 or P1 Ig could be obtained by passing the unfractionated antiserum over a column of Sepharose beads linked to pure 5563 Ig or P1 Ig. This would not decrease the size (amount) of primary complexes formed, but would reduce considerably the amount of goat antiserum required to precipitate these primary complexes. This would help reduce the amount of protein precipitated/unit of radioactivity precipitated. A lowering of the protein:radioactivity ratio would mitigate the overloading problems found with immunoprecipitates on PAG's.

Alternative/

Alternative methods of relieving this problem are discussed below.

3.2. Cell-free system.

As was demonstrated, (Results 4.5.) the M.D.L. cell-free system is highly efficient in its translation of added poly(A) RNA and also translates added polysomes well. Initiation and re-initiation of protein synthesis appeared to be no problem with the M.D.L. (Results 4.5.). One drawback to the system was the presence of large pools of endogenous amino acids in the system. These were observed as a result of amino acid analysis studies (Results 4.3) although only the level of methionine was measured (700 pmoles/ 50µl).

These pools reduced considerably the specific activity of added radioactive amino acids and were, moreover, of variable size (data not shown).

Various procedures could help to overcome this problem. The use of much higher quantities of radioactively labelled L(^{35}S) methionine (e.g. 250µCi/50µl assay rather than ∿25µCi/50µl assay). would increase the added methionine to a similar order of magnitude to the endogenous methionine pool size, e.g.

Specific activity of L-(^{35}S) methionine = 1000 Ci/mmole

= 1µCi/pmole

∴ 250 µCi = 250 pmoles

Endogenous pool size = 700 pmoles

In this way, a much higher percentage of the added L-(^{35}S) methionine would/

would be incorporated, assuming total protein synthesis remained constant.

e.g. L-(^{35}S) methionine incorporated (25μCi/assay) = 5 pmoles

" unlabelled " " " = $\frac{700}{25}$ x 5

= 140 pmoles

Total incorporation = 145 pmoles (20%) of total methionine present) but if 250μCi (= 250pmoles) L(^{35}S) methionine added, and total incorporation was 20% of the methionine added = 190 pmoles

pmoles L-(^{35}S) methionine incorporated = $\frac{250}{700}$ x 190 = 68 pmoles

= 68 μCi (∿33%)

In situations such as this, account must be taken of solutes present in the L-(^{35}S) methionine solution. KCl is present at 20mM concentration; adding 5μl (∿25μCi) to a 50μl assay adds 1.8mM to the KCl concentration which is relatively small (normal KCl concentration 95mM). Addition of 250μCi of an L-(^{35}S) methionine solution (lyophilised) to a 50μl assay, however, increases the KCl concentration by ∿20mM which would significantly affect incorporation (Results 4.2.). Moreover, lyophilisation must also remove small quantities of 2-mercaptoethanol present in the solution. It was noted, although not systematically investigated, that assays containing diluted L-(^{35}S) methionine or containing a lower percentage of L-(^{35}S) methionine solution, by volume, generally gave higher levels of incorporation of radioactivity/pmole mRNA added. It was thought that 2-mercaptoethanol might have a depressive effect on protein synthesis.

An alternative method of increasing incorporation of radioactivity incorporated would be to reduce the volume of M.D.L. used/

used, whilst keeping the amount of L-(^{35}S) methionine (lyophilised) constant. Hence a 10µl assay should increase the final assay specific activity of L-(^{35}S) methionine 5-fold. A combination of the two methods described above should have an even greater effect.

3.3. Gel filtration of M.D.L.

Another method of increasing the specific activity of the assay methionine concentration was, however, sought. This was done with a view towards preparing a cell-free system which would allow radioactive labelling of all the amino acids to a constant level. This step would allow amino acid sequencing studies to be carried out.

Analogously with various other cell-free systems, (e.g. wheat germ, Krebs II ascites), the use of a G-25 Sephadex filtration step was investigated. As described in Results 15, the removal of small molecular species (including amino acids) by this method was only partially successful as a means of increasing incorporation of radioactivity. Gel-filtered M.D.L. (cM.D.L.), in general, displayed a marked stimulation in the level of incorporation of radioactivity using polysomes compared to unfiltered M.D.L. but relatively little, if any, stimulation using poly(A) RNA. This suggested the removal, by gel filtration, of some low MW component (or components) necessary to promote initiation of translation.

ATA/

ATA inhibition studies of polysome translation in cM.D.L. (Results 15.6.), showing 33% of synthesis was sensitive to inhibition of initiation, suggested that gel filtration did not completely remove this component (alternatively the polysomes contained this component) as did the demonstration of translation of poly(A) RNA, although translation was at a low level. As the experiments described in Results 15.7 and 15.9 suggested, several low MW species can be ruled out as the required molecules, e.g. ATP, GTP, reducing agents (DTT).

The possibility of damage or inactivation of various components of protein synthesis had been ruled out in several experiments (tRNA, 0.5M salt wash factors, haemin concentration). That the inhibition of initiation was concentration-dependent was demonstrated by mixed M.D.L.:cM.D.L. assays of reticulocyte mRNA (Results 15.10). Only above levels of 50% (v/v) M.D.L./assay was significant incorporation of radioactivity detected. The role of the gel filtration step in causing the inhibition (e.g. by leaching out of an inhibitory substance) was checked by an alternative method of removing low MW species (dialysis) and found to be unimportant (Results 15.11).

Consequently, the conclusion drawn was that some low MW component (or components), which were partially removed by gel filtration (but were not added back to the lysate before making cM.D.L.) were required for initiation of protein synthesis. Such a requirement was concentration dependent; below a certain concentration, initiation of protein synthesis decreased abruptly. This implied a possible allosteric mechanism, perhaps involving cooperative/

cooperative effects, acting on a protein synthesis component.

After the completion of the work in this project, two pieces of information pertinent to this discussion came to light. Firstly, it was reported briefly in a review by Hunt (1976) that G-50 Sephadex filtration of reticulocyte lysate removed the capacity for protein synthesis. This capacity, however, was found to be restored by the addition to the filtered lysate of any of a range of intermediary metabolites (glucose, citrate, fructose-1,6 diphosphate). Also particularly helpful was DTT (not found so in this study), while c-AMP removed the inhibition completely. This finding was subsequently confirmed by D. Wilde (personal communication) who also found that polyamines (spermine, spermidine) were essential for activity.

It can be envisaged that in a system, such as the rabbit reticulocyte, which is dependent on a constant flow of intermediary metabolites for its energy supply, any shortage of an intermediary metabolite would require a rapid cut-back in non-essential energy-requiring processes in order to maintain essential processes (e.g. maintaining correct ion levels by "ion pumps").

Protein synthesis is a relatively non-crucial process in reticulocytes (at least in the short term) compared, for example, to the maintenance of ion gradients. It requires, moreover, large quantities of energy for its functioning. It would therefore seem a convenient process to inhibit during energy starvation. Conditions mimicking starvation are brought about by gel filtration. Although an energy source is then supplied (creatine phosphate/ creatine/

creatine kinase/ATP/GTP), the absence of normal levels of intermediary metabolites may cause inhibition. This suggests a normal situation of feed-forward activation of protein synthesis, by one or more of the metabolic intermediates of glycolysis, may obtain. Alternatively, some less direct control of protein synthesis via {AMP},for example, may be exerted.

The initiation of translation was not entirely inhibited. As Results 15.12 demonstrated 5563 L chain mRNA appeared to be translated in cM.D.L. in the absence of any other translation. This may indicate that 5563 L chain mRNA is an extremely efficient initiator of protein synthesis (Discussion 2). It may offer a method of easily isolating 5563 L chain mRNA from myeloma cell mRNA mixtures. Myeloma mRNA could be added to cM.D.L. and after a short incubation, polysomes could be removed by centrifugation. These polysomes should contain only mRNA for L chain. If this is a general phenomenon, it might be applied to mRNA of other cell types (e.g. Pl.17).

4. Future experiments.

The way towards deciding the question of whether Ig H chain contains a precursor sequence when synthesized in cell-free systems is now clear. Preliminary comparisons of cell-free synthesized H chain with cellular H chain lacking carbohydrate should provide qualitative mobility and peptide-mapping data on the nature of differences between the two products.

Development of the cM.D.L., with the improvements noted by other/

other workers, should allow the use of the system as a means of producing Ig H and L chains, radioactively labelled with amino acids of equal specific activity, and suitable for amino acid sequence analysis. This will provide quantitative evidence to corroborate any findings from the above experiments.

The translation of myeloma mRNA in the M.D.L. also provides a system for further investigating translational control of protein synthesis, and particularly the control of the synthesis of Ig H and L chains. A wide range of initiation (ATA, pactamycin, dsRNA, 7-methyl guanosine, polynucleotides) and elongation (emetine, cycloheximide) inhibitors could be investigated and results interpreted in the light of current theories of translational control. These experiments would test and possibly extend the validity of Lodish's hypothesis. The use of more physiological inhibitors of initiation, such as starvation, could be mimicked in the cell-free system by 2-deoxyglucose. The effect of 2-deoxyglucose in M.D.L. will be interesting in view of the potential role of intermediary metabolites in affecting protein synthesis in the M.D.L.

REFERENCES

Adamson,S.D., Herbert,E., Godchaux III, W. Arch. Biochem. Biophys. (1968). 125, 671-683.

Adesnik, M. & Darnell, J.E. (1972). J. Mol. Biol. 67, 397-405.

Adesnik, M., Salditt, M., Thomas, W. & Darnell, J.E. (1972). J. Mol. Biol. 71, 21-30.

Arlinghaus, R. & Schweet, R. (1962). Biochem. Biophys. Res. Commun. 9, 482-485.

Arnstein, H.R.V., Cox, R.A. and Hunt, J.A. (1962). Nature 194, 1042-1044.

Arnstein, H.R.V., Cox, R.A. and Hunt, J.A. (1964). Biochem. J. 92, 648-661.

Ascione, R. & Arlinghaus, R.B. (1970). Biochim. Biophys. Acta 204, 478-488.

Askonas, B.A. & Williamson, A.R. (1967). Nature 216, 264-267.

Aviv, H., Boime, I. & Leder, P. (1971). Proc. Nat. Acad. Sci. U.S. 68, 2303-2307.

Awdeh, Z.L., Williamson, A.R. & Askonas, B.A. (1970). Biochem. J. 116, 241-248.

Balkow, K., Hunt, R.T. & Jackson, R.J. (1975). Biochem. Biophys. Res. Commun. 67, 366-375.

Baralle, F.E. (1977). Cell 10, 549-558.

Bard, E., Efron, D., Marcus, A., Perry, R.P. (1976). Cell 1, 101-106.

Beato, M., & Nieto, A. (1976). Eur. J. Biochem. 64, 15-25.

Bedard, D. & Huang, R-C.C., (1977). J. Biol. Chem. 252, 2592-2598.

Berridge, M.V., Farmer, S.R., Green,C.D., Henshaw, E.C. & Tata,J.R. (1976). Eur. J. Biochem. 62, 161-171.

Blobel, G. (1972). Biochem. Biophys. Res. Commun. 47, 88-95.

Blobel, G. (1973). Biochem. Biophys. Res. Commun. 68, 1-7.

Blobel, G.& Dobberstein, B. (1974a). J. Cell. Biol. 67, 835-851.

Blobel, G. & Dobberstein, B. (1974b). J. Cell. Biol. 67, 852-862.

Blobel, G. & Sabatini, D.D. (1971) in "Biomembranes" (ed. Manson,L.A.) 2, pp. 193-195.

Boime, I., Boguslawski, S. & Caine, J. (1975). Biochem. Biophys. Res. Commun. 62, 103-109.

Boime, I., Szczesna, E. & Smith, D. (1977). Eur. J. Biochem. 73, 515-520.

Bollum,F.J. (1968) in Methods in Enzymology (Grossman, L. and Moldave, K., eds.) 12B, pp. 169-173. Academic Press Ltd., London and New York.

Bonanou-Tzedaki, S.A. and Arnstein, H.R.V. (1976). Eur. J. Biochem. 61, 397-407.

Bonner, W.M. & Laskey, R.A. (1974). Eur. J. Biochem. 46, 83-88.

Borsook, H., Fisher, E.H. & Keighley, G. (1957). J. Biol. Chem. 229, 1059-1070.

Brawerman, G. (1974a) in Methods in Enzymology (Moldave, K. and Grossman, L., eds.) 30, pp. 605-612. Academic Press, London and New York.

Brawerman, G. (1974b). Ann. Rev. Biochem. 43, 621-642.

Brawerman, G. (1976). Prog. Nuc. Acids Res. Mol. Biol. 17, 118-148.

Brawerman, G., Mendecki, J. & Lee, S.Y. (1972). Biochem. 11, 637-641.

Brecher, G. (1949). Am. J. Clin. Path. 19, 895-896.

Buckingham, M.E., Caput, D., Cohen, A., Whalen, R.G. & Gros, F. (1974). Proc. Nat. Acad. Sci. U.S. 71, 1466-1470.

Burr, H. & Lingrel, J.B. (1971). Nature New Biol. 233, 41-43.

Burstein, Y. & Schechter, I. (1976). Biochem. J. 157, 145-151.

Burstein, Y., Kantor, F. & Schechter, I. (1976). Proc. Nat. Acad. Sci. U.S. 73, 2604-2608.

Burstein, Y. and Schechter, I. (1977). Biochem. J. 165, 355-365.

Campbell, P.N. & Blobel, G. (1976). FEBS Letts. 72, 215-224.

Chan, S.J., Keim, P. & Steiner, D.F. (1976). Proc. Nat. Acad. Sci. U.S. 73, 1964-1968.

Chantrenne, H. (1970). Eur. J. Biochem. 64, 115-121.

Chantrenne, H., Burny, A. & Marbaix, G. (1967). Prog. Nuc. Acid Res. Mol. Biol. 7, 173-194.

Cheung, G.P., Stewart, M.L. & Gupta, N. (1973). Biochem. Biophys. Res. Commun. 54, 1092-1101.

Cowan, N.J. & Milstein, C. (1972). Biochem. J. 128, 445-454.

Cowan, N.J. & Milstein, C. (1973). Eur. J. Biochem. 36, 1-7.

Craig, R.K., Brown, P.A., Harrison, O.S., McIlreavy, D. & Campbell, P.N. (1976). Biochem. J. 160, 57-74.

Croft, L.R., (Ed.) Handbook of Protein Sequences (1973). Joynson-Bruvvers, Ltd., Oxford.

Crumpton, M.J. & Parkhouse, R.M.E. (1972). FEBS Letts. 53, 95-98.

Crystal et al.(1972) see end.

Darnell, J.E. (1968). Bact. Revs. 32, 262-290.

Darnell, J.E., Philipson, L., Wall, R. & Adesnik, M. (1971). Science, 174, 507-510.

Delovitch, T.L., Davis, B.K., Holme, G. & Sehon, A.H. (1972). J. Mol. Biol. 69, 373-386.

Devilliers-Thiery, A., Kindt, T., Scheele, G. & Blobel, G. (1975). Proc. Nat. Acad. Sci. U.S. 72, 5016-5020.

Dobberstein, B. & Blobel, G. (1977). Biochem. Biophys. Res. Commun. 74, 1675-1682.

Dobberstein, B., Blobel, G. & Chua, N. H.(1977). Proc. Nat. Acad. Sci. U.S. 74, 1082-1085.

Doel, M.T., & Carey, N.H. (1976). Cell, 8, 51-58.

Eagon, P.K., & Heath, E.C. (1977). J. Biol. Chem. 252, 2372-2383.

Efstratiadis, A., Kafatos, F.C., & Maniatis, T. (1977). Cell 10, 571-586.

Evans, G.E. & Rosenfeld, M.G. (1976). J. Biol. Chem. 251, 2842-2847.

Evans, M.J., & Lingrel, J.B. (1969). Biochem. 8, 829-831.

Finegold, I., Fahey, J.L., & Dutcher, T.F. (1968). J. Immunol. 101, 366-373.

Fougereau, M., Bourgois, A., de Preval, C., Rocca-Serra, J. & Schiff, C. (1976). Ann. Immunol. (Inst. Past.) 127C, 607-631.

Gedamu, L., Iatrou, K. & Dixon, G.H. (1977). Cell 10, 443-452.

Gierer, A. (1963). J. Mol. Biol. 6, 148-157.

Gilmore-Herbert, M.A., & Heywood, S.M. (1976). Biochim. Biophys. Acta. 454, 55-66.

Goldberg, I., Stewart, M.L., Ayuso, M., & Kappen, L. (1973). Fed. Proc. 32, 1688-1697.

Goldman, E., & Lodish, H.F. (1975). Biochem. Biophys. Res. Commun. 64, 668-672.

Gray, W.R., (1972) in Methods in Enzymology (Hirs,C.H.W. & Timasheff, S.N., eds.) 25B, pp. 121-138. Academic Press, London & New York.

Green,M., Zehavi-Willner, T., Graves, P.N., McInnes, J. & Pestka, S. (1976). Arch. Biochem. Biophys. 172, 74-89.

Greenberg, J.R., & Perry, R.P. (1972). J. Mol. Biol. 72, 91-98.

Gross, M. (1977). Arch. Biochem. Biophys. 180, 121-129.

Gross, M., & Rabinowitz, M. (1972). Proc. Nat. Acad. Sci. U.S. 69, 1565-1568.

Habener, J.F., Kemper, B., Potts, J.T.,Jr., & Rich, A. (1975). Biochem. Biophys. Res. Commun. 67, 1114-1121.

Hardesty, B., Arlinghaus, R., Schaeffer, J., & Schweet, R. (1963). Cold Spring Harb. Symp. Quant. Biol. 28, 215-222.

Hartley, M.R., Wheeler, A. & Ellis, R.J. (1975). J. Mol. Biol. 91, 67-77.

Heywood, S.M. & Kennedy, D.S. (1976). Prog. Nuc. Acid Res. Mol. Biol. 19, 477-484.

Heywood, S.M., & Nwagwa, M. (1969). Biochem. 8, 3839-3845.

Huez, G., Marbaix, G., Hubert, E., Leclerq., M., Nudel, U., Soreq, H., Saloman, R., Lebleu, B., Revel, M., & Littauer, U.Z. (1974). Proc. Nat. Acad. Sci.U.S. 71, 3143-3146.

Hunt(1974) see end.
Hunt, R.T. (1976). Brit. Med. Bull. 32, 257-261.

Hunt, R.T., Hunter, A., & Munro, A.J. (1968). Nature 220, 481-483.

Jackson, R.J., & Hunter, A. (1970). Nature 227, 672-676.

Jacob, F., & Monod, J. (1961). J. Mol. Biol. 3, 318-356.

Jacobs-Lorena, M., & Baglioni, C. (1972a). Proc. Nat. Acad. Sci. U.S. 69, 1425-1428.

Jacobs-Lorena, M., & Baglioni, C., (1972b). Biochem. 11, 4970-4974.

Kates, J., (1970). Cold Spring Harb. Symp. Quant. Biol. 35, 743-752.

Kates, J., (1974). Methods Cell. Biol. 7, 53-65.

Kaufman, V., Goldstein, E., & Penman, S. (1976). Proc. Nat. Acad. Sci. U.S. 73, 1834-1838.

Kedes, L.H., (1976). Cell 8, 321-331.

Kemper,B., Habener, J.F., Mulligan, R.C., Potts, J.T.,Jr., & Rich, A. (1974). Proc. Nat. Acad. Sci. U.S. 71, 3731-3735.

Kennedy, D.S., & Heywood, S.M. (1976). FEBS Letts. 72, 314-318.

Koch, G., Pawlowski, P.J., & Lukas, L.N. (1976). Arch. Biochem. Biophys. 178, 373-380.

Kruh, J., Schapira, G., Lareau, J., & Dreyfus, J.C. (1964). Biochim. Biophys. Acta. 87, 669-681.

Labrie, F., (1969). Nature 221, 1217-1222.

Laemmli, U.K., (1970). Nature 227, 680-685.

Lamfrom, H., & Knopf, P.M., (1964). J.Mol. Biol. 9, 558-575.

Lane, C.D., Gurdon, J.B., & Woodland, H.R., (1974). Nature 251, 436-437.

Lanyon, W.G., Paul, J. & Williamson, R., (1972). Eur. J. Biochem. 31, 38-43.

Lebleu, B., Marbaix, G., Huez, G., Temmerman, J., Burny, A. & Chantrenne, H., (1971). Eur. J. Biochem. 19, 264-269.

Lee, S.Y., Mendecki, J., & Brawerman, G. (1971). Proc. Nat. Acad. Sci. U.S. 68, 1331-1335.

Legon, S., Brayley, A., Hunt, R.T. & Jackson, R.J., (1974). Biochem. Biophys. Res. Commun., 56, 745-752.

Legon, S., Jackson, R.J., & Hunt, R.T., (1972). Nature New Biol. 241, 150-152.

Levenson, R.G., & Marcu, K.B., (1976). Cell 9, 311-322.

Lim, L., & Cannellakis, E.S., (1970). Nature 227, 710-713.

Lingrel, J.B., & Borsook, H., (1963). Biochem. 2, 309-314.

Lingrel, J.B., Lockard, R.E., Jones, R.F., Burr, H.G., & Holder, J.W., (1971). Ser. Haematol. 4(3), 37-69.

Lingrel, J.B., Morrison, M., Gorski, J., & Merkel, C.G., (1974). Ann. N.Y. Acad. Sci. 241, 156-169.

Live, T.R., & Kaminskas, E. (1975). J. Biol. Chem. 250, 1786-1789.

Lockard, R., & Lingrel, J.B. (1969). Biochem. Biophys. Res. Commun. 37, 204-212.

Lodish, H.F., (1971). J. Biol. Chem. 246, 7131-7138.

Lodish, H.F., (1974). Nature 251, 385-388.

Lodish, H.F., (1976). Ann. Rev. Biochem. 45, 39-72.

Lodish, H.F., & Jacobsen, M., (1972). J. Biol. Chem. 247, 3622-3629.

Lodish, H.F., & Small, B., (1976). Cell 7, 59-65.

London, I.M., Shemin, D., & Rittenberg, D., (1950). J. Biol. Chem., 183, 749-765.

Mach, B., Faust, C., & Vassalli, P., (1973). Proc. Nat. Acad. Sci. U.S. 70, 451-455.

Maniatis, T., Kee, S.G., Efstratiadis, A., Kafatos, F.C., (1976). Cell 8, 163-182.

Marcu, K., & Dudock, B., (1974). Nuc. Acids Res. 1, 1385-1397.

Marcus & Feeley (1964) see end.

Mathews, M.B., (1973) in Essays in Biochemistry (Campbell, P.N. & Dickens, F., eds.) 9, pp. 59-102. Academic Press, London and New York.

Mathews, M.B., and Korner, A.,(1970). Eur. J. Biochem. 17, 328-338.

Mathews, M.B., Hunt, R.T., & Brayley, A., (1973). Nature New Biol. 243, 230-233.

Mathias, A.P., Williamson, R., Huxley, H.E., & Page, S., (1964). J. Mol. Biol. 9, 154-167.

Maxwell, C.R., Kemper, C.S., & Rabinowitz, M., (1971). J. Mol. Biol. 58, 317-327.

McDowell, M.J., Joklik, W.K., Villa-Komaroff, L., & Lodish, H.F., (1972). Proc. Nat. Acad. Sci. U.S. 69, 2649-2653.

McIntosh, P.R., & O'Toole, K., (1976). Biochim. Biophys. Acta., 457, 171-212.

Melchers, F., (1973). Biochem. 12, 1471-1476.

Mendecki, J., Lee, S.Y., & Brawerman, G. (1972). Biochem. 11, 792-798.

Metafora, S., Terada, M., Dow, L.W., Marks, P.A., & Bank, A., (1972). Proc. Nat. Acad. Sci. U.S. 69, 1299-1303.

Milcarek, C., Price, R., & Penman, S., (1974). Cell 3, 1-10.

Milstein, C., Brownlee, G.G., Harrison, T.H., & Mathews, M.B., (1972). Nature New Biol. 239, 117-120.

Molloy, G.R., Sporn, M.B., Kelley, D.E., & Perry, R.P., (1972). Biochem. 11, 3256-3260.

Morel, C., Kayibanda, B., & Scherrer, K., (1971). FEBS Letts., 18, 84-88.

Morrison, M.R., Brinkley, S.A., Gorski, J., & Lingrel, J.B. (1974). J. Biol. Chem. 249, 5290-5295.

Murphy, W., & Attardi, G., (1973). Proc. Nat. Acad. Sci. U.S. 70, 115-119.

Nakanishi, S., Taii, S., Hirata, Y., Matsukura, S., Imura, H., & Nama, S., (1976). Proc. Nat. Acad. Sci. U.S. 73, 4319-4323.

Nakazato, H., Kopp, D.W., & Edmonds, M., (1973). J. Biol. Chem. 248, 1472-1476.

Nathans, D., Notani, G., Schwartz, J.H., & Zinder, N.D., (1962). Proc. Nat. Acad. Sci. U.S. 48, 1424-1431.

Nemer, M., Graham, M., & Dubroff, L.M., (1974). J. Mol. Biol. 89, 435-454.

Nemer, M., & Surrey, S., (1976). Prog. Nuc. Acid Res. Mol. Biol. 19, 119-122.

Nirenberg, M.W., Matthai, J.H., Jones, O.W., Martin, R.G. & Barondes, S.H., (1963). Fed. Proc. 22, 55-61.

Nudel, U., Soreq, H., Littauer, U.Z., Marbaix, G., Huez, G., Leclerq, M., Hubert, E., & Chantrenne, H., (1976). Eur. J. Biochem. 64, 115-121.

Nuss, D.L., & Koch, G., (1976). J. Mol. Biol. 102, 601-612.

Opperman, H., & Koch, G., (1976). J. Gen. Virol., 32, 261-273.

Osborn, M., & Mathews, M.B., (1974). Biochim. Biophys. Acta. 340, 147-152.

Palacios, R., Sullivan, D., Summers, N.M., Kiely, M.L., & Schimke, R.T., (1975). J. Biol. Chem. 248, 540-548.

Palmiter, R.D., (1973). J. Biol. Chem. 248, 2095-2106.

Palmiter, R.D., (1974). Biochem. 13, 3606-3615.

Palmiter, R.D., Palacios, R., & Schimke, R.T., (1973). J. Biol. Chem. 248, 3296-3304.

Palmiter, R.D., Thibodeau, S.N., Gagnon, J., & Walsh, K.A., (1977). FEBS Meeting, Copenhagen, Abstract Ab-2/L3/4.

Parkhouse, B., Ph.D. Thesis, 1969.

Pelham, H.R.B., & Jackson, R.J., (1976). Eur. J. Biochem. 67, 247-256.

Penman,S., Rosbach, M., & Penman, M., (1970). Proc. Nat. Acad. Sci. U.S. 67, 1878-1885.

Penman, S., Vesco, C., & Penman, M., (1968). J. Mol. Biol. 34, 49-69.

Perry(1976) see end.

Potter, M., (1972). Phys. Revs. 52, 631-719.

Potter, M. (1975) in Cancer: a comprehensive treatise I (Becker, F.F., Ed.). Plenum Press, New York.

Potter, M., (1976). in Meth. Canc. Res. II (Bush, H., ed.). pp. 105-157. Academic Press, London and New York.

Potter, M., Sklar,M.D., & Row, W.P., (1973). Science 182, 592-594.

Peterson(1976) see end.

Rabbitts, T.H., & Milstein, C., (1977) in Cont. Top. Mol. Immunol. (Porter, R.R. & Ada, G.L., eds.) 6, 117-144.

Ranu, R.S., & London, I.M., (1976). Proc. Nat. Acad. Sci. U.S. 72, 4849-4853.

Rapoport, T.A., Höhne, W.E., Klatt, D., Prehn, S., & Hahn, V., (1976). FEBS Letts. 69, 32-36.

Rhoads, R.E., McKnight, G.S., & Schimke, R.T., (1971). J. Biol. Chem. 246, 7407-7410.

Rhoads, R.E., McKnight, G.S., & Schimke, R.T., (1973). J. Biol. Chem. 248, 2031-2039.

Rich, A., Warner, J.R., & Goodman, H.M., (1963). Cold Spring Harb. Symp. Quant. Biol. 28, 269-285.

Robbins, E., & Borun, T.W., (1967). Proc. Nat. Acad. Sci. U.S. 57, 409-416.

Roberts, B.E., & Paterson, B.M., (1971). Proc. Nat. Acad. Sci. U.S. 70, 2330-2334.

Rosen, J.M., Woo, S.L.C., & Comstock, J.P., (1975). Biochem. 14, 2895-2903.

Rothman, J.E., & Lodish, H.F., (1977). Nature 269, 775-780.

Rourke, A.W., & Heywood, S.M., (1972). Biochem. 11, 2061-2066.

Rupniak, H.T.R., & Quincey, R.V., (1975). FEBS Letts. 58, 99-101.

Schaeffer, J., Favelukes, G., Schweet, R., (1964).

Biochim. Biophys. Acta 80, 247-255.

Schechter, I., (1973). Proc. Nat. Acad. Sci. U.S. 70,

2256-2260.

Schechter, I., (1974). Biochem. 13, 1875-1885.
Schechter(1975) see end.
Schechter, I., (1977). FEBS Congress, Copenhagen, Abstract

A6-2/L4/4.

Schechter, I., & Burstein, Y., (1976a). Biochem. J.

153, 543-550.

Schechter, I., & Burstein, Y., (1976b). Proc. Nat. Acad.

Sci. U.S. 73, 3273-3277.

Schechter, I., McKean, D.J., Guyer, R., & Terry, W., (1974).

Science, 188, 160-162.

Schmeckpeper, B.J., Adams, J.M., & Harris, A.W., (1975).

FEBS Letts. 53, 95-98.

Schmeckpeper, B.J., Cory, S., & Adams, J.M., (1974).

Mol. Biol. Reps. 1, 355-363.

Schmeckpeper, B.J., Cory, S., & Adams, J.M., (1977).

Biochim. Biophys. Acta 476, 303-320.

Schutz, G., Kieval, S., Groner, B., Suppel, A.E., Kurtz,

D.T., & Feigelson, P., (1977). Nuc. Acids Res. 4,

71-83.

Scott, A.C., & Wells, J.R.E., (1975). Biochem. Biophys. Res.

Commun. 64, 448-455.
Shafritz et al.(1973) see end
Shapiro, D.J., Taylor, J.M., McKnight, G.S., Palacios, R.A.,

Gonzalez, C., Kiely, M.L., & Schimke, R.T., (1974).

J. Biol. Chem. 249, 3665-3671.
Sharma et al.(1976) see end.
Shatkin, A.J., (1976). Cell 9, 45-53.

Shih & Kaesberg(1973) see end

Shore, G.C., & Tata, J.R., (1977). Biochim. Biophys. Acta 472, 197-236.

Singer, M.F., & Leder, P., (1966). Ann. Rev. Biochem. 35, 195-230.

Singer, R.H., & Penman, S., (1973). J. Mol. Biol. 78, 321-333.

Sippel et al.(1974) see end.

Sonenshein,G.E., & Brawerman, G., (1976a). Biochem. 15, 5497-5501.

Sonenshein, G.E., & Brawerman, G., (1976b). Biochem. 15, 5501-5506.

Soreq, H., Nudel, V., Salomon, R., Revel, M., & Littauer, U.Z., (1974). J. Mol. Biol. 88, 233-245.

Stavnezer, J., & Huang, R-C.C., (1971). Nature New Biol. 230, 172-176.

Stevens, R.H., & Williamson, A.R., (1973). J. Mol. Biol. 78, 505-516.

Strauss, A.W., Donohue, A.M., Bennett, C.D., Rodkey, J.A., & Alberts, A.W., (1977). Proc. Nat. Acad. Sci. U.S. 74, 1358-1362.

Suchanek, G., Kindas-Mügge, I., & Kreil, G., (1975). Eur. J. Biochem. 60, 309-315.

Suchanek, G., & Kreil, G., (1977). Proc. Nat. Acad. Sci. U.S., 74, 975-978.

Summers, D.F., Maizel, J.V., & Darnell, J.E., (1965). Proc. Nat. Acad. Sci. U.S. 54, 505-513.

Sussman, M., (1970). Nature 225, 1245-1246.

Sussman, P.M., Tushinski, R.J., & Bancroft, F.C., (1976). Proc. Nat. Acad. Sci. U.S. 73, 29-33.

Suzuki, H., (1976). FEBS Letts. 72, 309-313.

Swan, D., Aviv, H., & Leder, P., (1972). Proc. Nat. Acad. Sci. U.S. 69, 1967-1971.

Szczesna, E., & Boime, I., (1976). Proc. Nat. Acad. Sci. U.S. 73, 1179-1183.

Taylor & Schimke(1974)see end

Temple, G., & Lodish, H.F., (1975). Biochem. Biophys. Res. Commun. 63, 971-979.

Temple, G.F., & Housman, D.E., (1972). Proc. Nat. Acad. Sci. U.S. 69, 1574-1577.

Tyler, A., & Tyler, B.S., (1972) in Cell Differentiation (Schjeide, O.A., & de Villis, J., eds.). pp. 42-118, Van Nostrand Reinhold Co., Amsterdam.

Uenoyama, K., & Ono, T., (1972). J. Mol. Biol. 65, 75-89.

Vaquero, C., Rubel, L., & Schapira, G., (1975). FEBS Letts. 58, 325-329.

Waxman, H.S., & Rabinowitz, M., (1965). Biochem. Biophys. Res. Commun., 19, 538-545.

Waxman, H.S., & Rabinowitz, M., (1966). Biochim. Biophys. Acta. 129, 369-379.

Weissbach, H., & Ochoa, S., (1976). Ann. Rev. Biochem. 45, 467-500.

Wetekam, W., Mullinix, K.P., Deeley, R.G., Kronenberg, H.M., Eldridge, J.D., Meyers, M., & Goldberger, R.F., (1975). Proc. Nat. Acad. Sci. U.S. 72, 3364-3368.

Williamson, A.R., (1976). Ann. Rev. Biochem. 45, 467-500.

Williamson, A.R., Hausmann, E., Heintz, E., & Schweet, R., (1967). J. Mol. Biol. 26, 267-278.

Williamson, R., Crossley, J., Humphries, S., (1974).
Biochem., 13, 703-707.

Woods, K.R., & Wang, K-T., (1967). Biochim. Biophys.
Acta. 133, 169-170.

ADDITIONAL REFERENCES.

tal,R.G.,Nienhuis,A.W.,Elson,N.A.&Anderson,W.F.(1972)
J. Biol. Chem. 247 5357-5368.

t,R.T. (1974) Ann. N. Y. Acad. Sci. 241 223-231.

cus,A.& Weeley,J. (1964) Proc. Nat. Acad. Sci U.S. 51 1075-1079.

ry,R.P. (1976) Ann. Rev. Biochem. 46 605-629.

terson,J.A. (1976) Nuc. Acdi Res. 3 1427-1436.

hechter,I. (1975) Biochem. Biophys. Res. Commun. 67 [illegible].

afritz,D.A., Drysdale,J.W.,& Isselbacher,K.J. (1973)
J. Biol. Chem. 248 3220-3227.

harma,O.P.,Beezley,D.N.,& Roberts,W.K. (1976) Biochem. 15 4313-4317

hih,D.S. & Kaesberg,P.,(1973) Proc. Nat. Acad. Sci. U.S. 70
1799-1803.

Sippel,A.E.,Starrianopolous,J.G.,Schutz,G.& Fiegelson,P.
(1974) Proc. Nat. Acad. Sci. U.S. 71 4635-4639.

Taylor,J.M.& Schimke,R.T. (1974) Proc. Nat. Acad. Sci.U.S.
71 4327-4331.