THE INTRACELLULAR SITE OF SYNTHESIS OF FRUCTOSE 1,6-BISPHOSPHATASE IN RAT LIVER

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ABSTRACT

Fructose-1,6-bisphosphatase (FBPase, E.C. 3.1.3.11) has been purified from rat liver cytoplasm by a new purification procedure. Monospecific antibodies were raised to FBPase in rabbits to enable the immunochemical isolation and quantitation of FBPase in protein homogenates.

Pulse labelling of rat liver *in vivo* showed that the synthesis of FBPase amounted to 0.89% of total soluble protein synthesis. Newly synthesized FBPase was found almost entirely in liver cytoplasm in contrast to serum albumin which was associated only with microsomes, and amounted to 16.7% of total microsomal protein synthesis.

Isolated free and bound polysomes from liver synthesized almost equal amounts of FBPase when incubated *in vitro*, whereas albumin synthesis was confined to bound polysomes. Premature termination of translation led to the release of partial protein transcripts which were specifically immunoprecipitated.

Affinity-purified ^{125}I-labelled antibodies to FBPase and albumin were used to quantitate nascent protein chains on free and bound polysomes. FBPase antibody bound to both classes of polysome with almost equal affinity but albumin antibody was only associated with bound polysomes.

Poly(A)$^+$ RNA isolated from free and bound polysomes by poly(U)-Sepharose chromatography was translated in a cell-free protein-synthesizing system derived from wheat germ. RNA from both classes of polysome synthesized FBPase, but albumin was only synthesized in response to bound polysomal RNA. Some full lengths transcripts corresponding to both proteins were produced in wheat germ extracts, but a large amount of smaller molecular weight material was specifically immunoprecipitated. Total translation products from both classes of polysome also produced considerable abnormally short molecular weight material, but only full length transcripts were produced when the system was programmed with TMV RNA.

Since liver cells contain twice as many bound polysomes as free, it has been concluded that about 70% of intracellular FBPase synthesis takes place on bound polysomes. In contrast, albumin synthesis was almost totally confined (>97%) to bound polysomes and newly synthesized

albumin was segregated into microsomes. The partitioning of synthetic activity for albumin is therefore a result of the distribution of mRNA coding for this protein.

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LIST OF ABBREVIATIONS

AMP	Adenosine 5'-monophosphate
ATP	Adenosine 5'-triphosphate
BPB	Bromophenol blue
CM-	Carboxymethyl-
DE-	Diethylaminoethyl-
DNA	Deoxyribonucleic acid
cDNA	Copy DNA
DTT	Dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid
ER	Endoplasmic reticulum
FBP	D-Fructose 1,6-bisphosphate
FBPase	Fructose-1,6-bisphosphatase
F6P	D-Fructose 6-phosphate
GTP	Guanosine 5'-triphosphate
Hab	Immunoglobulin heavy chain
Hepes	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HK(x)M	50 mM Hepes, x mM KCl, 5 mM $MgCl_2$, pH 7.4
HNa(x)M	50 mM Hepes, x mM NaCl, 5 mM $MgCl_2$, pH 7.4
Lab	Immunoglobulin light chain
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
PB	Phosphate buffer (10 mM Na_2HPO_4, 15 mM NaCl, pH 7.2)
PBS	Phosphate buffered saline (10 mM Na_2HPO_4, 150 mM NaCl, pH 7.4)
PEG	Polyethylene glycol
PFK	Phosphofructokinase
PMSF	Phenylmethyl sulphonyl fluoride
poly(A)$^+$	RNA having a polyadenylate tract at its 3' terminus
poly(A)$^-$	RNA lacking a polyadenylate tract at its 3' terminus
RNA	Ribonucleic acid
mRNA	Messenger RNA
rRNA	Ribosomal RNA
tRNA	Transfer RNA
RNase	Ribonuclease
SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid
TEMED	N,N,N",N"-Tetramethylethylenediamine
TMV	Tobacco Mosaic Virus
Tris	tris(Hydroxymethyl)aminomethane

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CHAPTER ONE

INTRODUCTION AND HISTORICAL REVIEW

1.1 The intracellular sites of protein synthesis in animal cells

The liver is one of a number of tissues which exhibit a high level of protein synthesis in the adult animal. Many different proteins are produced by the hepatocyte, and these fall into two major groups, namely plasma proteins which are secreted, and proteins which are required for the internal functions of the cell. These two groups are produced in about equal amounts (Ryoo and Tarver, 1968). Examples of secretory proteins are serum albumin, the major product of liver protein synthesis (Campbell and Stone, 1957; Peters, 1962), glycoproteins (Redman and Cherian, 1972), high- and low-density lipoproteins (Bungenberg de Jong and Marsh, 1968), transferrin (Masuya et al., 1963) and fibrinogen (Straub, 1963).

The group of proteins destined to remain within the cell can be subdivided into several groups: (a) those which will become components of the endoplasmic reticulum membrane; (b) those transferred to cellular organelles after synthesis, such as mitochondrial cytochrome c and peroxisomal catalase, and (c) those which function in the cytoplasm.

One of the most striking ultrastructural features of many types of animal cells, including liver cells, is the presence of an extensive network of intracellular membranes which form the endoplasmic reticulum (ER). The general structure of the ER is that of a 'network of parallel membranes making up tubules, cisternae and vesicles, somewhat varying in diameter and form, and all enclosing spaces or channels separated from the cytoplasm' (Glaumann, 1973). A study of adjacent serial sections of the ER has demonstrated that the membranes are connected with one another to form a continuum which separates the cytoplasm from the intermembrane space of the ER (Palade, 1956; Palade and Siekevitz, 1956). Another prominant intracellular membrane structure is the Golgi apparatus which is composed of a series of flattened or rounded, smooth-surfaced cisternae, vesicles and vacuoles, often located near the bile canaliculi in hepatocytes. The Golgi complex seems to be functionally related to the ER in as much as both organelles participate in the migration of secretory proteins. In addition the intermembrane space of the developing Golgi vesicles appears to be continuous with the intermembrane compartment of the ER.

Protein synthesis in animal cells takes place on ribosomes, which are organised into polysomes by their association with messenger RNA (mRNA) during protein synthesis. Two distinct populations of polysomes are recognised in all animal cells: those bound to the ER membrane (forming the rough ER), and those that do not appear to be bound to any membrane but are free in the cytoplasm, Both classes of polysome are active in protein synthesis.

Siekevitz and Palade (1960) observed from electron microscope studies that in cells active in protein secretion a high proportion of the ribosomes were bound to the ER membrane forming an extensive rough ER. In contrast, cells which did not secrete their synthesized protein showed fewer membrane bound ribosomes, but numerous ribosomes free in the cytoplasm. Extreme examples of secretory cells are the exocrine or acinar cells of pancreas which export zymogen granules and plasma cells which are the site of immunoglobulin synthesis (Porter, 1961). In these cells most of the ribosomes are associated with membranes. The opposite extreme is found in rapidly growing embroynic cells and in de-differentiated tumour cells. Very little endoplasmic reticulum is evident in these cells and very few ribosomes seem to be associated with membrane structures. The parenchymal cells of liver are an example of a cell type intermediate between these two extremes (Porter, 1961; Porter and Bonneville, 1968). These observations have led to the concept that proteins destined for export are made on bound polysomes, and proteins retained by the cell are made on free polysomes. However, certain observations could not be accounted for by this suggestion. The increase in the synthesis of protein for the internal needs of cells occuring after partial hepatectomy (Zweig and Grisham, 1971), or whole body exposure to ionizing radiation (Ekren and Yatvin, 1972) was not accompanied by a change in the ratio of free to membrane-bound polysomes. HeLa cells appear morphologically to be de-differentiated with comparatively little endoplasmic reticulum, prominant Golgi complexes and smooth endoplasmic reticulum (Epstein, 1961; Fuse et al., 1963). Nevertheless about 15% of the ribosomes are found associated with membranes (Attardi et al., 1969), although HeLa cells export less than 2% of their newly synthesized protein (Rosbash, 1970). Andrews and Tata (1971a,b) found that brain, a non-secretory tissue, contained about 20% bound polysomes, and isolated brain microsomes released nascent protein into the incubation medium on treatment with puromycin. In contrast, liver microsomes discharged nascent protein to predominantly inside the ER membrane vesicles (presumably for transport outside the cell).

The intracellular site of synthesis has been reported for a number of proteins. Rat liver microsomes, incubated in vitro, synthesized albumin and other serum proteins (Campbell et al., 1960, van der Decken and Campbell, 1962; Ganoza and Williams, 1969). Isolated bound polysomes incubated in vitro were also found to be the major site of albumin and other serum protein synthesis (Takagi and Ogata, 1968; Redman, 1968, 1969; Takagi et al., 1969, 1970; Hicks et al., 1969; Glaumann, 1970; Tanaka and Ogata, 1972). The binding of ^{125}I-labelled anti-albumin to nascent proteins on polysomes was predominantly associated with bound polysomes (McLaughlin and Pitot, 1976a). Albumin mRNA activity was restricted to poly(A)$^+$ RNA isolated from bound polysomes, when translated in a cell-free protein-synthesizing system derived from wheat germ (Zähringer et al., 1977), or rabbit reticulocytes (Goldman and Blobel, 1978). Similarly a copy DNA (cDNA) specific for albumin mRNA confirmed the association of albumin mRNA with only bound polysomes (Yap et al., 1977). As a quantitative assay of mRNA the latter method is probably the best, since it does not rely on the relative translational efficiency of various mRNA templates, or the complete function integrity of the mRNA. A cDNA specific for immunoglobulin light chain mRNA has been used to quantitate the distribution of light chain mRNA in a mouse plastocytoma (Okuyama et al., 1977). Bound polysomes contained 3 times more light chain mRNA than free, but since this plastocytoma contained more free than bound polysomes, bound polysomes as a whole synthesized only twice as much light chain as free. Other secreted proteins shown to be predominantly synthesized by bound polysomes (by in vitro protein synthesis or quantitation of nascent protein chains), include rat liver glycoproteins (Hallinan et al., 1968), mouse myeloma immunoglobulins (Sheer and Uhr, 1970), sheep mammary gland β-lactoglobulin, α-lactalbumin, α_{s1}-, α_{s2}-, β- and κ- caseins (Gaye and Denamur, 1970; Gaye et al., 1972, 1973a,b;

Gay and Houdebine, 1975), and horse thyroid thyroglobulin (Vassart and Dumont, 1973).

A growing number of intracellular proteins appear to be synthesized on both free and bound polysomes. Incubation of isolated free and bound polysomes and microsomes or quantitation of nascent protein chains have shown no preferred site of synthesis for rat liver mitochondrial cytochrome c (González-Cadavid et al., 1974), rat liver membrane NADP-cytochrome c reductase (Ragnotti et al., 1969), rabbit reticulocyte globin (Woodward et al., 1973), rat liver ferritin (Hicks et al., 1969; Puro and Richter, 1971), and rat liver peroxisomal catalase (Higashi and Peters, 1963; Takagi et al., 1970; Kashiwagi et al., 1971; Higashi et al., 1972; Sakamoto and Higashi, 1973). Goldman and Blobel (1978) however reported that rat liver catalase and peroxisomal uricase were exclusively synthesized on free polysomes when isolated polysomes were incubated in vitro, or when isolated poly(A)$^+$ RNA was translated in a rabbit reticulocyte cell-free protein synthesizing system. Konijin et al. (1973) reported that ^{125}I-labelled anti-ferritin bound to different size classes of isolated free and bound polysomes. They postulated that each class of polysome was involved in the synthesis of the 2 separate subunits. Ferritin mRNA activity was equally abundant in both classes of polysome when isolated poly(A)$^+$ RNA was translated in vitro in wheat germ extracts (Zähringer et al., 1977). Rat liver serine dehydratase was synthesized to a greater extent by rough microsomes than free polysomes during in vitro incubations (Pitot and Jost, 1968). The induction of this enzyme by glucagon and high protein diets was associated initially with an increase in synthesis of the enzyme on bound polysomes. These results were confirmed by studies on the binding of ^{125}I-labelled antibody to nascent serine dehydratase protein chains on isolated polysomes (McLaughlin and Pitot, 1976b).

Proteins, the synthesis of which seems to be solely associated with free polysomes (when polysomes are incubated in vitro) include rat liver arginase (Tanaka and Ogata, 1971), rat brain S-100 protein (Amaldi et al., 1973), and rat skeletal muscle myosin (Nihei, 1971). Mouse myeloma mRNA for histone was found only in free polysomal RNA after pulse labelling mouse myeloma RNA in vivo, and subsequent identification of histone mRNA by sucrose gradient analysis (Zauderer et al., 1973).

In some minimal deviation rat hepatomas, which synthesize albumin yet do not secrete it, albumin synthesis is associated exclusively with free polysomes (Uenoyama and Ono, 1972; Schreiber et al., 1976; McLaughlin and Pitot, 1976a).

These findings suggest that the functional differentiation of ribosomes into two classes is not as clear-cut as was first thought. From the evidence available so far it can be concluded that proteins for export from the cell are made predominantly if not exclusively on membrane-bound polysomes, and proteins destined for intracellular use are made on both free and bound polysomes.

1.2 The segregation of secretory proteins during protein synthesis

For several groups of proteins, passage across an intracellular membrane is an obligatory step in the pathway from synthesis to enclosure within a membrane-bound compartment. Examples are, secretory proteins transferred to the intermembrane space bounded by the endoplasmic reticulum, nuclear, mitochondrial and peroxisomal proteins. Two distinct mechanisms have so far been uncovered for the transfer of proteins across intracellular membranes. In 'co-translational' transfer, passage across the membrane is tightly coupled to translation and proceeds only during synthesis of the protein by membrane-bound polysomes (Blobel and Dobberstein, 1975). In 'post-translational' transfer, passage through the membrane occurs after protein synthesis and is not mediated by a ribosome-membrane junction (Dobberstein et al., 1977). Transmembrane transport is also encountered in the entrance of toxic peptides such as abrin, ricin and some colicins into cells. In each case there are presumably specific membrane receptors that recognize the proteins to be transported, and direct them through the membrane.

However, protein synthesis on membrane-bound polysomes does not necessarily imply transport of the newly synthesized protein into the cisternae of the endoplasmic reticulum. When rough microsomes were incubated *in vitro* at least half of the newly synthesized protein was transported inside the vesicles during translation (Redman and Sabatini, 1966; Andrews and Tata, 1971b; Bevan, 1971). Secreted proteins were wholly segregated into microsomes in these experiments.

The biochemical mechanisms by which the synthesis of specific proteins is partitioned between free and bound polysomes is unknown. It is not known whether the association of ribosome and membrane is a consequence of the synthesis of a particular protein or whether the corresponding mRNA seeks out ribosome-membrane complexes prior to peptide chain initiation. It is known, however, that the 60S ribosome subunit bearing the peptidyl tRNA is bound to the endoplasmic reticulum membrane (Sabatini et al., 1966), while the 40S subunit which binds the mRNA is not. It is likely therefore, that the 60S subunit or the nascent protein chain on the polysome recognises the membrane. The 60S subunit of rat liver bound polysomes contains 4 extra proteins not found in free polysomes, and lacks 2 proteins found on free polysomes. There is no difference in 40S protein components (Ramsey and Steele, 1977).

It has been proposed that there is a channel between the large ribosomal subunit and the cisternae through which the nascent chain passes. This passage has been shown to be non-enzymatic and not dependent on any energy source (Redman, 1969). Studies with liver albumin have demonstrated that newly synthesized protein passes from the rough ER to the smooth ER to the Golgi complex, where it is probably concentrated before release into the circulation (Peters, 1962; Glaumann et al., 1968; Redman, 1969; Peters et al., 1971; Redman and Cherian, 1972).

The translation of the mRNAs for numerous secretory proteins in cell-free translation systems which lack membranes showed that typically the synthesized protein was larger (by up to 30 amino acids) at its amino terminus than the corresponding intracellular or serum form of these proteins. Milstein et al. (1972) originally proposed that these extensions might act as 'signal sequences' which might participate in the transport of nascent chains of proteins destined for secretion acorss the rough ER. Blobel and Dobberstein (1975) developed this suggestion into a detailed 'signal hypothesis'. They postulated that the translation of a unique set of codons located after the initiation codon of a mRNA resulted in a special amino terminal sequence of the nascent polypeptide chain. This signal sequence would trigger binding of the ribosome-mRNA-nascent polypeptide complex to the membrane, and the sequence would 'thread' the nascent polypeptide into the ER. This signal sequence would be proteolytically cleaved shortly after it had crossed the membrane, allowing proper folding of the emerging peptide chain.

Sequence analysis has been carried out on the amino termini of many secretory proteins synthesized in cell-free systems. These include: mouse immunoglobulin light chains (Schechter et al., 1975; Schechter and Burstein, 1976a,b; Burstein and Schechter, 1976. 1977; Burstein et al., 1976, 1977), dog pancreatic zymogens (Devillers-Thierry et al., 1975), bovine parathyroid hormone (Kemper et al., 1974, 1976), rat proinsulin (Chan et al., 1976), bovine proinsulin (Lomedico et al., 1977), fish proinsulin (Shields and Blobel, 1977), human placental lactogen (Birken et al., 1977), rat prolactin (Maurer et al., 1977), rat proalbumin (Strauss et al., 1977a,b), chick ovalbumin, lysozyme, ovomucoid and conalbumin (Palmiter et al., 1977, 1978; Thibodeau et al., 1978), and sheep α-lactalbumin, β-lactoglobulin, α_{s1}-, α_{s2}-, β-, and κ-caseins (Gaye et al., 1977; Mercier et al., 1978).

These additional amino terminal sequences were characterized by a predominance and a clustering of hydrophobic residues, two features which were consistent with the proposed role of the transient amino terminal extension in binding to presumed hydrophobic membrane receptors. The one notable exception was ovalbumin, which lacked any hydrophobic extension and did not have a naturally hydrophobic amino terminus. The simplest explanation for ovalbumin is that the normal amino terminal sequence may function as a signal: it may interact with membrane receptors in a manner analogous to other 'signal peptides' but is not cleaved during membrane transport. The finding that ovalbumin is not synthesized with a transient hydrophobic sequence indicates that a major secretory pathway in the oviduct does not require the typical 'signal peptide' that is subsequently removed. It may be that special mechanisms have evolved for secretion of ovalbumin, which represents nearly 50% of total protein synthesis in the oviduct.

The signal hypothesis predicted that proteolytic cleavage of pre-proteins would occur during or immediately following translation (Milstein et al., 1972; Blobel and Dobberstein, 1975). Several investigators have shown that processing of pre-proteins by a rough ER-associated protease occurs both in vivo and in vitro, yielding nascent peptides with the amino terminal sequence of the mature protein or pro form (Blobel and Dobberstein, 1975; Schmekpeper, 1975; Campbell and Blobel, 1976; Habener et al., 1976; Lingappa et al., 1977; Birken et al., 1977; Jackson and Blobel, 1977; Shields and Blobel, 1977; Shields and Blobel, 1978). Post-translational cleavage was not obtained when completed pre-proteins were incubated with intact microsomal vesicles. However the isolated membrane proteases cleaved pre-proteins when added either co- or post-translationally.

The sequencing of 'signal peptides' of several secretory proteins from a single tissue has provided additional information on the nature of such sequences. In ovine mammary gland the α_{s1}-, α_{s2}-, and β-casein signal peptides show a high degree of homology, but those of α-lactalbumin, β-lactoglobulin and κ-casein are significantly different in length and sequence. Cleavage of the pre-protein to the secreted analogue involves the cleavage of an alanyl-(arginyl, lysyl, glutaminyl, glutyl or isoleucyl) bond. The cleavage site is obviously a signal for the specific removal of the amino terminal extension, although other factors must play a role since secreted lactoproteins contain several peptide bonds of the above type. A comparison of the precurosr peptides of chick oviduct proteins lysozyme and ovomucoid indicate 6 identities in the sequence of amino acids preceding the cleavage site, but this homology is not shared with conalbumin. The amino acids at the cleavage sites are alanyl-alanine (pre-conalbumin), glycyl-lysine (pre-lysozyme), and glycyl-alanine (pre-ovomucoid), and suggest that the protease(s) involved have something of an elastase-like specificity.

In some minimal deviation rat hepatomas, albumin is synthesized on free polysomes but not secreted (Uenoyama and Ono, 1972; Schreiber et al., 1976; McLaughlin and Pitot, 1976a). Albumin mRNA from hepatoma 5123TC directed the synthesis in vitro of a protein containing a pre-piece identical to that of the normal rat liver pre-proalbumin (Strauss et al., 1977c). These results suggest that some other component for binding albumin synthesizing polysomes to the rough ER is deficient in this hepatoma, even though the ultrastructure of the hepatoma is similar to that of liver obtained from the host animal.

The rat liver peroxisomal enzymes catalase and uricase are synthesized entirely on free polysomes (Goldman and Blobel, 1978) and are not co-translationally segregated into microsomes. These results were surprising because ultrastructural studies had shown that peroxisomes bud from the ER (Novikoff and Shin, 1964). Transfer of peroxisomal proteins across the membrane must therefore be by a post-translational mechanism. Although the possibility of a precursor was not resolved, such a precursor was considered likely by these authors, since malate dehydrogenase, a glyoxysomal enzyme (the plant equivalent of peroxisomes) was synthesized in wheat germ extracts as a precursor (Walk and Hock, 1978).

1.3 Objectives of this investigation

The aim of this project was to investigate the subcellular site of synthesis of a rat liver cytoplasmic enzyme. The reported variability of the synthetic capacity of free and bound polysomes for specific intracellular proteins has not provided enough information to enable any conclusions to be made regarding the segregation of specific messengers coding for this class of protein.

Previous investigations of two rat liver cytoplasmic enzymes, arginase (Tanaka and Ogata, 1971) and serine dehydratase (Pitot and Jost, 1968; McLaughlin and Pitot, 1976b) demonstrated that arginase was synthesized predominantly on free polysomes, and serine dehydratase on both classes of polysome. However, only with the latter enzyme were several independent techniques used to identify enzyme synthesis on the two polysome types.

Fructose-1,6-bisphosphatase (FBPase, E.C. 3.1.3.11) was chosen for further work, as this enzyme plays an essential role in gluconeogenesis, a major function of liver *in vivo*.

Several different techniques were utilized to quantitate the synthesis of FBPase:

(a) The rate of enzyme synthesis during pulse labelling *in vivo* and the subcellular distribution of newly synthesized enzyme were studied.

(b) Isolated free and bound polysomes were tested for their capacity to synthesize FBPase *in vitro*.

(c) The binding of affinity-purified, ^{125}I-labelled anti-FBPase to nascent polysomal protein chains was investigated.

(d) Poly(A)$^+$ RNA isolated from free and bound polysomes was translated *in vitro* in a cell-free protein-synthesizing system derived from wheat germ, and its capacity for FBPase synthesis was investigated.

Albumin synthesis was studied in conjunction with FBPase, as the distribution and site of synthesis of this protein are well documented.

A preliminary attempt to purify the mRNA for FBPase by immunochemical isolation of polysomes was also made.

1.4 The discovery and isolation of fructose-1,6-bisphosphatase

A specific phosphatase, catalysing the hydrolysis of fructose 1,6-bisphosphate (FBP) was first reported in 1943 by Gomori. The enzyme was separated from other phosphatases in liver and kidney extracts from several mammalian species and was shown to require a divalent cation such as Mg^{2+} or Mn^{2+} for activity. The importance of a specific FBPase in carbohydrate metabolism was not recognized until several years after Gomori's original isolation of the enzyme. Previously phosphatases were generally regarded as primarily catabolic enzymes. The enzyme was shown to specifically hydrolyze the 1-phosphate group of FBP to yield fructose 6-phosphate (F6P) (Roche and Bouchilloux, 1950; Pogell and McGilvery, 1952), and a role in mammalian fructose metabolism was established (Hers and Kusaka, 1953; Leuthardt et al., 1953). A specific key role in gluconeogenesis was proposed by Krebs (1954), and an increase in enzyme activity was found under gluconeogenic conditions in rabbit liver (Mokrasch et al., 1956).

The discovery of FBPase activity in skeletal muscle (Salas et al., 1964; Krebs and Woodford, 1965) was unexpected, since its function was through to be confined to gluconeogenesis. It has since been found in the muscles of a wide variety of animals (Opie and Newsholme, 1967a,b; Newsholme et al., 1972), and the activity in many of these tissues is remarkably high, suggesting that the enzyme has a special function in these tissues.

The enzyme preparation described by Gomori was characterized by an alkaline pH optimum and showed little or no activity in the neutral pH range. Similar pH-activity profiles were later reported for partially-purified enzyme preparations from rabbit liver (Pogell and McGilvery, 1954; Mokrasch and McGilvery, 1956) and pig kidney (Mendicino and Vasarhely, 1963), and for crystalline enzyme from rabbit liver (Pontremoli et al., 1965). Although these results indicated that an alkaline pH optimum was a common property of mammalian FBPases, evidence was accumulating to suggest that this pH optimum was not an intrinsic property but rather was a result of structural modification of the enzyme during the isolation procedure. In the Gomori isolation procedure, tissue was extracted for 3 days at $0^{o}C$ at pH 3.5 followed by incubation of the extract at the same pH for 6 hours at 37°C. During the autolysis at 37°C, the enzyme activity at pH 9.2 increased severalfold while the activity at pH 7 decreased (Pogel and McGilvery, 1952; Hers and Kusaka, 1953; Byrne et al., 1961). This change was attributed to the action of proteolytic enzymes, and a similar activation at alkaline pH was observed when extracts were incubated with papain (Pogell and McGilvery, 1952) or with a particulate fraction from rabbit liver (Pogell, 1961).

Improved purification procedures employing heat steps at neutral pH to destroy proteolytic activity, and biospecific elution of FBPases from phospho- or CM-cellulose have enabled the isolation of undegraded FBPases with neutral pH optima from a variety of mammalian tissues: pig kidney (Marcus, 1967; Mendicino et al., 1968), ox liver (Nimmo and Tipton, 1975), sheep liver (Chang et al., 1974), rabbit liver (Traniello et al., 1971; Carlson et al., 1973), rabbit kidney (Tashima et al., 1972), rabbit muscle (Black et al., 1972), rat liver (Traniello, 1974; Tejwani et al., 1976; Riou et al., 1977). FBPase has recently been reported in brain and has been purified from this source (Majumder and Eisenberg, 1977).

1.5 Physical and enzymic properties of fructose-1,6-bisphosphatase

All mammalian FBPases isolated to date appear to be composed of 4 identical subunits, 30 000-40 000 daltons in subunit molecular weight. Preliminary X-ray diffraction studies on the rabbit liver enzyme confirmed the subunit identity (Soloway and McPherson, 1978). Rabbit liver and kidney enzymes have been shown to be identical by immunological techniques (Enser et al., 1969) and peptide analysis (Horecker et al., 1975). The muscle enzyme on the other hand showed little antigenic cross-reaction and yielded distinctly different peptide patterns. Similarly rat liver and kidney enzymes were shown to be immunologically identical and distinct from the rat muscle enzyme (Tejwani et al., 1976). Antisera against purified brain enzyme was shown to quantitatively precipitate FBPase activity from brain, liver and kidney (Majumder and Eisenberg, 1977). Blocked amino termini have been found in FBPases from livers of ox (Byrne et al., 1971; Nimmo and Tipton, 1975), rat (Tejwani et al., 1976) and rabbit (Pontremoli et al., 1973, 1975). In the case of rabbit liver, the amino terminal residue has been identified as acetylalanine (El-Dorry et al., 1977).

Rabbit liver FBPase is another in a growing list of proteins having acetylated amino termini (Jörnvall, 1975) and conforms to the general pattern of having alanine, glycine, methionine, serine or threonine as the amino terminal residue. In the present case the presence of the blocking group cannot be attributed to the need to stabilize the enzyme against the action of intracellular proteases, since FBPases containing blocked amino termini are still very sensitive to proteolytic cleavage. Such a function has been implicated for α-crystallin from calf lens cells, whose half-life is several decades in a tissue with a high level of aminopeptidase (Bloemendal, 1977). Thus the function of the blocking group in FBPases remains unknown. The acetylation of α-crystallin and ovalbumin has been shown to take place on the nascent polypeptide from an acetyl coenzyme A donor (Berns et al., 1972a,b, 1973; Granger et al., 1976; Smeets et al., 1977; Palmiter et al., 1978).

FBPases from liver, kidney and muscle are highly sensitive to inhibition by adenosine 5'-monophosphate (AMP) and FBP (Krebs, 1963; Mendicino and Vasarhely, 1963; Tageta and Pogell, 1963, 1965; Underwood and Newsholme, 1965b; Gancedo et al., 1965) and this is considered to be one of the essential control mechanisms in the regulation of gluconeogenesis (Atkinson, 1966; Horecker et al., 1966). However AMP was without effect on the brain enzyme (Majumder and Eisenberg, 1977). Zn^{2+} has been shown to be an allosteric regulator of liver and muscle enzymes, and the commonly observed activation at neutral pH by EDTA, histidine and other chelators is due to the removal of endogenous Zn^{2+} by these agents (Tejwani et al., 1976; Frey et al., 1976). The physiological significance, if any, of inhibition by Zn^{2+} remains to be established. The fact that it is reversed by concentrations of histidine found in livers of fasting rabbits (Pontremoli et al., 1974) suggests that the inhibition by Zn^{2+} and its reversal by histidine may play a role in gluconeogenesis. Oleate and other fatty acids have been reported to activate rabbit liver enzyme (Baxter et al., 1972; Carlson et al., 1973, 1975). Ca^{2+} has been reported to be a potent inhibitor of rat liver and rabbit skeletal muscle FBPases (Van Tol et al., 1972; Tejwani et al., 1976).

1.6 Metabolic regulation by fructose-1,6-bisphosphatase in vivo

Subcellular fractionation studies revealed that FBPase was present only in rat liver cytoplasm (Hers et al., 1951). In isolated suspended liver cells evidence has been obtained for the simultaneous action of glycolysis and gluconeogenesis (Katz and Jungermann, 1976), the net process being dependent on relative substrate concentrations. This mode of action of liver cells, and the histochemically determined heterogeneous distribution of glucose-6-phosphatase (Sasse et al., 1975) and of glycogen metabolism between periportal and perivenous zones of liver parenchyma (Sasse, 1975), led to the proposal of metabolic zonation of the organ. It was proposed that in the periportal zone, glucose formation by gluconeogenesis and glycogenolysis would be the predominant process, while in the perivenous zone glycolysis and lipogenesis should prevail. The further demonstration that phosphoenolpyruvate carboxykinase (Guder et al., 1976) and FBPase (Katz et al., 1977) were predominantly located in the periportal region and pyruvate kinase (Guder et al., 1976) and glucokinase (Katz et al., 1977) in the perivenous zone lent support to this hypothesis.

A major effect of glucagon *in vivo* is the mobilization of liver glycogen to maintain blood glucose levels. It has been reported that the activity of, and the flux through pyruvate kinase, glucokinase, phosphofructokinase (PFK) and FBPase is affected by glucagon (Taunton et al., 1974; Clark et al., 1974; Pilkis et al., 1976; Riou et al., 1976; Feliu et al., 1976; Rognstad and Katz, 1976; Blair et al., 1976; Dunn et al., 1976). Within minutes of an intravenous infusion of glucagon in humans (Greene et al., 1974) or rats (Taunton et al., 1972), the activities of these enzymes increased. Glucagon and insulin had opposite effects on these enzyme activities. Furthermore, the glucagon-induced changes were completely reversed within 3 minutes of insulin infusion. In rats infusion of cyclic AMP caused changes similar to those seen after glucagon administration (Greene et al., 1974). The mechanism whereby these enzyme activities are altered by glucagon is not known. It is conceivable that in addition to the activation of liver phosphorylase, certain other key enzymes are activated or inactivated by a similar mechanism. Both pyruvate kinase and PFK are phosphorylated *in vitro* by the catalytic subunit of cyclic AMP-dependent protein kinase (Brand and Soling, 1975; Titanji et al., 1976; Riou et al., 1977), however neither enzyme has yet been shown to be phosphorylated *in vivo*. Rat liver FBPase on the other hand was shown to be phosphorylated *in vivo* and *in vitro*, the latter phosphorylation being associated with a 40% increase in enzyme activity (Riou et al., 1977). Enzyme phosphorylation both *in vivo* and *in vitro* was specific for a single serine in each subunit.

In rabbits and rats, the administration of adrenal glucocorticoid hormones causes an increased synthesis of glucose in liver, accompanied by increases in the levels of a number of gluconeogenic enzymes including FBPase (Kvam and Parks, 1960; Weber et al., 1961). These increases in activity were prevented by inhibitors of protein synthesis (Kvam and Parks, 1960; Weber et al., 1962. 1964a,b; Shrago et al., 1963). Diets with low carbohydrate content (Mokrasch and McGilvery, 1956; Friedland and Harper, 1959), fasting or alloxan diabetes (Weber et al., 1965) also caused increaes in levels of FBPase in liver. However the induction of FBPase in rat liver by dietary and hormonal manipulation has been disputed (Zalitis and Pitot, 1977).

The proteolysis of FBPase during isolation noted earlier, has been shown to be associated with lysosomal enzymes (Pontremoli et al., 1973; Nakashima and Ogino, 1974). A number of observations suggested that similar proteolytic modifications might be associated with gluconeogenic conditions. Traces of proteolysis were found in enzyme purified from livers of rabbits exposed to cold, fasting, triamcinolone or alloxan. Under such conditions, a 2- to 3-fold increase in FBPase levels was noted (Pontremoli et al., 1974, 1975). However, it was not demonstrated whether enzyme proteolysis was an *in vivo* or an *in vitro* phenomenon associated with enzyme purification. Enzyme proteolysis was only associated with an increase in activity at alkaline pH and *not* with an increase at neutral pH.

When two enzymes, catalysing opposing non-equilibrium reactions are present in the same cell compartment, substrate ('futile') cycling is a possibility. The was first considered for liver with respect to PFK and FBPase (Cahill et al., 1959). Hepatic PFK was shown to be ihnibited by ATP. citrate and this inhibition was relieved by AMP, FBP, NH_4^+ and phosphate (Underwood and Newsholme, 1965a), whereas FBPase was inhibited by AMP and FBP (Underwood and Newsholme, 1965b). On the basis of these properties it was proposed that the changes in the activity of one enzyme would lead to a change in the concentration of FBP that would cause an opposite change in the activity of the other enzyme. Atkinson (1966) drew an analogy with an electronic flip-flop, and summarized the current view of the problem of the simultaneous activity of the two enzymes: 'Simultaneous activity of these two enzymes would obviously short-circuit the cell's energy metabolism by catalysing the hydrolysis of ATP'.

The flip-flop mechanism had to be abandoned when experiments with kidney-cortex slices revealed that fatty acids or ketone bodies could inhibit glycolysis without any change in the intracellular concentration of FBP or adenine nucleotides. This inhibition was explained by the increased concentration of citrate (Newsholme and Underwood, 1966; Underwood and Newsholme, 1967), leading to an increased net rate of FBP hydrolysis, and hence an increase in the rate of gluconeogenesis, i.e. a substrate cycle existed between F6P and FBP. Gevers and Krebs (1966) obtained isotopic evidence for the existence of this cycle in pigeon liver homogenates.

The significance of substrate cycles in providing sensitivity and flexibility in metabolic control was first discussed in a review by Newsholme and Gevers (1967). However the significant find in relation to the development of the theory was the discovery of high activities of FBPase in insect flight muscle (Newsholme and Crabtree, 1970). This tissue is characterised by high rates of glycolysis during mechanical activity. Consequently, the control mechanism for F6P phosphorylation has to be very sensitive and capable of a high degree of amplification, so that it was proposed that greater amplification of regulatory signals was obtained through the F6P-FBP substrate cycle. Additionally, this substrate cycle in insect flight muscle has been implicated in the maintenance of thoracic temperature by the controlled hydrolysis of ATP (Newsholme et al., 1972; Newsholme and Crabtree, 1973, 1976).

However there is still great debate concerning this recycling in the normal liver, where the predominant direction of metabolism lies toward gluconeogenesis (Kneer et al., 1974; Hue and Hers, 1974a,b; Clark et al., 1975; Katz et al., 1975; Rognstad et al., 1975). The evidence for recycling based on isotopically labelled substrates (Clark et al., 1973, 1974; Kneer et al., 1974) has been subject to criticism (Hue, 1976). Other evidence against any important recycling was the demonstration that [1-^{14}C]glucose (Cook and Lorber, 1952; Hers, 1955), and [1-^{14}C]galactose (Topper and Stetten, 1951) were converted into [1-^{14}C]glycogen without specific racemization of carbon between positions 1 and 6. The preferred anomeric specificity of PFK and FBPases pose a constraint on any recycling, since their preferred substrates are of the opposite anomeric configuration (β-D-fructose 6-phosphate for PFK; α-D-fructose 1,6-bisphosphate for FBPase). This means that α-D-fructose 6-phosphate produced by FBPase will be a potentially better substrate of glucose-6-phosphate isomerase than it will be for PFK (Shray et al., 1973; Benkovic et al., 1974).

CHAPTER TWO

GENERAL METHODS

2.1 Introduction

Unless otherwise stated, all chemicals were of reagent-grade quality. Aqueous solutions were made up in deionized glass-distilled water. Buffers were cooled to the temperature of use before being adjusted to the required pH.

In experiments involving RNA, glassware was rinsed in 0.1% diethylpyrocarbonate and dried at 130°C. Solutions were treated with diethylpyrocarbonate at 0.1% final concentration at least 24 hours before use.

2.2 Experimental animals

Sprague-Dawley rats were obtained from the Small Animal Production Unit at Massey University. Animals were maintained at 23°C in a room illuminated from 6.00 a.m. to 6.00 p.m. daily. Food and water were available *ad libitum*. Rabbits (New Zealand White) were from the Small Animal Production Unit, and sheep (Romney) were from the Animal Physiology Unit at Massey University.

2.3 Centrifugation

Ultracentrifugation was carried out on a Beckman Model L2-65B or L5-50 ultracentrifuge. Low speed centrifugation was performed in a Sorvall RC-2B centrifuge. All centrifugal forces are quoted as $\underline{g}_{max}$ values.

2.4 Ultrafiltration

Protein solutions were concentrated by ultrafiltration in a 50 cm^3 cell (ChemLab Instruments, Ilford, Essex) fitted with an XM-50 membrane (Amicon, Lexington Massachusetts).

2.5 Spectrophotometry

All spectrophotometric determinations were made on a Unicam SP 500 Series 2 recording spectrophotometer.

2.6 Protein and RNA determinations

Protein concentrations were determined by the Biuret procedure (Gornall et al., 1949), by protein dye binding using Coomassie Blue G 250 (Bradford, 1976), or by ultraviolet absorbance measurement (see text). Bovine serum albumin was used as the standard protein in the Biuret and protein dye binding procedures. Solutions of bovine serum albumin were prepared in 0.01 M phosphate buffer, pH 7.2 and standardized assuming an $A^{1\%}_{1\ cm}$ at 279 nm of 6.67 (Foster and Sherman, 1956).

RNA determinations were all made by absorbance measurement (see text).

2.7 Polyacrylamide gel electrophoresis of proteins

Polyacrylamide gel electrophoresis was carried out in 0.7 x 10 cm tubes and on 13 x 15 x 0.15 cm slabs.

2.7.1 Discontinuous non-denaturing gels

These were prepared essentially as described by Davis (1964).

RESOLVING GEL

Sufficient acrylamide and N,N'-methylene-bis-acrylamide to give the desired gel concentration and an acrylamide to bis-acrylamide ratio of 30:1 were dissolved in small pore buffer [0.48 M HCl, Tris to pH 8.9](12.5 cm^3 per 100 cm^3), N,N,N',N'-tetramethylethylenediamine [TEMED](50 mm^3 per 100 cm^3), and water to give the required volume.

STACKING GEL

Sufficient acrylamide and bis-acrylamide to give a gel concentration of 2.5% and an acrylamide to bis-acrylamide ratio of 4:1 were dissolved in large pore buffer [0.495 M Tris, pH to 6.7 with HCl](12.5 cm^3 per 100 cm^3), TEMED (50 mm^3 per 100 cm^3), sucrose (1.0 g per 100 cm^3), and water to give the required volume.

POLYMERIZATION

Gels were polymerized with ammonium persulphate (100 mg per 100 cm^3) which was dissolved in the gel solution just before pouring.

ELECTRODE RESERVOIR BUFFER

Electrode reservoir buffer [38.5 mM glycine, Tris to pH 8.3] was used in both upper and lower reservoirs, with the addition of bromophenol blue (5 cm^3 of 0.001% per 100 cm^3) to the upper reservoir.

SAMPLE PREPARATION

Protein samples (5-150 μg in up to 150 mm^3) were applied in a solution containing 20% glycerol and the same ionic components as the stacking gel. Samples were applied by layering between the upper reservoir buffer and the stacking gel.

2.7.2 Sodium dodecyl sulphate continuous denaturing gels

RESOLVING GEL

Sufficient acrylamide and bis-acrylamide to give the desired gel concentration and an acrylamide to bis-acrylamide ratio of 30:1 were dissolved in TEMED (50 mm^3 per 100 cm^3), and denaturing buffer [0.1 M Tris, 0.1 M glycine, 0.1% sodium dodecyl sulphate (SDS)] to give the required volume.

POLYMERIZATION

Gels were polymerized with ammonium perulphate (100 mg per 100 cm^3), which was dissolved in the gel solution just before pouring.

ELECTRODE RESERVOIR BUFFER

Denaturing buffer (see above) was used in both upper and lower reservoirs.

SAMPLE PREPARATION

Protein samples (5-150 μg in up to 100 mm^3) were made up in a solution containing 1% SDS, 1% dithiothreitol (DTT), 6 M urea, bromophenol blue (5 mm^3 of 0.001% per 100 mm^3), and the same ionic components as the resolving gel. Before application to the gel, the sample was heated to 100°C for 5 minutes to denature the protein.

Electrophoresis

Electrophoresis was performed at room temperature. For disc gels a current of 2 mA per tube was maintained until the dye front had entered the resolving gel, when the current was increased to 4 mA per tube for the remainder of the run. At the conclusion of the run, gels were removed from the tubes and the dye front marked by insertion of a thin piece of wire into the gel.

Slab gels were electrophoresed at 50 V until the dye front had entered the resolving gel after which time the voltage was increased to 150 V for the remainder of the run.

Gel staining

Gels were stained for 1 hour at $50^{o}C$ in freshly prepared 0.125% Coomassie Brilliant Blue R 250, in glacial acetic acid-methanol-water (1:9:10, v/v/v). Gels were destained for several hours at $50^{o}C$ in the same solvent and stored in 7% acetic acid.

Gel scanning

Stained gels were scanned at 600 nm on a Beckman ACTA III spectrophotometer fitted with a gel scanning attachment.

2.8 Estimation of protein subunit molecular weights

Subunit molecular weights of proteins were estimated from SDS gels calibrated with proteins of known subunit molecular weight (Weber and Osborn, 1969; Darnall and Klotz, 1972). Mixtures of standard proteins (each 10 μg) were electrophoresed on gels and stained. The migration of each protein relative to the bromophenol blue dye front was plotted against log subunit molecular weight. The subunit molecular weight of unknown proteins was estimated by interpolation. Standards used were: bovine serum albumin (68 000), rat serum albumin (65 000), bovine liver catalase (60 000), rabbit gamma globulin heavy chain (50 000), hen ovalbumin (43 000), bovine pancreas carboxypeptidase A (34 600), rabbit gamma globulin light chain (23 500) bovine β-lactoglobulin (18 400), TMV coat protein (17 500), horse heart myoglobin (17 200), and bovine α-lactalbumin (15 500).

2.9 Polyacrylamide gel electrophoresis of RNA

2.9.1 Continuous non-denaturing gels

These were prepared according to Loening (1967). Electrophoresis was carried out in 0.6 x 10 cm quartz tubes with rubber rings inserted into the bases to prevent the soft gels from sliding out. Acrylamide and bis-acrylamide (electrophoresis grade) were obtained from Biorad Laboratories, Richmond, California. High purity reagents were necessary as detection of the RNA was by direct scanning of the gels *in situ* at 280 nm.

RESOLVING GEL

Sufficient acrylamide and bis-acrylamide to give a gel concentration of 2.4% and an acrylamide to bis-acrylamide ratio of 20:1 were dissolved in resolving gel buffer [0.4 M Tris, 0.2 M sodium acetate, 0.02 M EDTA, pH to 7.8 with acetic acid](10 cm^3 per 100 cm^3), TEMED (80 mm^3 per 100 cm^3), and water to give the required volume.

POLYMERIZATION

After degassing for 20 seconds, gels were polymerized by the addition of ammonium persulphate (800 mm^3 of 10% per 100 cm^3). Water was carefully layered over the gel solution after pouring into the quartz tubes.

ELECTRODE RESERVOIR BUFFER

Electrode reservoir buffer [0.4 M Tris, 0.02 M sodium acetate, 2 mM EDTA, 0.2% SDS, acetic acid to pH 7.8] was used in both upper and lower reservoirs.

SAMPLE PREPARATION

RNA samples (5-100 µg in up to 100 mm^3) were dissolved in 1:10 diluted reservoir buffer containing 10% glycerol, and heated to 65°C for 1 minute to denature the RNA (Groner et al., 1977).

ELECTROPHORESIS

Gels were electrophoresed for 3 hours at 5 mA per tube before application of samples. After sample application, gels were electrophoresed for 90 minutes at 5 mA per tube.

GEL SCANNING

After electrophoresis, gels were immediately scanned at 280 nm on a double beam gel scanner (ISCO Model 1310).

2.9.2 Formamide denaturing gels

These were prepared according to Pinder et al. (1974) and run in an apparatus employing reversible external electrodes as described by the authors. Formamide was deionized by stirring 50 cm^3 with 2 g of mixed-bed ion-exchange resin (Amberlite MB-1, BDH, Poole, Dorset) for 2 hours, during which time the conductivity dropped from 500 to 3 μmho.

RESOLVING GEL

Gels were prepared by dissolving 3.4 g of acrylamide, 0.6 g of bis-acrylamide, 0.368 g of diethylbarbituric acid and 240 mm^3 of TEMED in deionized formamide. The solution was adjusted to an apparent pH of 9.0 (glass/calomel electrodes) with 1 M HCl and made up to 100 cm^3 with formamide.

POLYMERIZATION

Gels were polymerized by the addition of freshy prepared ammonium persulphate (0.8 cm^3 of 18% per 100 cm^3). Gels were overlaid with 70% aqueous formamide. After polymerization, the aqueous formamide was replaced with 1 cm of 0.02 M diethylbarbituric acid in formamide at an apparent pH of 9.0 (adjusted with 1 M NaOH).

RESERVOIR BUFFER

In both upper and lower reservoirs 0.02 M sodium chloride was used.

SAMPLE PREPARATION

RNA samples (10-100 μg in up to 100 mm^3) were dissolved in 0.02 M diethylbarbituric acid in formamide (pH 9.0) also containing 5% glycerol and 0.0005% bromophenol blue. Samples were applied by layering between the gel and buffered formamide.

ELECTROPHORESIS

Gels were electrophoresed at 5 mA per tube until the bromophenol blue dye front had almost reached the bottom of the tube.

GEL STAINING

Gels were stained for 1 hour in 0.2% Toluidine Blue O (Eastman Kodak, Rochester, New York) in 0.4 M acetate buffer, pH 4.7, and destained in water (Peacock and Dingman, 1967).

2.10 Determination of radioactivity

Beta emitors were determined by liquid scintillation spectrometry in a Beckman Model LS-350 scintillation spectrometer. Aqueous samples were counted in Triton X-100-toluene (1:2,v/v), containing 0.4% 2,5-diphenyloxazole (PPO) and 0.01% 1,4-di-(2-(5-phenyloxazoyl))benzene (POPOP). Non-aqueous samples were counted in toluene containing 0.4% PPO and 0.01% POPOP. Counting efficiency was determined by automatic external standardization.

Gamma emitors were measured on either a Packard Model 5258 or an LKB-Wallac Model 1280 scintillation spectrometer.

2.10.1 Gel slices

Disc gels were sliced into 2 mm sections with a multiple razor blade cutter. Each slice was transferred to a scintillation vial and 1 cm^3 of concentrated ammonia-30% hydrogen peroxide (1:99, v/v) was added. Vials were loosely capped and incubated at 60°C overnight, after which time the gels had completely dissolved. Radioactivity was determined by liquid scintillation spectrometry after the addition of 10 cm^3 of Triton-toluene scintillation fluid.

2.10.2 Trichloroacetic acid-precipitable protein

Incorporation of radioactivity into trichloroacetic acid (TCA) insoluble products was determined by plating aliquots onto 2 x 2 cm squares of Whatman 3MM paper. Paper squares were dried under infrared lamps and then washed sequentially in the following solvents (10 cm^3 per square): 30 minutes at 0°C in 7.5% TCA, 10 mM methionine, and then for 10 minutes in TCA-methionine, in TCA-methionine at 100°C, in TCA-methionine, in ethanol, in ethanol-ether (1:1, v/v), and finally in ether. Air-dried squares were placed in scintillation vials and counted in 10 cm^3 of toluene scintillation fluid.

CHAPTER THREE

PURIFICATION OF FRUCTOSE-1,6-BISPHOSPHATASE FROM RAT LIVER

3.1 Introduction

A procedure for the purification of FBPase from rat liver has been published by Traniello (1974). This procedure was attempted but was found difficult to reproduce and did not give pure enzyme. A new purification procedure was developed incorporating a heat step at neutral pH to inactivate proteases, and a biospecific elution of the enzyme from phosphocellulose with fructose 1,6-bisphosphate. These two steps have been used extensively in the purification of mammalian FBPases. This purification scheme gave highly-purified, homogeneous enzyme in good yield.

During the course of this work, a further purification scheme was published by Tejwani et al. (1976), which had also been developed in response to the non-reproducibility of the Traniello procedure. In this purification, FBPase was eluted from CM-cellulose with fructose 1,6-bisphosphate. Highly-purified enzyme was obtained of specific activity comparable to that reported in this work.

Glucocorticoid hormones, particularly triamcinolone, and alloxan diabetes have been reported to increase FBPase levels in rat liver (Kvam and Parks, 1960; Weber et al., 1961, 1964). However these findings have been disputed (Monder and Coufalik, 1972; Zalitis and Pitot, 1977). Both these treatments were investigated as a possible means of increasing FBPase levels in liver to facilitate purification.

3.2 Materials

Glucosephosphate isomerase (E.C. 5.3.1.9, yeast), glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49, Torula yeast), $NADP^+$ (sodium salt, yeast), D-fructose 1,6-bisphosphate (tetrasodium salt), triamcinolone (9α-fluoro-11β, 21-dihydroxy-16α, 17α-isopropylidenedioxypregna-1,4-diene-3,20-dione), phenylmethyl sulphonyl fluoride (PMSF) and alloxan were obtained from the Sigma Chemical Company, St Louis, Missouri.

Phosphocellulose (P11) was obtained from Whatman Limited, Maidstone, Kent. The dry powder was stirred into 15 volumes of 0.5 M NaOH and left for 30 minutes. The fines were decanted and the resin washed with water on a sintered glass funnel until the filtered effluent reached pH 8. The resin was then stirred into 15 volumes of 0.5 M HCl and left 30 minutes, then washed with water until the filtered effluent was near neutral. The resin was discarded after use.

3.3 Methods

3.3.1 Assay of fructose-1,6-bisphosphatase

Fructose-1,6-bisphosphatase was assayed spectrophotometrically by following the rate of reduction of $NADP^+$ at 340 nm in the coupled enzyme system of Traniello (1974). Coupling enzyme concentrations were optimized according to McClure (1969).

The reaction mixture of 1.0 cm^3 contained: 0.04 M Tris-HCl, pH 7.4, 0.1 mM $NADP^+$, 0.1 mM D-fructose 1,6-bisphosphate, 0.1 mM EDTA, 2 mM $MgCl_2$, 6 units of glucosephosphate isomerase and 1 unit of glucose-6-phosphate dehydrogenase. The concentration of ammonium sulphate introduced with the coupling enzymes was 9.1 mM [rat liver FBPase has been shown to be activated by monovalent cations, especially ammonium ion (Tejwani et al., 1976)]. The reaction was started by the addition of enzyme. One unit of enzyme activity was defined as that amount that catalyses the formation of 1 μmole of product per minute at 30°C.

3.3.2 Purification of fructose-1,6-bisphosphatase

Female rats, 200-300 g were stunned and killed by cervical dislocation. The livers were removed, rinsed in water and stored on ice. All further operations were performed at 4°C. The bulked livers were blotted dry and minced in a chilled stainless-steel tissue press. After weighing the minced livers were homogenized in 2.2 volumes of 0.25 M sucrose, 1 mM Tris HCl, 0.1 mM EDTA, 1 mM PMSF, pH 7.0 with 5 strokes of a Potter-Elvehjem homogenizer (clearance 0.23 mm, rotating at 1700 rpm). The homogenate was centrifuged for 30 minutes at 35 000 *g* (Sorvall SS-34 rotor, 17 000 rpm). The supernatant was filtered through 'Miracloth' to trap fat particles, diluted 3-fold with 0.1 mM EDTA, 2 mM $MgCl_2$, and the pH adjusted to 7.3 with Tris base.

The diluted homogenate was heated to 60°C in an 80°C water bath and incubated at 60°C for 2 minutes. Batches of 500 cm^3 were treated in this fashion and then quickly cooled to 4°C. The solution was brought to pH 6.3 with acetic acid, and centrifuged for 10 minutes at 14 000 g (Sorvall GSA rotor, 9500 rpm).

The heat-treated supernatant was adjusted to 2 mM EDTA and to pH 6.8 with Tris base. Wet phosphocellulose (100 g dry weight per 200 g of liver) was added, the pH being kept at 6.8 with Tris base. The dilute slurry was gently stirred for 40 minutes, the pH being adjusted to 6.8 as necessary, and centrifuged for 10 minutes at 4470 g (Sorvall HG-4L rotor, 4000 rpm). The phosphocellulose was discarded, and the enzyme-containing supernatant diluted with 1.5 volumes of 0.1 mM EDTA, and the pH adjusted to 6.3 with acetic acid. Fresh phosphocellulose (the same amount as used previously) was added, the pH being kept at 6.3. The slurry was stirred for 40 minutes, after which time almost all the enzyme had adsorbed onto the resin. The phosphocellulose was collected by centrifugation and resuspended in 20 mM Tris-acetate, 0.1 mM EDTA, 0.1 mM 2-mercaptoethanol, pH 6.3 (10 cm^3 per g of phosphocellulose), and resedimented. The resin was suspended in the minimum volume of 65 mM Tris-acetate, 0.1 mM EDTA, 0.1 mM 2-mercaptoethanol, pH 6.3, and deaerated for 15 minutes. The phosphocellulose was packed under gravity into a 5 x 50 cm column and washed overnight with 2500 cm^3 of 65 mM Tris-acetate buffer (flow-rate 2-3 cm^3 per minute).

The enzyme was eluted with 2 mM D-fructose 1,6-bisphosphate in 65 mM Tris-acetate buffer (2 cm^3 per gram of liver). High specific activity fractions were combined, brought to 20 mM $MgCl_2$ and pH 7.5, and allowed to stand 30 minutes at room temperature.

The enzyme was precipitated by overnight dialysis against 8 volumes of saturated ammonium sulphate. pH 7.0. The precipitate, when stored as a suspension in 80% ammonium sulphate at 4°C was stable for many months.

3.3.3 Treatment of animals with triamcinolone and alloxan

Rats were injected interperitoneally with 1 mg per 100 g body weight of triamcinolone (2 mg per cm^3 in 60% propane-1,2-diol) for 5 days prior to sacrifice. Alloxan diabetes was induced by the interperitoneal injection of 12 mg per 100 g body weight of alloxan (60 mg per cm^3 in water) 5 days prior to sacrifice.

3.3.4 Quantitation of purified enzyme protein

The protein content of purified enzyme solutions was related to the absorbance at 280 nm by dry weight determination. A solution of enzyme (approximately 30 mg, in 65 mM Tris-acetate buffer) was dialysed extensively against distilled water and lyophilized to constant weight

3.4 Results and Discussion

Rats treated with triamcinolone or made alloxan diabetic showed no increase in FBPase levels in crude homogenates, and purified enzyme from these animals had the same specific activity as that from animals not receiving these treatments.

The procedure for purifying the enzyme was repeated 8 times and was found to be very reproducible. A typical purification scheme is shown in Table 3.1. The specific activity of the centrifuged homogenate was increased approximately 13% after dilution with 0.1 mM EDTA, 2 mM $MgCl_2$. The heat step not only inactivated proteases, but was also found to be necessary for complete adsorption of the enzyme onto the phosphocellulose. This was ascribed to the presence of fructose 1,6-bisphosphate in crude homogenates, which would presumably have been hydrolysed under the incubation conditions employed. This suggestion would also account for the quite substantial rate of $NADP^+$ reduction by crude homogenates in enzyme assays without any added fructose 1,6-bisphosphate. After this activity had subsided, addition of exogenous substrate gave rise to a rate of $NADP^+$ reduction equivalent to that obtained when assays were started with addition of homogenate to reaction mixtures already containing fructose 1,6-bisphosphate.

The enzyme was eluted from the phosphocellulose at almost constant specific activity (Figure 3.1)

The purified enzyme, when dissolved in 65 mM Tris-acetate, 0.1 mM EDTA, 0.1 mM 2-mercaptoethanol, pH 6.3, and an $A_{280\ nm}/A_{260\ nm}$ ratio of 2.0 in agreement with Traniello (1974). The absorbance at 280 nm for a 1 mg per cm^3 solution in the above buffer was 0.63 as reported by Tejwani et al. (1976).

When purified enzyme was examined by gel electrophoresis under both non-denaturing (Figure 3.2) and denaturing conditions (Figure 3.3), a single band was observed when up to 100 μg of protein was applied to the gel. The subunit molecular wieght, when estimated by SDS gel electrophoresis was 40 000-41 000 daltons (Figure 3.4). This is in close agreement with the estimate obtained by Tejwani et al (1976), who reported a subunit molecular weight of 38 000-39 000 daltons.

When assayed in the presence of 2 mM Mg^{2+} and 0.1 mM EDTA, the purified enzyme had a single activity maximum at pH 7.5 (Figure 3.5). The rat liver enzyme may thus be classified as a 'neutral' FBPase.

Table 3.1

PURIFICATION OF FRUCTOSE-1,6-BISPHOSPHATASE FROM RAT LIVER

Fraction	Volume (cm^3)	Activity ($units/cm^3$)	Protein* (mg/cm^3)	Specific Activity (units/mg)	Recovery (%)	Purification (fold)
Diluted homogenate	2060	1.190	5.30	0.22	100.0	1.0
Heat treatment	1979	0.810	1.58	0.51	65.6	2.3
First P11 supernatant	2200	0.794	0.48	1.65	71.2	7.5
Second P11 supernatant	5351	0.038	0.11	0.34	8.3	-
P11 affinity elution	380	2.120	0.088	24.09	32.8	109.5
Mg^{2+} incubation	385	2.300	0.088	26.10	36.1	118.6

Results are presented for an initial extract from 250 g of liver.

*Protein concentrations were measured by the Biuret method except for the pure enzyme which was determined by absorbance measurement at 280 nm.

Figure 3.1

ELUTION OF FRUCTOSE-1,6-BISPHOSPHATASE FROM PHOSPHOCELLULOSE WITH FRUCTOSE 1,6-BISPHOSPHATE

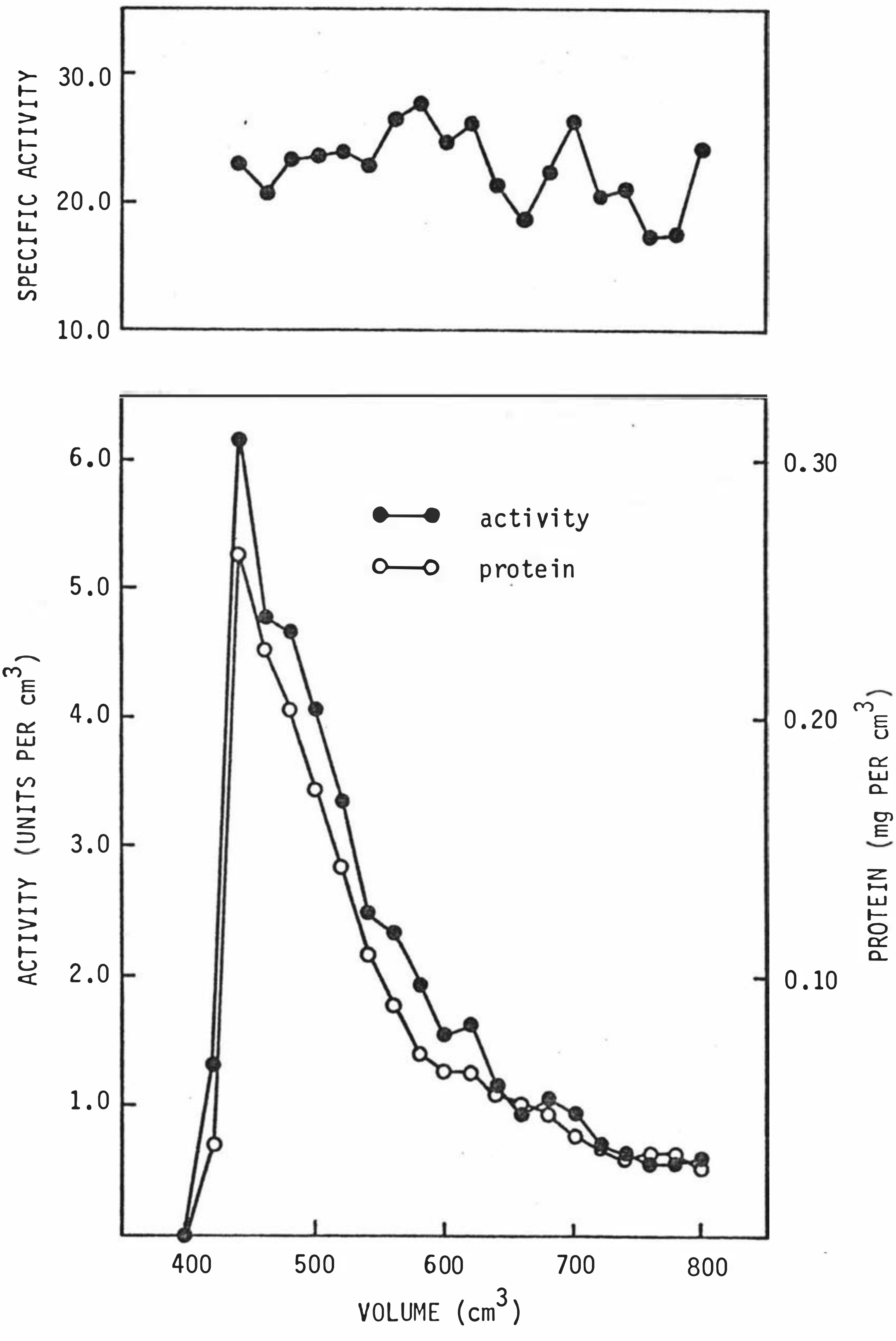


Figure 3.2

NON-DENATURING GEL (7%) ELECTROPHORESIS OF FRUCTOSE-1,6-BISPHOSPHATASE

100 μg of protein were applied to the gel

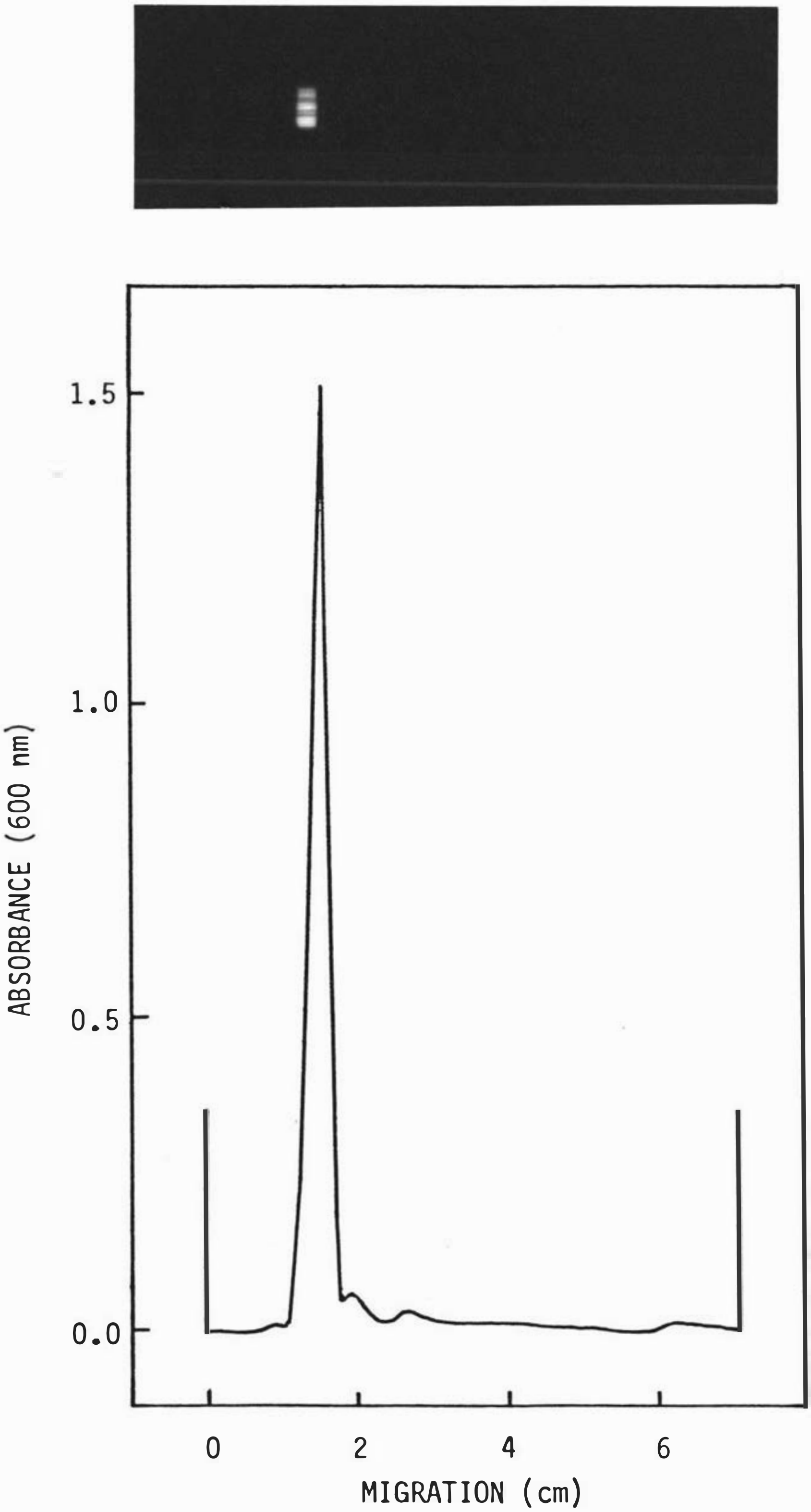


Figure 3.3

SDS GEL (10%) ELECTROPHORESIS OF FRUCTOSE-1,6-BISPHOSPHATASE

100 μg of protein were applied to the gel

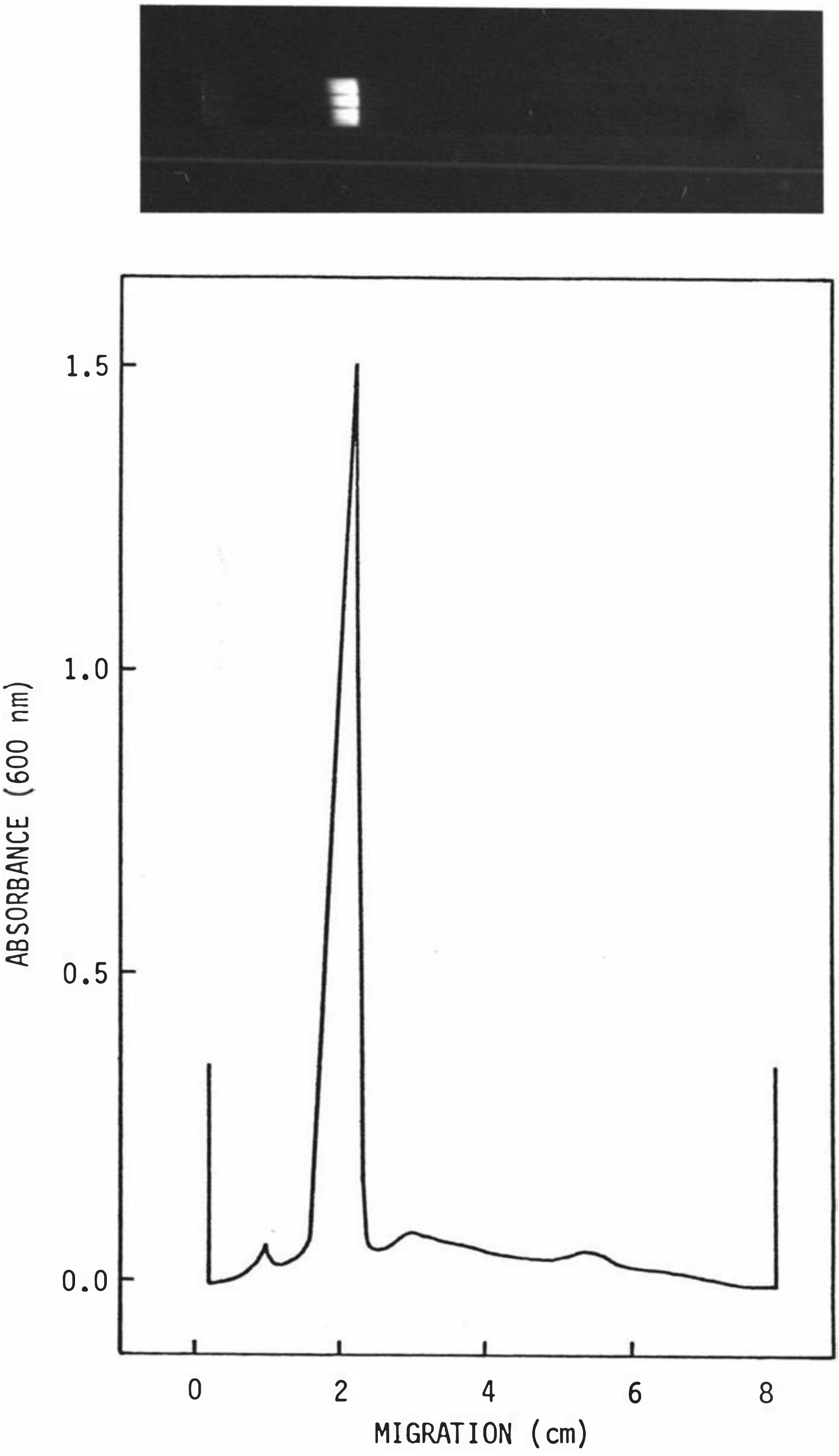


Figure 3.4

ESTIMATION OF FRUCTOSE-1,6-BISPHOSPHATASE SUBUNIT MOLECULAR WEIGHT BY SDS GEL (10%) ELECTROPHORESIS

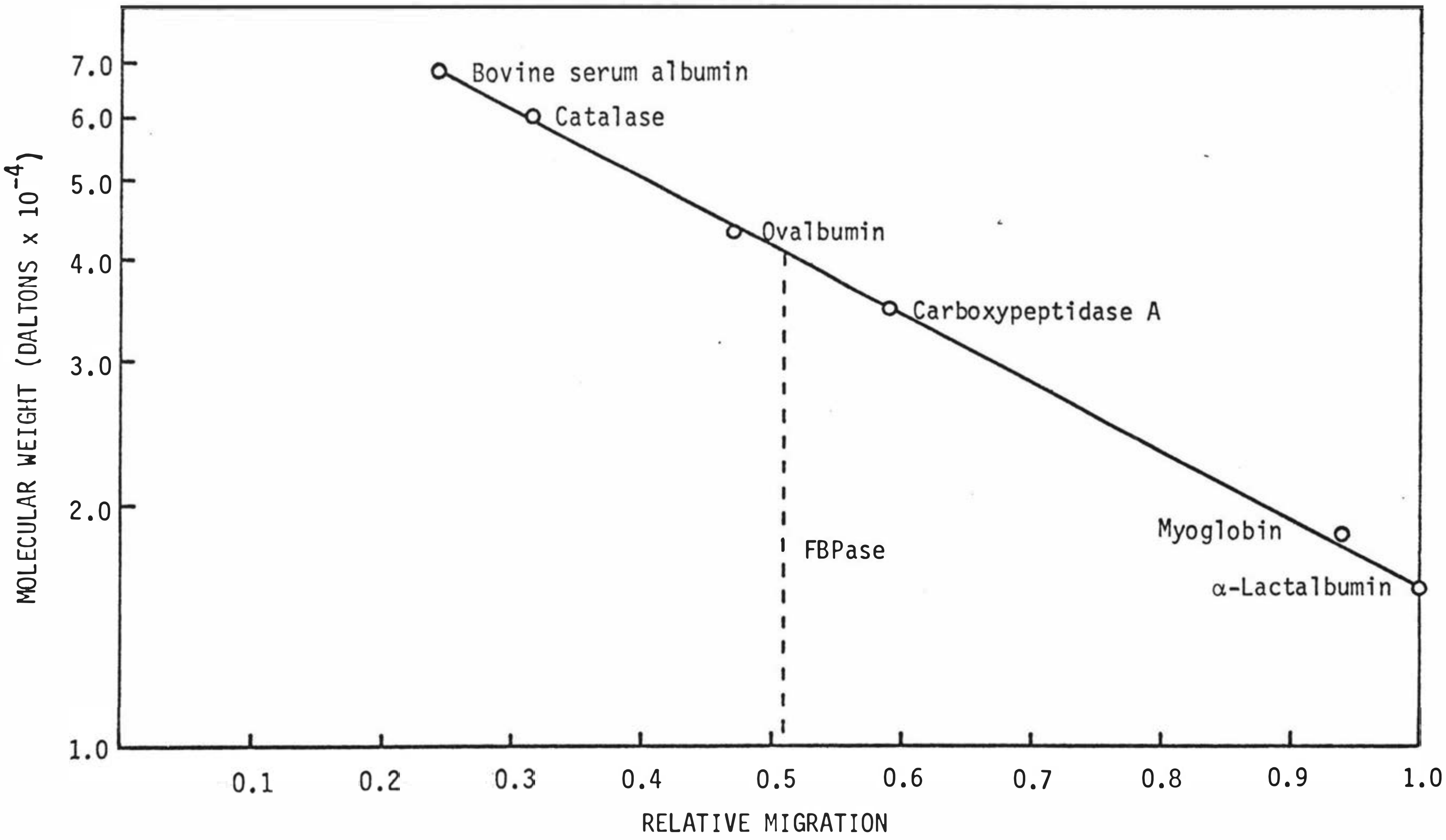
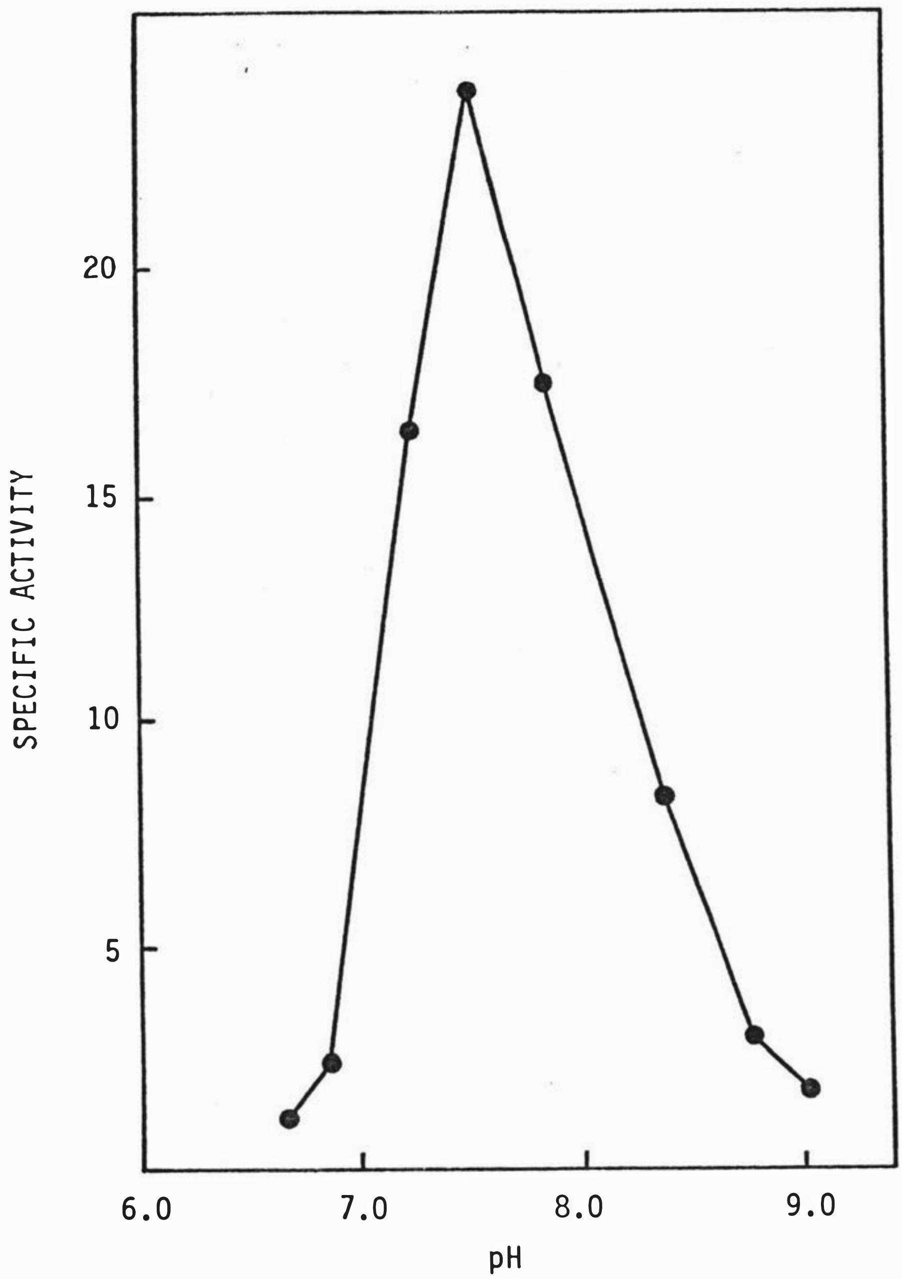


Figure 3.5

EFFECT OF pH ON FRUCTOSE-1,6-BISPHOSPHATASE ACTIVITY

Purified FBPase was assayed in 0.04 M Tris acetate over the entire pH range



CHAPTER FOUR

PURIFICATION OF OVALBUMIN, RAT ALBUMIN AND SHEEP GAMMA GLOBULIN

4.1 Introduction

Ovalbumin, rat albumin and sheep gamma globulin were purified to homogeneity for use in antibody production.

4.2 Materials

Ovalbumin and rat albumin were obtained from Sigma. DEAE-cellulose (DE-32) was obtained from Whatman, and precycled according to the manufacturers instructions.

4.3 Methods and Results

4.3.1 Ovalbumin

Commercial ovalbumin was further purified by preparative gel electrophoresis on an apparatus built as described by Furlong et al. (1973). A 25 mg sample of ovalbumin, dissolved in 2.0 cm^3 of sample application buffer was purified by electrophoresis through a 4.0 cm^3 stacking gel and an 8.0 cm^3 8% resolving gel (section 2.7.1). Electrophoresis was performed at 4^oC with a current of 20 mA. A buffer stream collected protein from the resolving gel at a flow rate of 20 cm^3 per hour. Fractions were collected and monitored at 280 nm for protein. Those fractions that contained a substantial amount of protein were examined by SDS gel electrophoresis (section 2.7.2) and homogeneous fractions combined (Figure 4.1).

4.3.2 Rat albumin

Commercial rat albumin was purified by preparative gel electrophoresis as described for ovalbumin (section 4.3.1). A 25 mg sample of rat albumin, dissolved in 2.0 cm^3 of sample application buffer was purified by electrophoresis through a 4.0 cm^3 stacking gel and an 8.0 cm^3 6% resolving gel (Figure 4.1).

4.3.3 Rabbit gamma globulin

Gamma globulin was separated from rabbit serum according to the ammonium sulphate fractionation procedure of Palmiter et al. (1971). Further purification, by chromatography on DEAE-cellulose was as described by Hudson and Hay (1976).

Blood was obtained from non-immune rabbits by venepuncture of a marginal ear vein. Blood was collected in plastic centrifuge tubes and allowed to clot 1 hour at room temperature and overnight at 4°C. Serum was separated from the clot by centrifugation at 35 000 g for 10 minutes (Sorvall SS-34 rotor, 17 000 rpm). Complement was inactivated by heating the serum to 54°C for 10 minutes.

The serum was diluted with an equal volume of 10 mM Na_2HPO_4, 15 mM NaCl, pH 7.2 (phosphate buffer, PB) and saturated ammonium sulphate (pH 7.0) was added to 40% saturation. After standing 30 minutes at 4°C, the precipitate was collected by centrifugation, dissolved in 2.5 volumes (compared to the original serum volume) of PB and reprecipitated with ammonium sulphate at 40% saturation. The precipitate was collected by centrifugation and dissolved in a volume of 0.005 M Na_2HPO_4, pH 8.0 to remove ammonium sulphate.

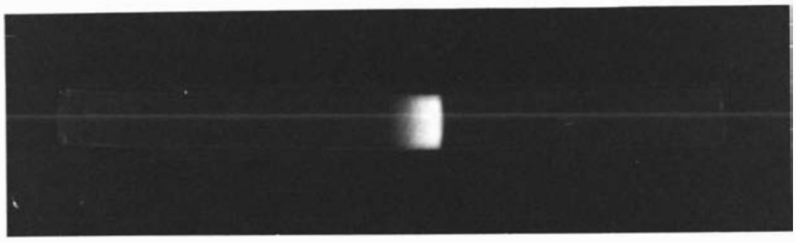
A solution containing 200 mg of crude gamma globulin was applied to a 2 x 50 cm column of DEAE-cellulose equilibrated in 0.005 M Na_2HPO_4, pH 8.0. Elution was by means of a convex salt gradient (limit buffer 0.18 M Na_2HPO_4, pH 8.0, mixing vessel volume 110 cm^3). A flow rate of 2 cm^3 per minute was maintained during loading elution of the protein. Those fractions that contained substantial amounts of protein were examined by SDS gel electrophoresis and homogeneous fractions combined (Figure 4.1).

Figure 4.1

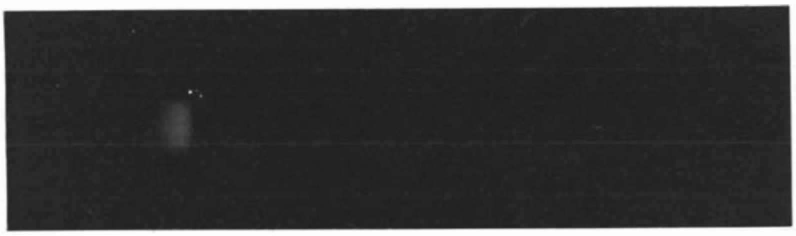
SDS GEL ANALYSIS OF PURIFIED OVALBUMIN, RAT ALBUMIN AND RABBIT GAMMA GLOBULIN

100 μg of protein were applied to each gel

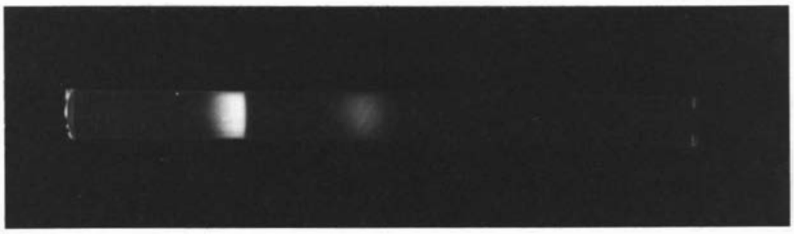
Ovalbumin (7% gel)



Rat albumin (7% gel)



Rabbit gamma globulin (10% gel)



CHAPTER FIVE

ANTIBODY PRODUCTION AND PURIFICATION

5.1 Introduction

Monospecific antibodies were raised in rabbits against rat liver fructose-1,6-bisphosphatase, rat albumin and hen ovalbumin and in sheep against rabbit gamma globulin.

Gamma globulin was prepared from immune serum by ammonium sulphate fractionation and further purification was by affinity chromatography on Sepharose-bound antigen.

5.2 Materials

Freund's complete adjuvant was from Difco Laboratories, Detroit, Michigan. Cyanogen bromide activated Sepharose 4B-200 was obtained from Sigma or prepared according to Porath (1970). Agarose was from Sigma.

5.3 Methods

5.3.1 Immunization protocols

Antibodies were raised in rabbits and sheep by procedures modified from those of Crowle (1973).

Rabbits

One mg of antigen, dissolved in 1 cm^3 of 10 mM Na_2HPO_4, 150 mM NaCl, pH 7.4 (phosphate buffered saline, PBS) was emulsified with an equal volume of Freund's complete adjuvant. On days 1 and 7, half the emulsion was injected subcutaneously into the back skin flap and half intramuscularly into a back leg. On day 21, 0.1 mg of antigen (in PBS) was injected subcutaneously into the back skin flap. On day 22, 1.0 mg of antigen was injected intravenously into a marginal ear vein. On day 23, 5.0 mg of antigen was injected into a marginal ear vein. Animals were bleed from a marginal ear vein at weekly intervals following the last intravenous injection. At each bleeding 30-40 cm^3

of blood were obtained.

Sheep

Five mg of antigen, dissolved in 2.5 cm^3 of PBS, were emulsified with an equal volume of Freund's complete adjuvant. On days 1 and 7 half the emulsion was injected subcutaneously into the back skin flap and half intramuscularly into a back leg. On day 21, 0.5 mg of antigen (in PBS) was injected subcutaneously into the back skin flap. On day 22, 5.0 mg of antigen was injected into the jugular vein. On day 23, 25.0 mg of antigen was injected into the jugular vein. Animals were bleed from the jugular vein at weekly intervals following the last intravenous injection. At each bleeding, 400-500 cm^3 of blood were obtained.

5.3.2 Separation of gamma globulin from serum

Gamma globulin was separated from serum by fractional ammonium sulphate precipitation (section 4.3.3). The final precipitate was dissolved in a volume of PB equal to half the original volume of serum. After overnight dialysis against PB to remove ammonium sulphate, any precipitated material was removed by centrifugation. Protein was quantitated by absorbance measurement at 280 using $E_{1\ cm}^{1\%} = 14.3$ (Hudson and Hay, 1976). After division into several aliquots, gamma globulin was quick-frozen and stored at -20^oC.

5.3.3 Antibody specificity

Antibody specificity was investigated by Ouchterlony double immunodiffusion.

Microscope slides were coated with 1 cm^3 of hot 0.2% agarose in distilled water and heated to dryness. Slides were then coated with 2.5 cm^3 of hot 1.0% agarose in sodium barbital, ionic strength 0.025, pH 8.2, and allowed to set on a level surface. Coated slides were stored at 4^oC in a water-saturated atmosphere, and wells punched after a least 4 hours storage.

After suitable aliquots of antigen and antibody had been placed in the wells, immunodiffusion was allowed to proceed at 4^oC in a water-saturated atmosphere. After precipitin lines had formed, slides were washed overnight in PBS then stained for 10 minutes in 0.125% Coomassie Brilliant Blue R 250 in glacial acetic acid-methanol-water (1:9:10, v/v/v), and destained in 7% acetic acid.

5.3.4 Preparation of Sepharose-bound antigen affinity columns

Proteins were coupled to cyanogen bromide activated Sepharose 4B-200 by methods derived from those recommended by Pharmacia.

Thirty mg of purified ovalbumin, rat albumin or rabbit gamma globulin were dissolved in 6 cm^3 of 0.1 M $NaHCO_3$, 0.5 M NaCl pH 8.3. A similar amount of fructose-1,6-bisphosphatase was dissolved in 15 cm^3 of 0.1 M $NaHCO_3$, 1.0 M $(NH_4)_2SO_4$, 0.05 M 2-mercaptoethanol, pH 8.0 because of its low solubility in buffers of low ionic strength without 2-mercaptoethanol.

One and a half grams of freeze-dried cyanogen bromide activated Sepharose 4B-200 was swollen by washing with 300 cm^3 of 1 mM HCl on a sintered glass funnel for 30 minutes. Activated Sepharose and buffered protein solution were mixed and rotated end-over-end for 2 hours at room temperature. The coupled Sepharose was collected by centrifugation and washed twice with 10 cm^3 of coupling buffer to remove unreacted protein. Excess active groups on the Sepharose were blocked by resuspension of the coupled Sepharose in 10 cm^3 of 1.0 M ethanolamine, 0.1 M Na_2CO_3, pH 9.0 and rotating end-over-end for 2 hours at room temperature. The final product was washed 3 times, alternately with 10 cm^3 of 0.1 M Tris-HCl, 0.5 M NaCl, pH 8.3 and 10 cm^3 of 200 mM glycine-HCl, pH 2.9. The Sepharose was equilibrated in PB and packed into a 10 cm^3 plastic syringe barrel fitted with a 3-way flow adaptor. When not in use, columns were stored at 4^oC in the presence of 0.02% sodium azide. No detectable deterioration of columns was found after 4 months use.

5.3.5 Affinity chromatography

Antibody was further purified on Sepharose-bound antigen following in general the method described by Taylor and Schimke (1974). All operations were performed at room temperature.

Gamma globulin (in PB) was applied to an appropriate Sepharose-antigen column at 5 cm^3 per hour. The column was then washed with the following buffers at 60 cm^3 per hour: 10 cm^3 of PB, 10 cm^3 of 0.1 M sodium acetate, 0.5 M NaCl, pH 4.0, 10 cm^3 of 0.1 M Tris-HCl, 0.5 M NaCl, pH 8.3, and 10 cm^3 of 0.1 M sodium acetate, 0.5 M NaCl, pH 4.0. The pure antibody was eluted from the matrix with 20 cm^3 of 200 mM glycine-HCl, pH 2.7. The column effluent was monitored for protein at 280 nm. Fractions of 3 cm^3 were collected and immediately neutralized with 5 M K_2HPO_4. Combined fractions were dialysed overnight against PB, and any precipitate removed by centrifugation. The protein was concentrated by ultrafiltration (section 2.4) to at least 1 mg per cm^3, and stored at -20°C.

5.3.6 Antibody titre

Crude gamma globulin fractions and affinity purified antibodies were quantitated by immunoprecipitation of ^{125}I-labelled antigens.

Purified ovalbumin, rat albumin, fructose-1,6-bisphosphatase and rabbit gamma globulin were labelled with 0.1 μCi of ^{125}iodine per 10 mg of protein and purified on Biogel P150 (section 8.3.2).

Discontinuous sucrose gradients were prepared in 500 mm^3 plastic centrifuge tubes. In each tube was placed 100 mm^3 of 1 M sucrose, 1% sodium deoxycholate, 1% Triton X-100 in PBS, 150 mm^3 of 0.5 M sucrose in the same detergent buffer, 100 mm^3 of PB, followed by 1-20 μg of ^{125}I-labelled antigen and a suitable amount of antibody. Tubes were incubated at 37°C for 1 hour and overnight at 4°C. Capped tubes were floated in water-containing 40 cm^3 centrifuge tubes and immunoprecipitates pelleted through the sucrose gradients by centrifugation at 27 000 g for 10 minutes (Sorvall HB-4 rotor, 13 000 rpm). After quick-freezing in liquid air, tubes were sheared just above and pellet, and both sections were placed in vials in preparation for gamma counting.

5.4 Results and Discussion

Monospecific antibodies were raised against all four proteins as shown by Ouchterlony double immunodiffusion on serum samples (Figure 5.1).

Gamma globulin was separated from other serum proteins by fractional ammonium sulphate precipitation. Further purification of antibodies by affinity chromatography on Sepharose-bound antigen is summarized in Table 5.1. A typical affinity elution of antibody is shown for anti-fructose-1,6-bisphosphatase (Figure 5.2). Non-specific gamma globulin was not bound to the antigen-Sepharose, but specific antibodies were completely removed from the sample applied to the column. Specific antibody was eluted at both pH 4.0 and pH 2.7, the antibody eluting at the lower pH being of higher specific activity. Crude and purified antibodies were shown to be immunologically identical by Ouchterlony double immunodiffusion (Figure 5.3). The low recoveries of both anti-ovalbumin and anti-fructose-1,6-bisphosphatase were caused by denaturation of the antibody during elution at pH 2.7, and its subsequent removal by centrifugation before quantitation of antibody yields.

Quantitative immunoprecipitation of ^{125}I-labelled antigens demonstrated a 3.6 to 14.6 fold purification of affinity-purified antibodies based on equivalence-point comparisons (Table 5.2). The precipitin curve for ^{125}I-labelled fructose-1,6-bisphosphatase with both crude and affinity-purified antibodies is shown in Figures 5.4 and 5.5. For all four antibodies, there was complete precipitation of antigen at all levels of antigen below the equivalence point. A noticeable characteristic of affinity-purified antibodies was their resistance to solubilization in antigen excess.

Denaturing gel electrophoresis in SDS (section 2.7.2) of affinity-purified antibodies revealed only two bands, corresponding to the heavy and light chains of gamma globulin (Figure 5.6).

Figure 5.1

OUCHTERLONY IMMUNODIFFUSION ANALYSIS OF ANTISERA AGAINST OVALBUMIN, RAT ALBUMIN, FBPase AND RABBIT GAMMA GLOBULIN

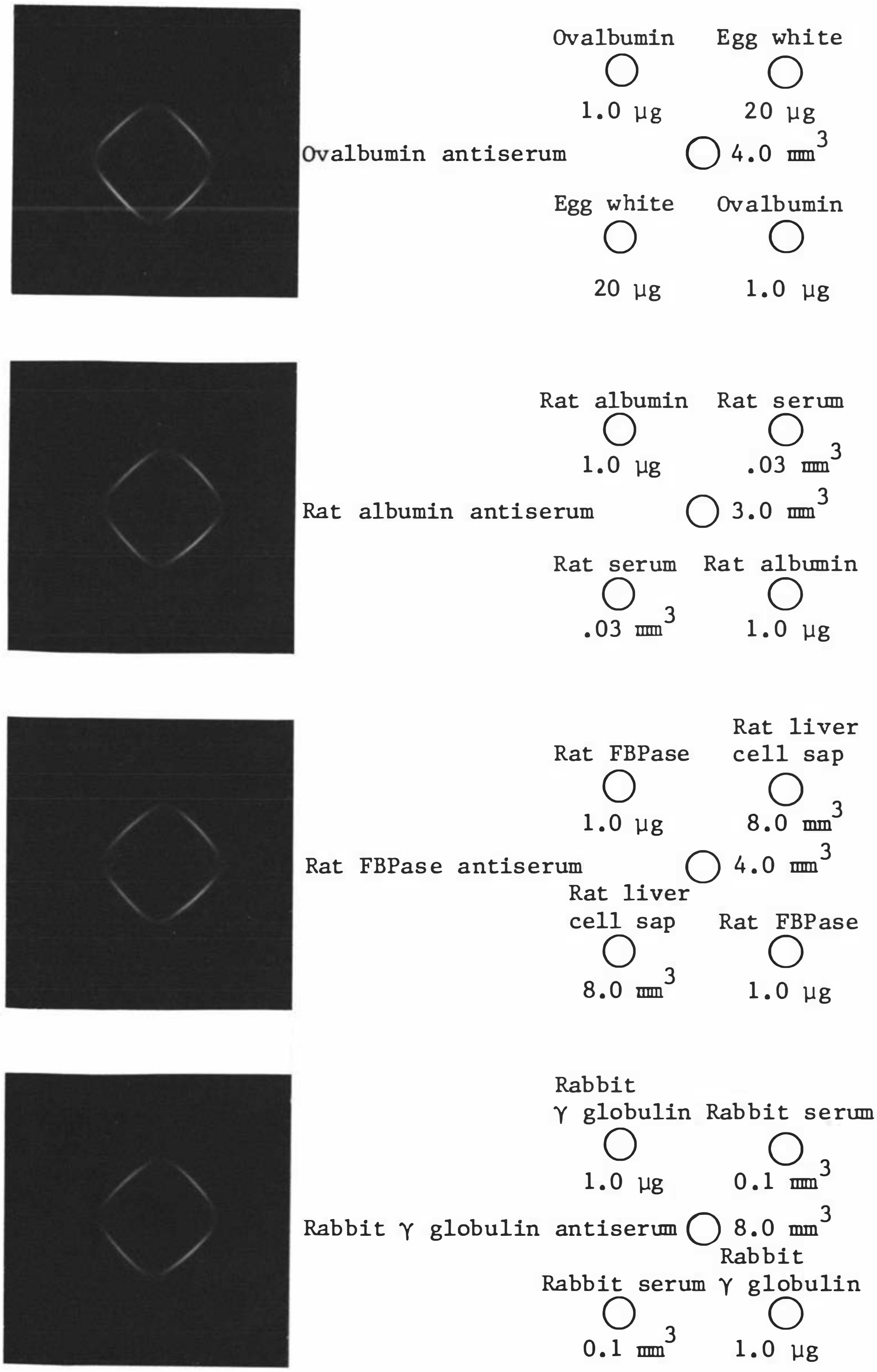


Table 5.1

ANTIBODY PURIFICATION BY AFFINITY CHROMATOGRAPHY

Antibody	Column fraction (mg)				Recovery
	Load	Unbound	Acetate/Tris	Glycine-HCl	(%)
anti-Fructose-1,6-bisphosphatase	300.0	226.0	22.1	10.0	87
anti-Ovalbumin	300.0	202.0	25.8	25.0	86
anti-Albumin	400.0	335.0	28.0	36.0	99
anti-Rabbit gamma globulin	1000.0	909.0	27.3	20.6	96

Figure 5.2

PURIFICATION OF ANTIBODIES TO FRUCTOSE-1,6-BISPHOSPHATASE BY AFFINITY CHROMATOGRAPHY ON SEPHAROSE-BOUND ANTIGEN

300 mg of crude anti-FBPase were applied to a 5 cm^3 column of Sepharose-bound FBPase

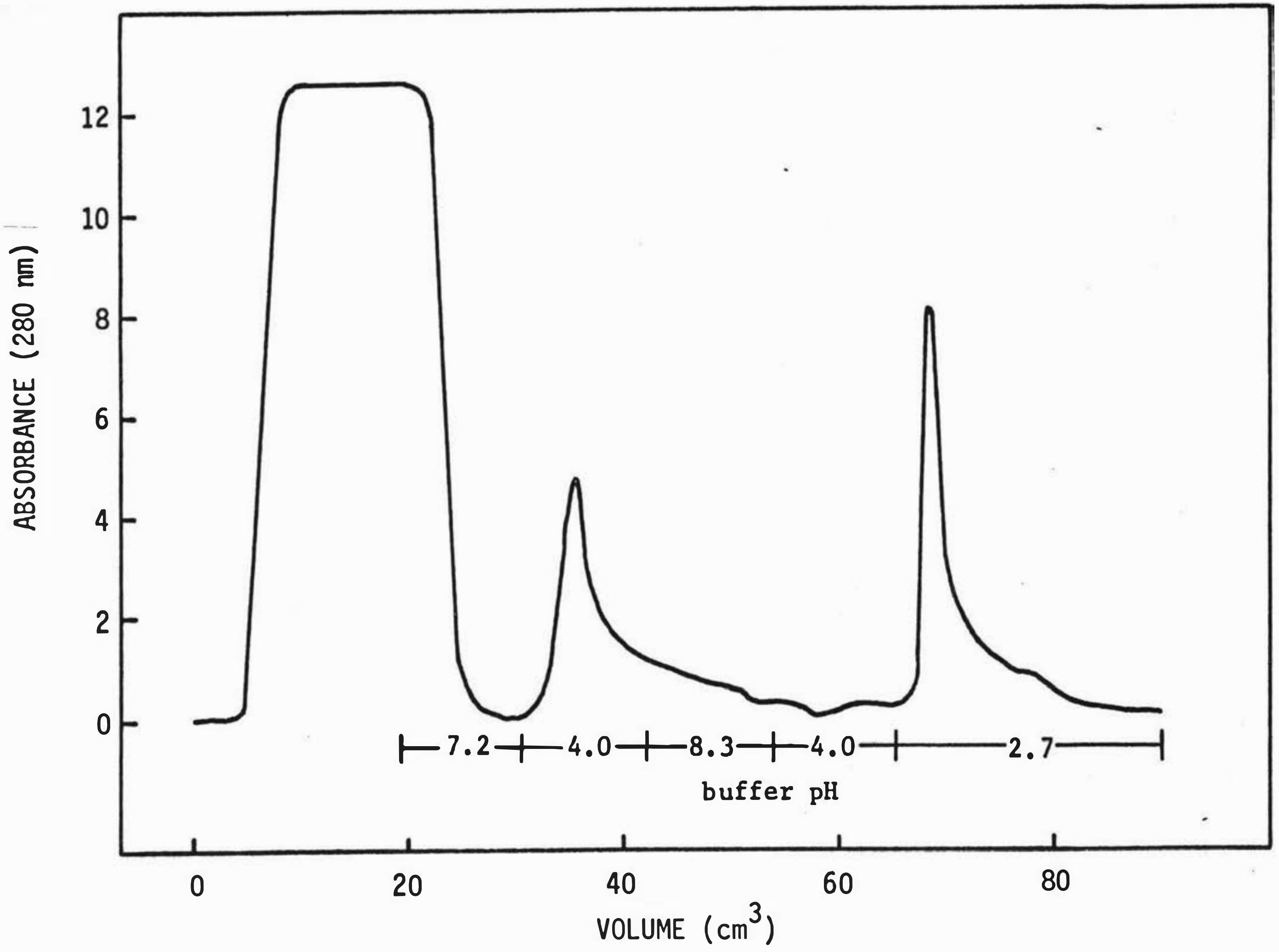


Figure 5.3

OUCHTERLONY IMMUNODIFFUSION ANALYSIS OF AFFINITY-PURIFIED ANTIBODIES

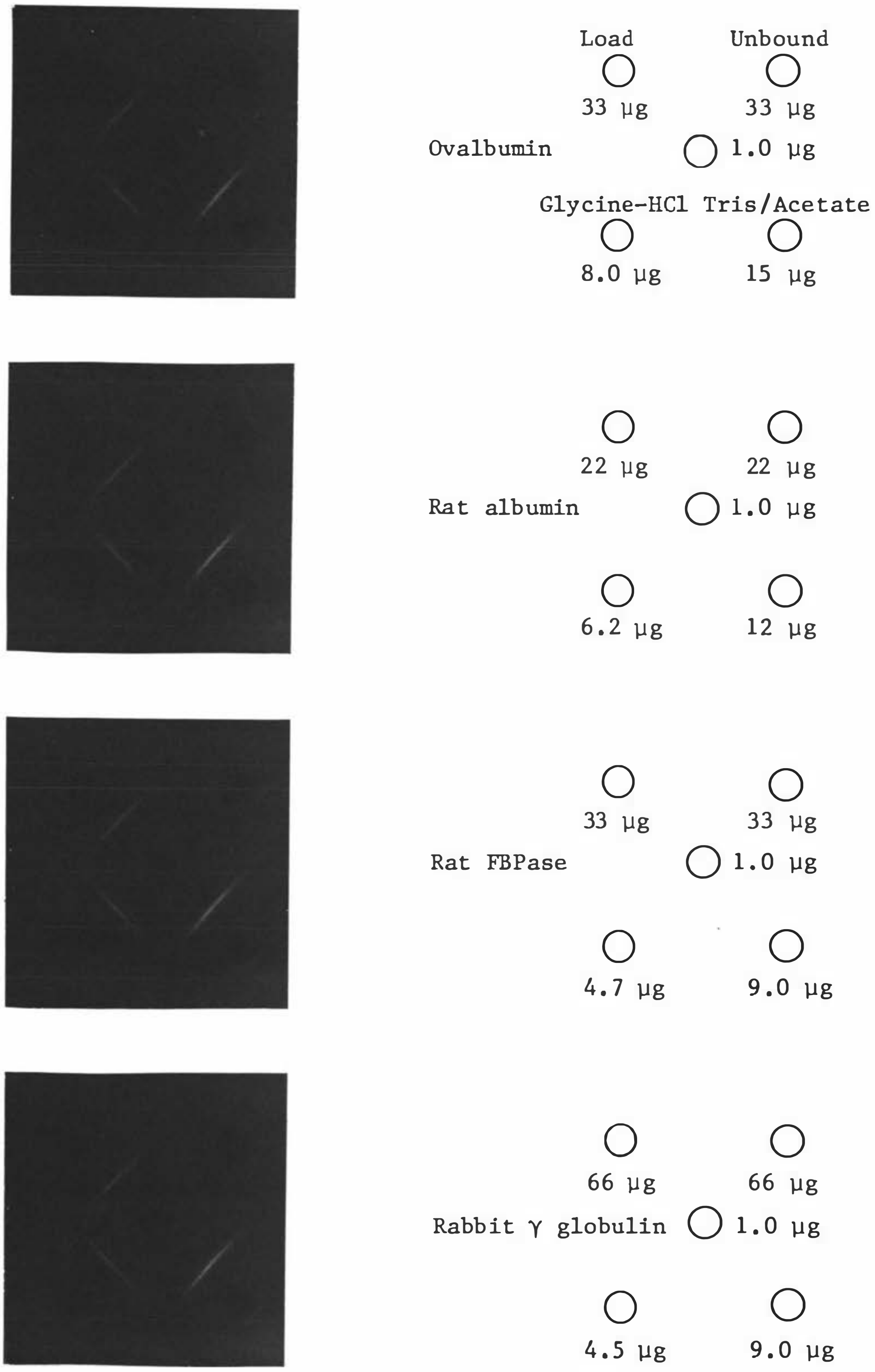


Table 5.2

ANTIBODY QUANTITATION

Antibody	µg of antigen precipitated by 1 mg of antibody at equivalence		Purification (fold)
	crude gamma globulin	affinity-purified antibody	
anti-Fructose-1,6-bisphosphatase	30	214	7.1
anti-Ovalbumin	30	124	4.1
anti-Albumin	44	160	3.6
anti-Rabbit gamma globulin	15	220	14.6

Figure 5.4

IMMUNOPRECIPITATION OF ^{125}I-LABELLED ANTI-FRUCTOSE-1,6-BISPHOSPHATASE BY CRUDE GAMMA GLOBULIN

500 µg of gamma globulin was added per incubation

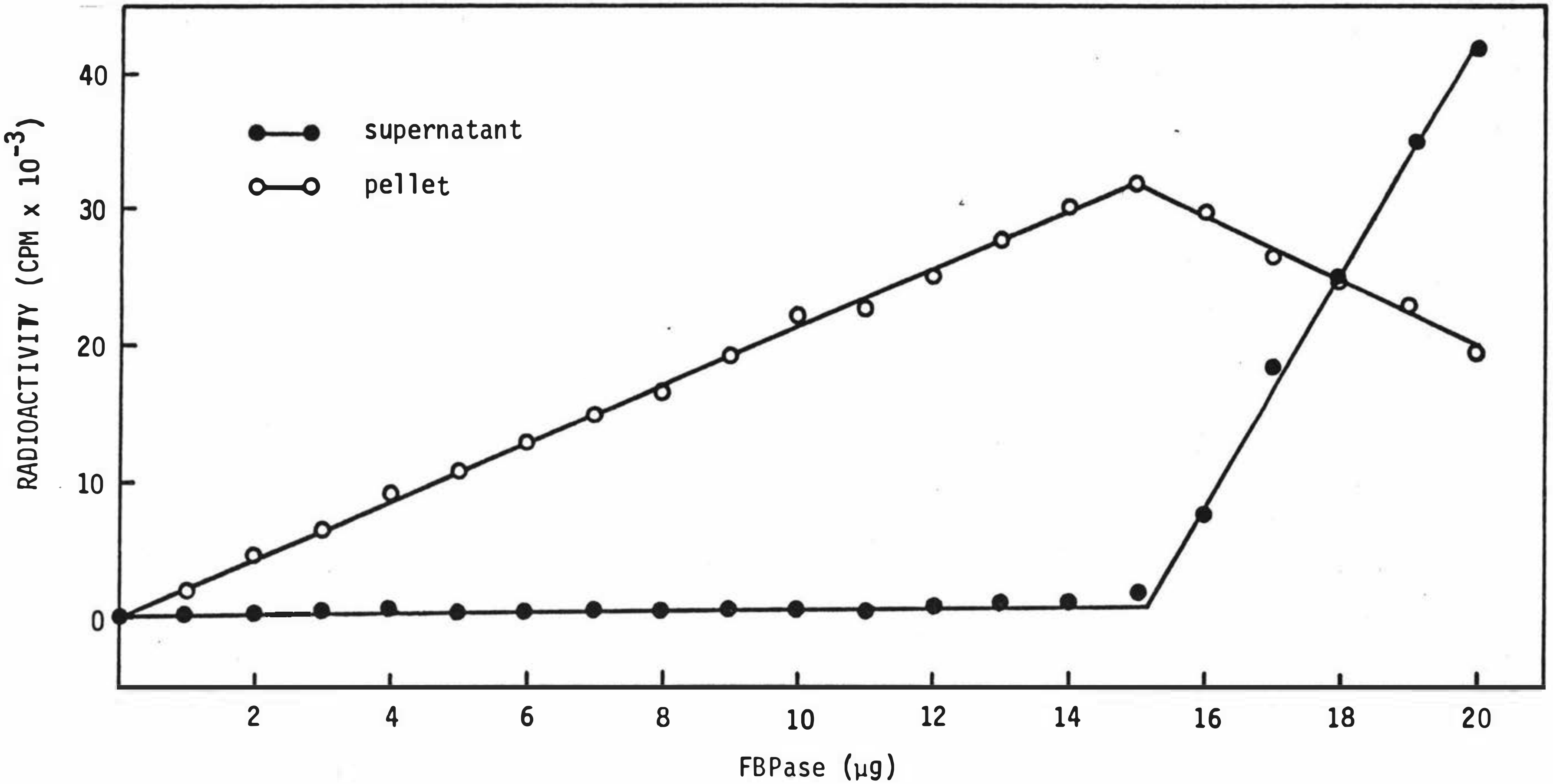


Figure 5.5

IMMUNOPRECIPITATION OF ^{125}I-LABELLED ANTI-FRUCTOSE-1,6-BISPHOSPHATASE BY AFFINITY-PURIFIED ANTIBODY

50 μg of affinity-purified antibody was added per incubation

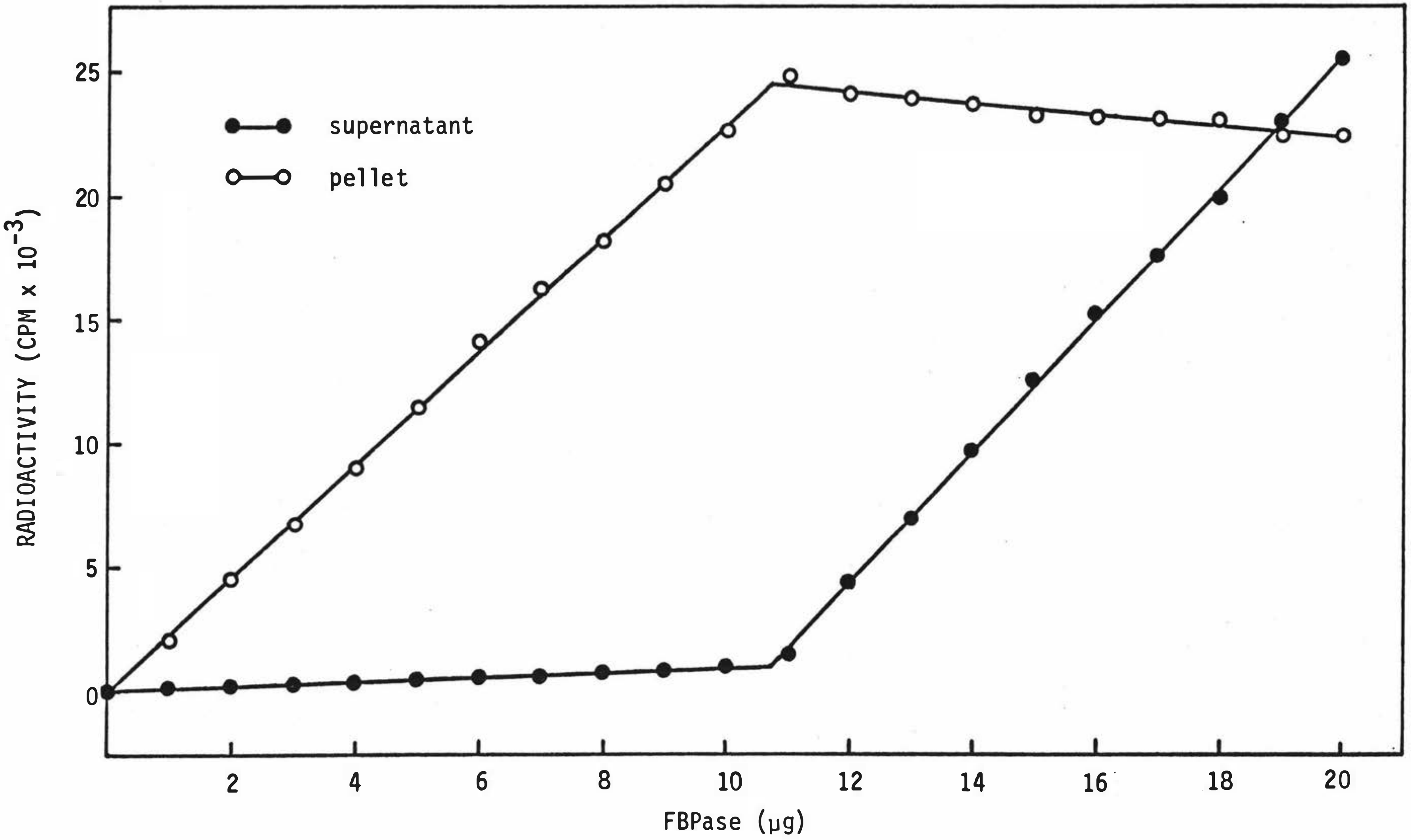
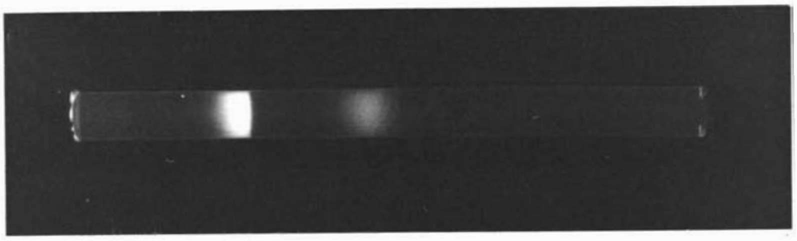


Figure 5.6

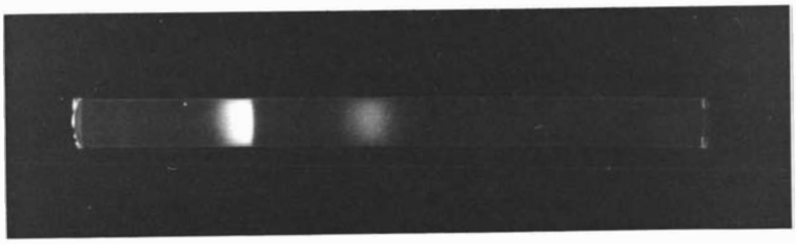
SDS GEL (10%) ANALYSIS OF AFFINITY-PURIFIED ANTIBODIES

100 μg of protein were applied to each gel

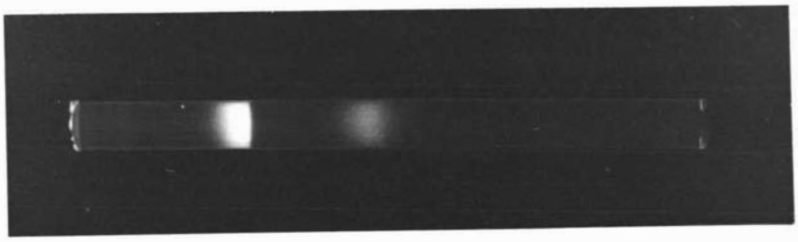
Anti-ovalbumin



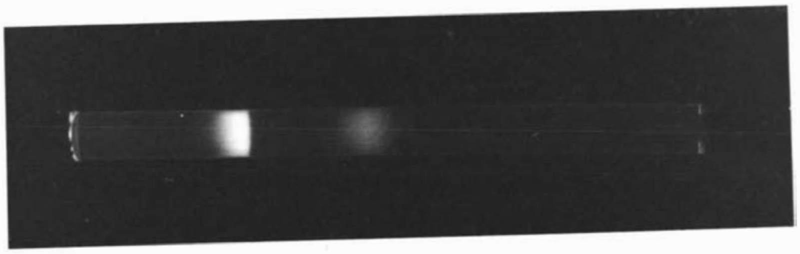
Anti-rat albumin



Anti-rat FBPase



Anti-rabbit gamma globulin



CHAPTER SIX

LABELLING OF LIVER PROTEINS IN VIVO

6.1 Introduction

The capacity of rat liver in vivo for the synthesis of albumin and FBPase was investigated by the immunochemical isolation of these proteins after administration of isotope.

The specificity of immunoprecipitation was examined by the isolation of albumin and FBPase from rat serum and liver homogenates after a long term (24 hour) interperitoneal administration of isotope. Non-specific trapping of labelled protein in antigen-antibdy complexes was minimised by the addition of 1% Triton X-100 and 1% sodium deoxycholate prior to immunoprecipitation (Schimke et al., 1974). Trapping was further decreased by centrifuging the immunoprecipitates through discontinuous sucrose gradients containing the above detergents. Ovalbumin and its antibody were chosen as a control since they do not cross-react with mammalian proteins (Palmiter et al., 1971) and provide an estimate of non-specific immunoprecipitation. An amount of ovalbumin and its antibody that gave a precipitate comparable in size to that obtained from albumin and FBPase immunoprecipitation was added to the labelled extract and carried through the same incubation and washing procedures. The specificity of immunoprecipitation was examined by electrophoresis of disrupted immunoprecipitates on denaturing SDS gels. This procedure is the most sensitive indicator of the specificity of the immunoprecipitation technique (Cashman and Pitot, 1971).

The rate of synthesis of albumin and FBPase was measured after short term incorporation of isotope in vivo. Additionally, the isolation of subcellular fractions from liver homogenates permitted the intracellular localization of newly synthesized FBPase and albumin. The incorporation of isotope into specific proteins was expressed as a percentage of the incorporation into total protein in a particular subcellular fraction. This assumes that all proteins are synthesized from the same amino acid pool and that the specific proteins in question do not have a significantly different amino acid composition to the total subcellular proteins with respect to the labelled amino acid.

The maximum permissible time between isotope administration and subcellular fractionation is imposed by the secretion time of albumin from the liver. Newly synthesized albumin is retained within the liver for approximately 15 minutes before being released into the bloodstream (Peters et al., 1971). A labelling time of less than this must be used to prevent contamination of supernatant preparations with radioactive albumin secreted into the blood, and to enable an estimate of relative albumin synthesis to be made, since proteins are secreted at different rates from the liver (Peters et al., 1971).

Isotope administration was by intraportal injection as this has been shown to be an excellent route (Glaumann, 1970; Schreiber et al.,1970). The high specific activity of the isotope used gives a true tracer dose after intravenous injection (Morgan and Peters, 1971), and there is a neglibible change in plasma methionine levels. Free isotope has been shown to be cleared from the blood within 5 minutes of injection (Peters et al., 1971), and maximal specific activity of nascent protein occurs within 1 to 1.5 minutes after injection (Takagi et al., 1970).

6.2 Materials

L-[^{35}S]Methionine (600 Ci per mmole) was obtained from the Radiochemical Centre, Amersham, and was stored at -196°C.

6.3 Methods

6.3.1 Interperitoneal (24 hour) labelling

A male rat (150 g) was starved overnight (16 hours) and injected interperitoneally at 9.00 a.m. with 100 μCi of L-[^{35}S]methionine in 0.4 cm^3 of PBS. Food was returned to the animal at 6.00 p.m.. At 9.00 a.m. on the following day the rat was etherized and exsanguinated from the jugular vein. Blood was collected and serum obtained (section 4.3.3) for the determination of incorporation of radioactivity into serum protein and immunoprecipitable albumin. The liver was perfused via the portal vein with 10 cm^3 of 250 mM sucrose, 1 mM $MgCl_2$ to remove blood from the liver. The large amounts of albumin in blood necessitates liver perfusion in order that albumin immunoprecipitates from liver supernatant fractions will not be too large. The liver was excised, blotted dry, weighed (5 g) and chopped up with scissors. The liver was homogenized with 3 volumes (15 cm^3) of 250 mM sucrose, 1 mM $MgCl_2$, 1 mM PMSF, with 5 strokes of a Potter-Elvehjem homogenizer (clearance 0.23 mm, rotating at 1700 rpm). The homogenate was centrifuged at 12 000 g for 10 minutes (Sorvall SS-34 rotor, 10 000 rpm). The supernatant was removed and centrifuged at 250 000 g for 2 hours (Beckman 60 Ti rotor, 50 000 rpm) to pellet microsomes. The supernatant was carefully removed, adjusted to 1% Triton X-100 and 1% sodium deoxycholate, and centrifuged at 250 000 g for 1 hour. The supernatant was removed, and used for the determination of incorporation of radioactivity into total protein and immunoprecipitable fructose-1,6-bisphosphatase.

6.3.2 Intraportal (10 minute) labelling

A male rat (150 g) was starved overnight and anaesthetized by the interperitoneal injection of 7.5 mg of sodium pentabarbital in 0.75 cm^3 of PBS. The peritoneal cavity was exposed and 50 µCi of L-[^{35}S]methionine in 0.4 cm^3 of PBS was injected into the portal vein. Ten minutes later, the liver was perfused and processed as described above (section 6.2.1). The microsomal pellet was resuspended in 20 cm^3 of cell sap containing 250 mM KCl (section 7.3.1) with 5 strokes of a Potter-Elvehjem homogenizer (clearance 0.36 mm, rotating at 1000 rpm). The microsomes were repelleted by centrifugation at 250 000 g for 1 hour. The supernatant was removed and the pellet resuspended by homogenization in 10 cm^3 of 50 mM Hepes, 250 mM KCl, 5 mM $MgCl_2$, pH 7.2 (HK(250)M), containing 250 mM sucrose and 1 mM PMSF. The homogenate was adjusted to 1% Triton X-100 and 1% sodium deoxycholate, and centrifuged at 250 000 g for 2 hours to pellet polysomes. The supernatant was removed and used for the determination of radioactive incorporation into total protein, immunoprecipitable albumin and fructose-1,6-bisphosphatase.

6.3.3 Analysis of labelled products

Protein was precipitated from duplicate aliquots of subcellular fractions (100 mm^3) by the addition of a 20 volume excess of 10% TCA, 10 mM methionine, heating to 90°C for 10 minutes to hydrolyse methionine-tRNA, and standing overnight at 4°C. Precipitated protein was collected by centrifugation and the pellet washed by suspension and recentrifugation twice in TCA-methionine, and once in ice-cold water. Pellets were dissolved in 1.0 cm^3 of 0.1 M NaOH in preparation for liquid scintillation counting (section 2.10), or analysed by SDS gel electrophoresis (section 2.7.2) and the radioactivity incorporated into individual gel slices determined (section 2.10.1).

6.3.4 Immunoprecipitation assay

A 1.5 fold excess of specific antibody was added to duplicate 0.25-1.0 cm^3 aliquots of liver subcellular homogenate fractions. Serum (2-3 mm^3) was diluted with PBS containing 1% Triton X-100 and 1% sodium deoxycholate to a volume of 0.25 cm^3 before the addition of antibody. Sufficient homogenate or serum was taken to give immunoprecipitates containing about 70 μg of antigen. Non-specific immunoprecipitation was quantitated with ovalbumin controls. Seventy μg of purified ovalbumin was added to 1.0 cm^3 of detergent-treated supernatant before the addition of its antibody. Immunoprecipitation was allowed to proceed at 37°C for 30 minutes and overnight at 4°C. Immunoprecipitates were layered over discontinuous sucrose gradients consisting of 2.0 cm^3 of 1.0 M and 3.0 cm^3 of 0.5 M sucrose in PBS containing 1% Triton X-100, 1% sodium deoxycholate, and 10 mM methionine. Immunoprecipitates were pelleted by centrifugation at 13 000 g for 10 minutes (Sorvall HB-4 rotor, 9000 rpm). Pellets were washed twice by suspension and recentrifugation in 2.0 cm^3 of PBS. Pellets were dissolved in 1.0 cm^3 of 0.1 M NaOH in preparation for liquid scintillation counting, or analysed by SDS gel electrophoresis and the radioactivity incorporated into individual gel slices determined. Radioactive albumin and fructose-1,6-bisphosphatase were identified by comparison with stained gels on which authentic protein had been electrophoresed.

6.4 Results and Discussion

SDS gel analyses of immunoprecipitates from rat liver homogenate and serum were carried out after a 24 hour interperitoneal labelling. Immunoprecipitates of FBPase (Figure 6.1), albumin (Figure 6.2) and ovalbumin (Figure 6.3) showed that for both liver-synthesized proteins each antibody precipitated a single radioactive protein that corresponded in mobility to the appropriate antigen and a major protein band on immunoprecipitate gels. The specificity of immunoprecipitation was also demonstrated by the absence of any radioactivity associated with ovalbumin immunoprecipitates. At least 90% of the precipitated radioactivity migrated with FBPase subunits, and an even higher percentage with albumin monomer. In addition all gels had two other major protein-staining bands, which probably represented heavy and light antibody chains. The presence of additional higher molecular weight materials in these gels was probably due to minor amounts of associated heavy and light chains (Cashman and Pitot, 1971), however little radioactivity was associated with these bands. The tentative band identifications were tested by plotting the log molecular weight versus their relative migration. As shown in Figure 6.4 the identification correlates well with the known molecular weights of these proteins.

The quantitation of radioactive immunoprecipitates is shown in Table 6.1 The specificity of immunoprecipitation with regard to ovalbumin controls was again demonstrated. The incorporation of isotope into FBPase relative to total supernatant protein (0.88%), gave some indication of the relative synthetic rate, although the labelling time (24 hours) was a significant fraction of the reported half-life of this enzyme (45.7 hours, Zalitis and Pitot, 1977).

A more accurate estimate of FBPase and albumin synthesis was achieved by pulse labelling the liver via the portal vein for 10 minutes. The subcellular fractionation scheme applied to liver homogenates is shown in Figure 6.5, and the distribution of radioactivity in TCA-precipitable protein in each fraction is summarized in Table 6.2. The large amounnt of radioactive protein non-specifically adsorbed to the microsomes and released by suspension in cell sap confirms the results of Glaumann and Dallner (1968). Removal of this protein was necessary to minimize contamination of microsomes with supernatant protein. TCA-precipitable protein in liver supernatant and microsome fractions was examined by SDS gel electrophoresis (Figure 6.6). Microsome-derived material showed a higher molecular weight distribution than supernatant material. The prominant peak at 65 000 daltons in microsomal fractions was almost certainly albumin.

FBPase and albumin were immunoprecipitated from liver supernatant and microsome fractions. The distribution of radioactivity is summarized in Table 6.3. It was necessary to add carrier FBPase (70 μg) to the microsome fraction to obtain any immunoprecipitate. Albumin was found to be almost exclusively associated with microsomal protein and FBPase with supernatant protein. On a relative synthetic basis, microsomes contained 63 times more albumin, and supernatant 25 times more FBPase. This distribution would not be significantly altered on an absolute basis, since the distribution of radioactivity into microsomal protein was only 26% higher than into supernatant protein. The specificity of the immunoprecipitation and the clear-cut segregation of albumin and FBPase into microsomal and supernatant fractions was also demonstrated by gel electrophoresis (Figures 6.7 and 6.8).

The relative rate of FBPase synthesis, 0.89% relative to total supernatant protein, agrees well with the 0.8-1.0% relative synthesis of this protein reported by Zalitis and Pitot (1977). Albumin synthesis, 16.7% of total microsomal protein represents 9.7% of total synthesis in combined microsomes and supernatant. This agrees favourably with the results of Keller and Taylor (1976) who reported a value of 11.3% of total synthesis in postmitochondrial supernatants.

Figure 6.1

SDS GEL (10%) ANALYSIS OF FBPase IMMUNOPRECIPITATED FROM LIVER POST-MICROSOMAL SUPERNATANT AFTER A 24 HOUR INTERPERITONEAL LABELLING WITH [^{35}S]METHIONINE

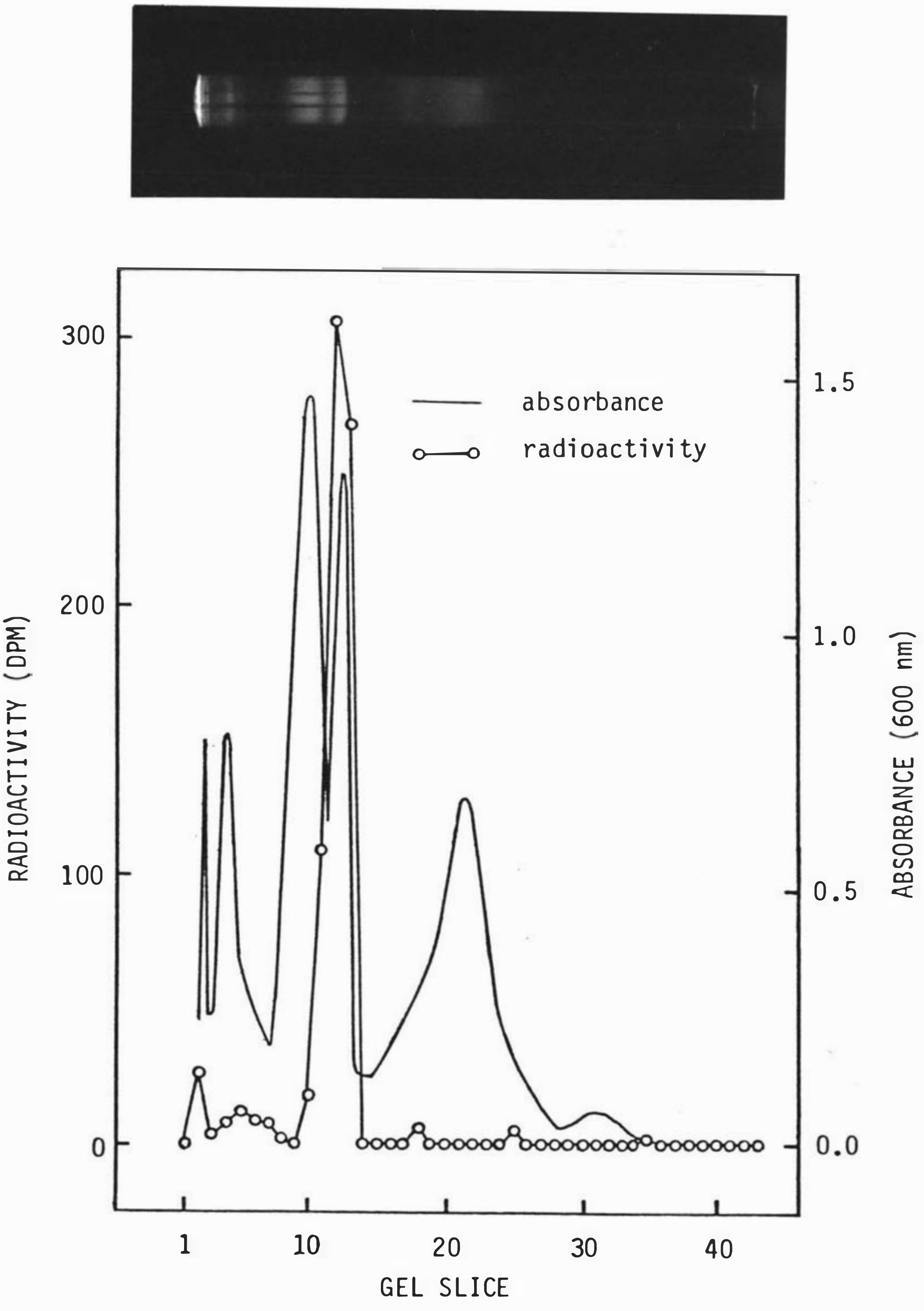


Figure 6.2

SDS GEL (10%) ANALYSIS OF ALBUMIN IMMUNOPRECIPITATED FROM SERUM AFTER A 24 HOUR INTERPERITONEAL LABELLING WITH [^{35}S]METHIONINE

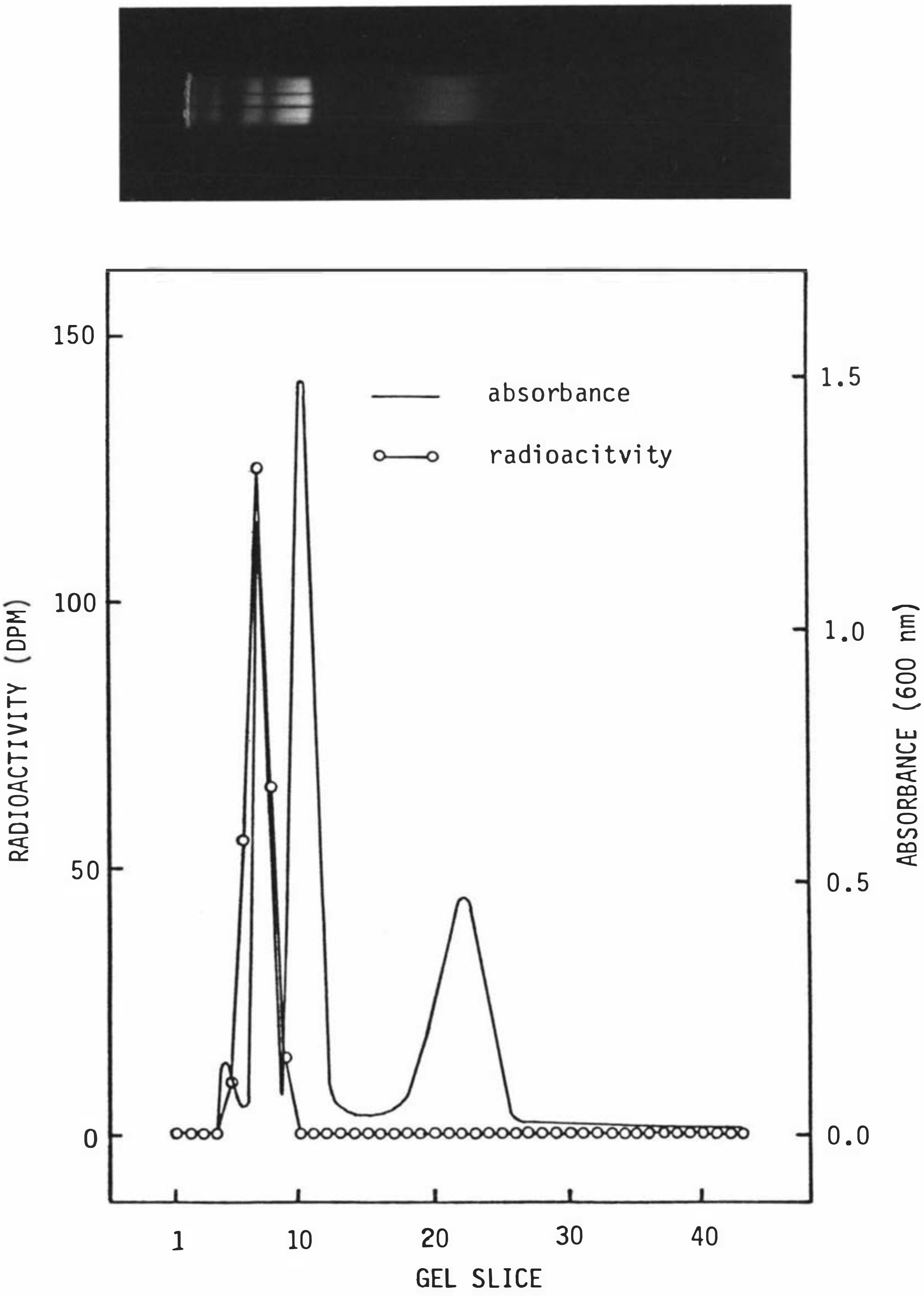


Figure 6.3

SDS GEL (10%) ANALYSIS OF OVALBUMIN ADDED TO AND IMMUNOPRECIPITATED FROM LIVER POST-MICROSOMAL SUPERNATANT AFTER A 24 HOUR INTERPERITONEAL LABELLING WITH [^{35}S]METHIONINE

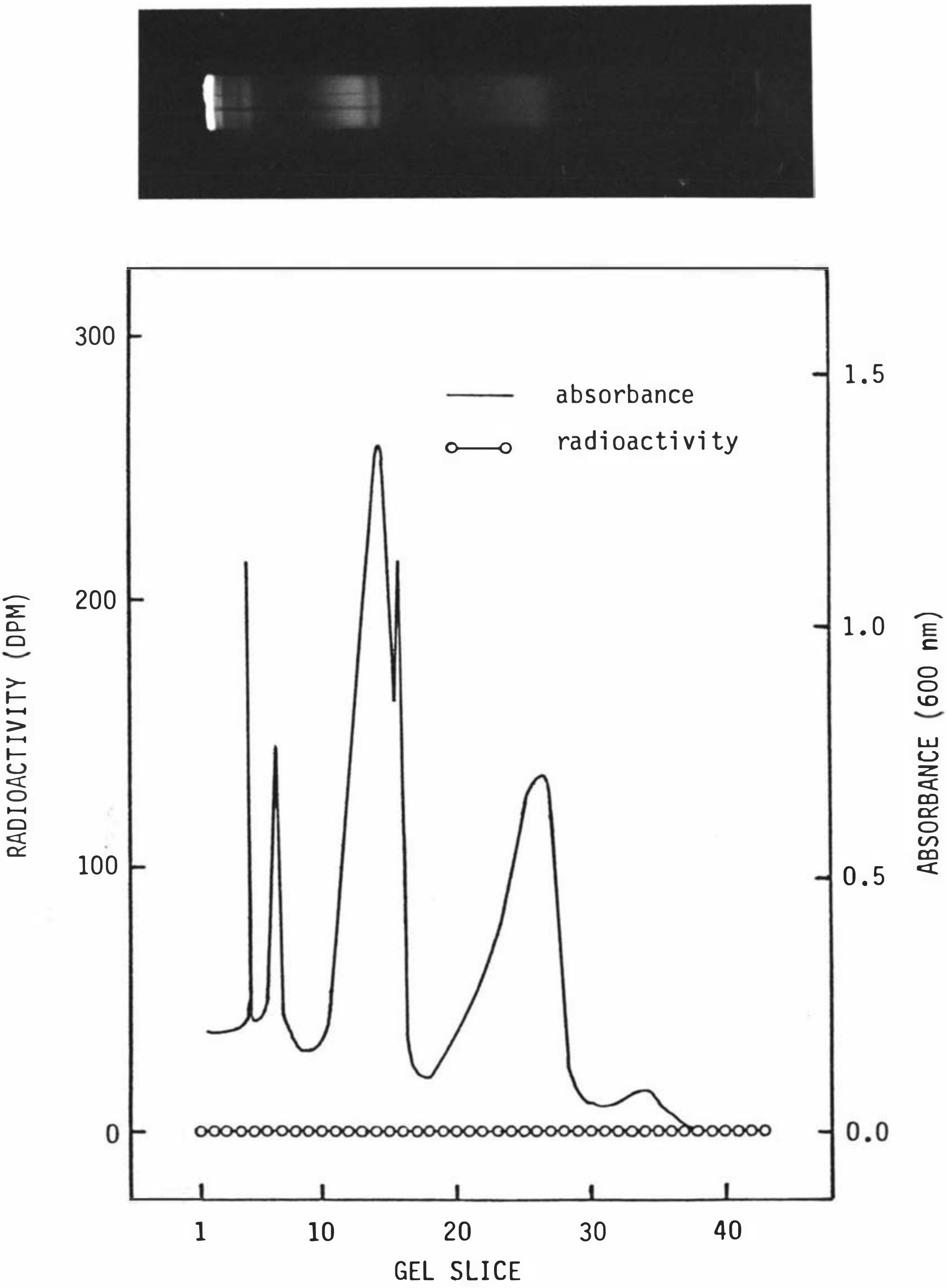


Figure 6.4

SEMILOG PLOT OF MOLECULAR WEIGHT VERSUS RELATIVE MIGRATION FOR PROTEIN SUBUNITS IN ALBUMIN, FBPase AND OVALBUMIN IMMUNOPRECIPITATES ON 10% SDS GELS

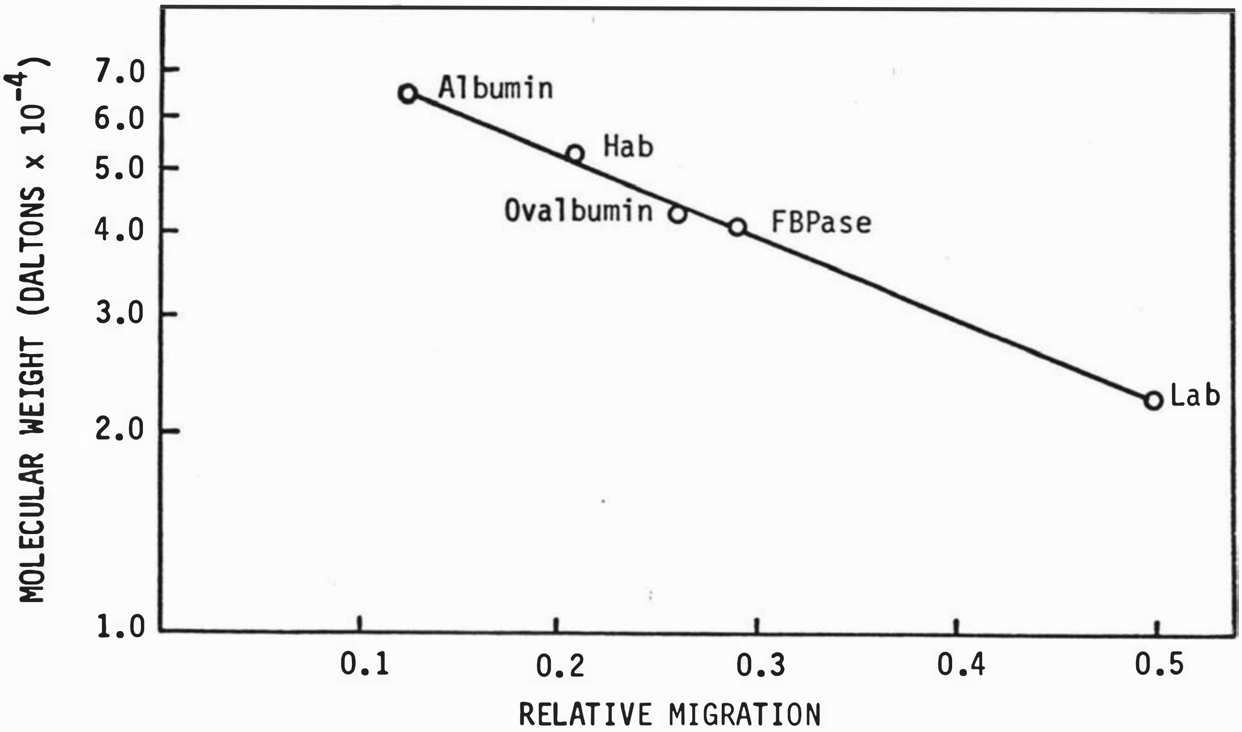


Table 6.1

IMMUNOPRECIPITATION OF ALBUMIN FROM SERUM, FBPase AND CARRIER OVALBUMIN FROM LIVER POST-MICROSOMAL SUPERNATANT AFTER A 24 HOUR INTERPERITONEAL LABELLING WITH [^{35}S]METHIONINE

Fraction	Total protein	Radioactivity in Albumin	(DPM/cm^3) FBPase	 Ovalbumin
Serum	231 680	168 750 (72.8%)	-	-
Post-microsomal supernatant	109 000	-	956 (0.88%)	16 (0.015%)

Figure 6.5

FRACTIONATION SCHEME FOR THE ISOLATION OF SUBCELLULAR FRACTIONS FROM RAT LIVER

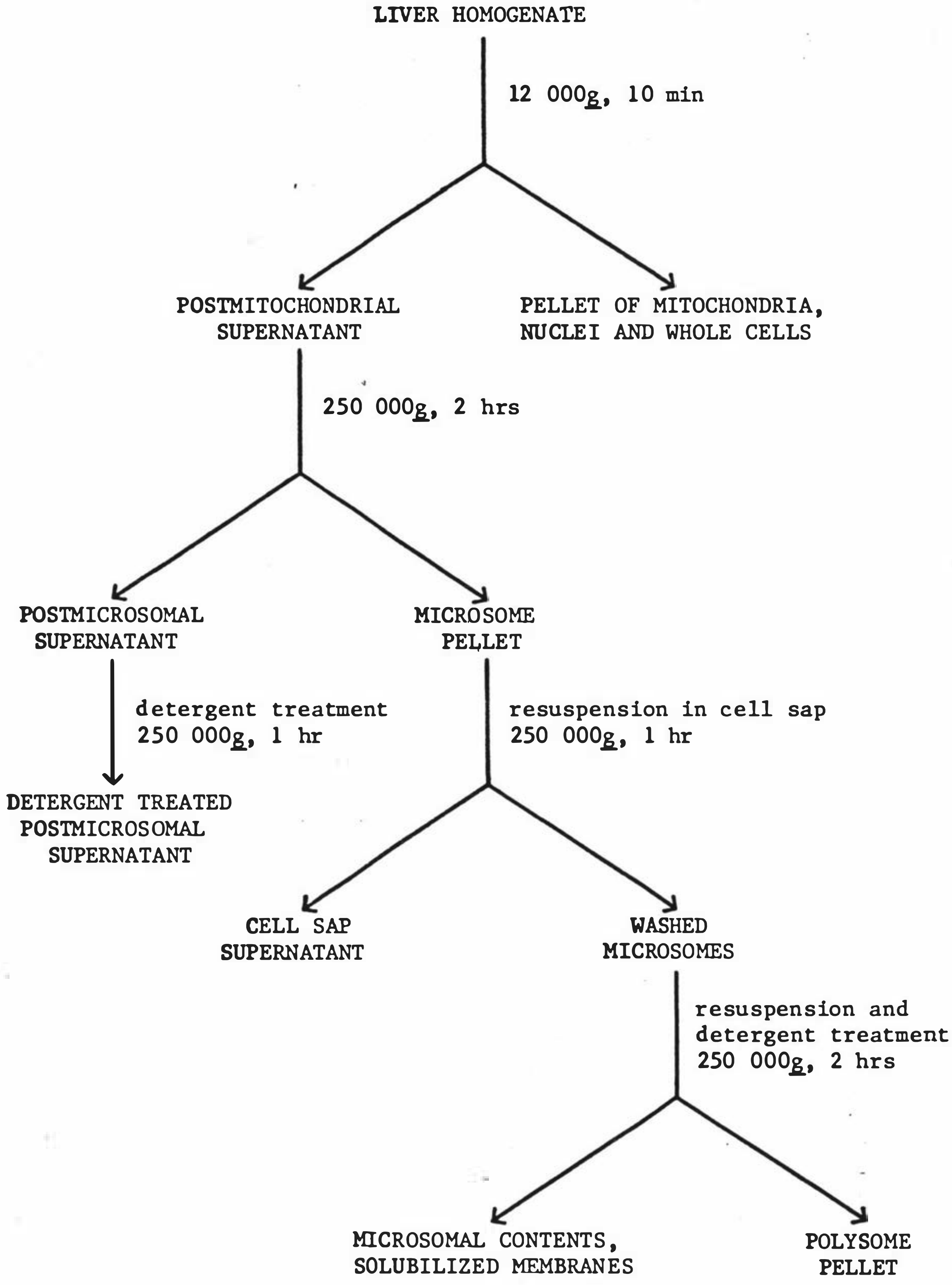


Table 6.2

DISTRIBUTION OF TCA-PRECIPITABLE PROTEIN IN LIVER SUBCELLULAR FRACTIONS AFTER A 10 MINUTE PULSE LABEL OF [^{35}S]METHIONINE VIA THE PORTAL VEIN

Subcellular fraction	Radioactivity (DPM)	Protein (mg)	Specific Activity (DPM/mg)
Pellet of mitochondria, nuclei and whole cells	5 115 250	-	-
Detergent-treated post-microsomal supernatant	1 160 820	215.0	5 399
Cell sap wash supernatant	232 120	1 000.5	232
Microsomal contents and solubilized membranes	1 566 870	83.6	18 734
Polysome pellet	116 200	9.3	12 495

Figure 6.6

SDS GEL (7%) ANALYSIS OF TCA-PRECIPITABLE PROTEIN IN LIVER SUPERNATANT AND MICROSOME FRACTIONS AFTER A 10 MINUTE PULSE LABEL OF [^{35}S]METHIONINE VIA THE PORTAL VEIN

Data has been normalized to 10 000 dpm per gel for each fraction

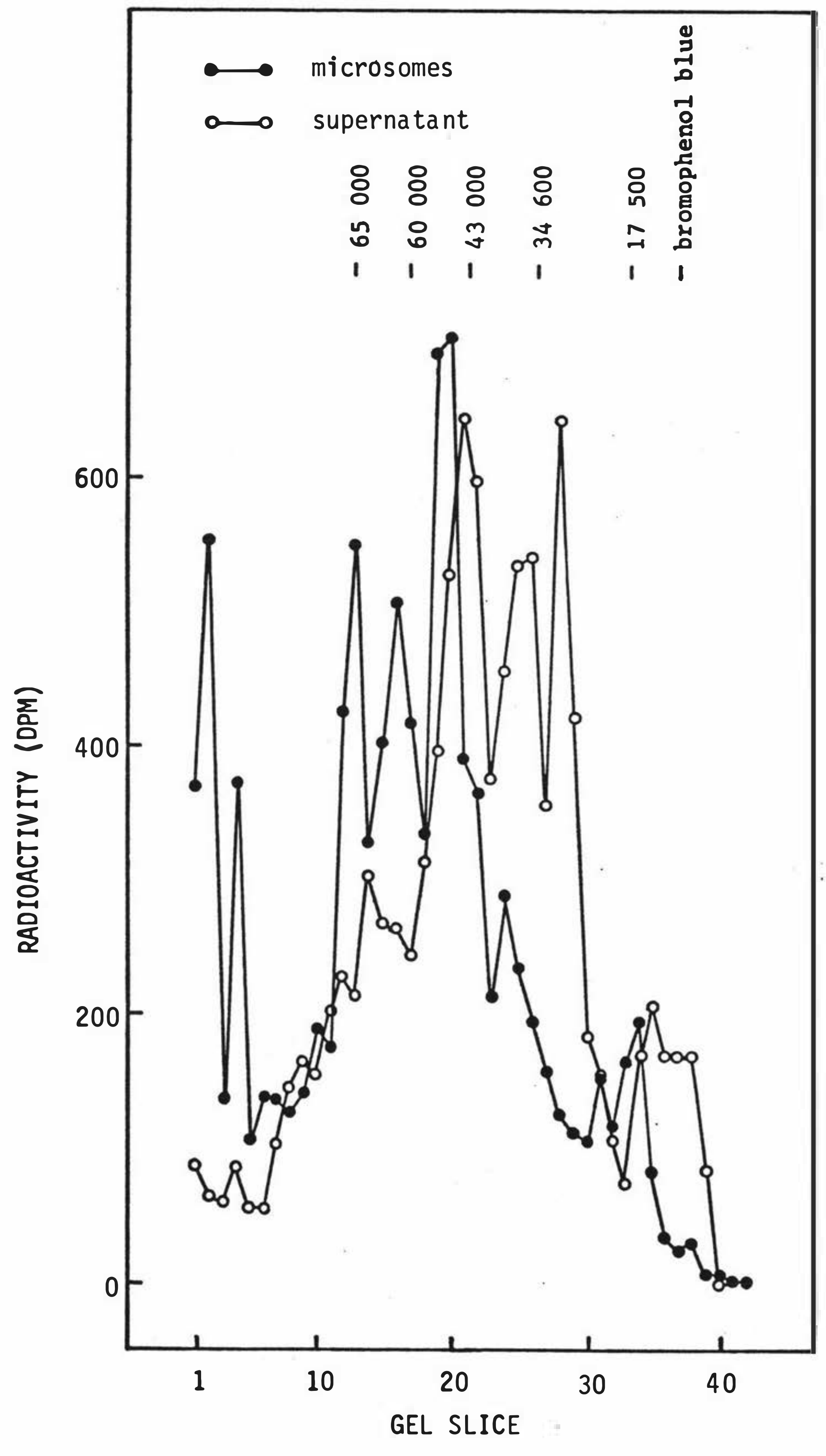


Table 6.3

DISTRIBUTION OF RADIOACTIVITY IN ALBUMIN AND FBPase IMMUNOPRECIPITATES FROM LIVER SUPERNATANT AND MICROSOME FRACTIONS AFTER A 10 MINUTE PULSE LABEL OF [^{35}S]METHIONINE VIA THE PORTAL VEIN

	Total protein	Radioactivity in Albumin	FBPase
		(DPM/cm^3)	
Detergent-treated post-microsomal supernatant	152 040	416 (0.27%)	1 348 (0.89%)
Microsomal contents and solubilized membranes	193 000	32 200 (16.7%)	67 (0.035%)

Figure 6.7

SDS GEL (7%) ANALYSIS OF FBPase IMMUNOPRECIPITATED FROM LIVER SUPERNATANT AND MICROSOME FRACTIONS AFTER A 10 MINUTE PULSE LABEL OF [^{35}S]METHIONINE VIA THE PORTAL VEIN

FBPase was precipitated from 83 680 dpm of total protein in each fraction

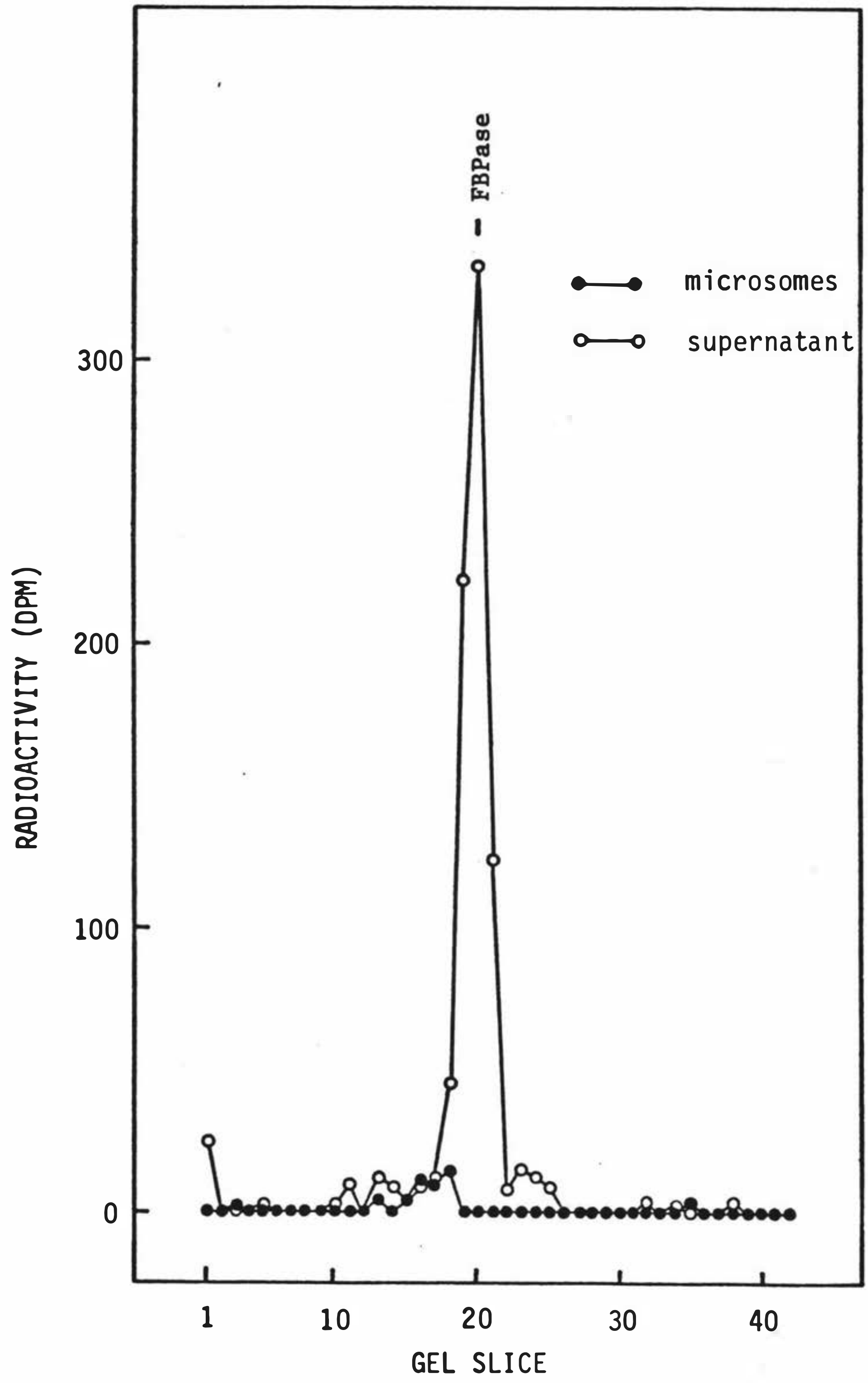
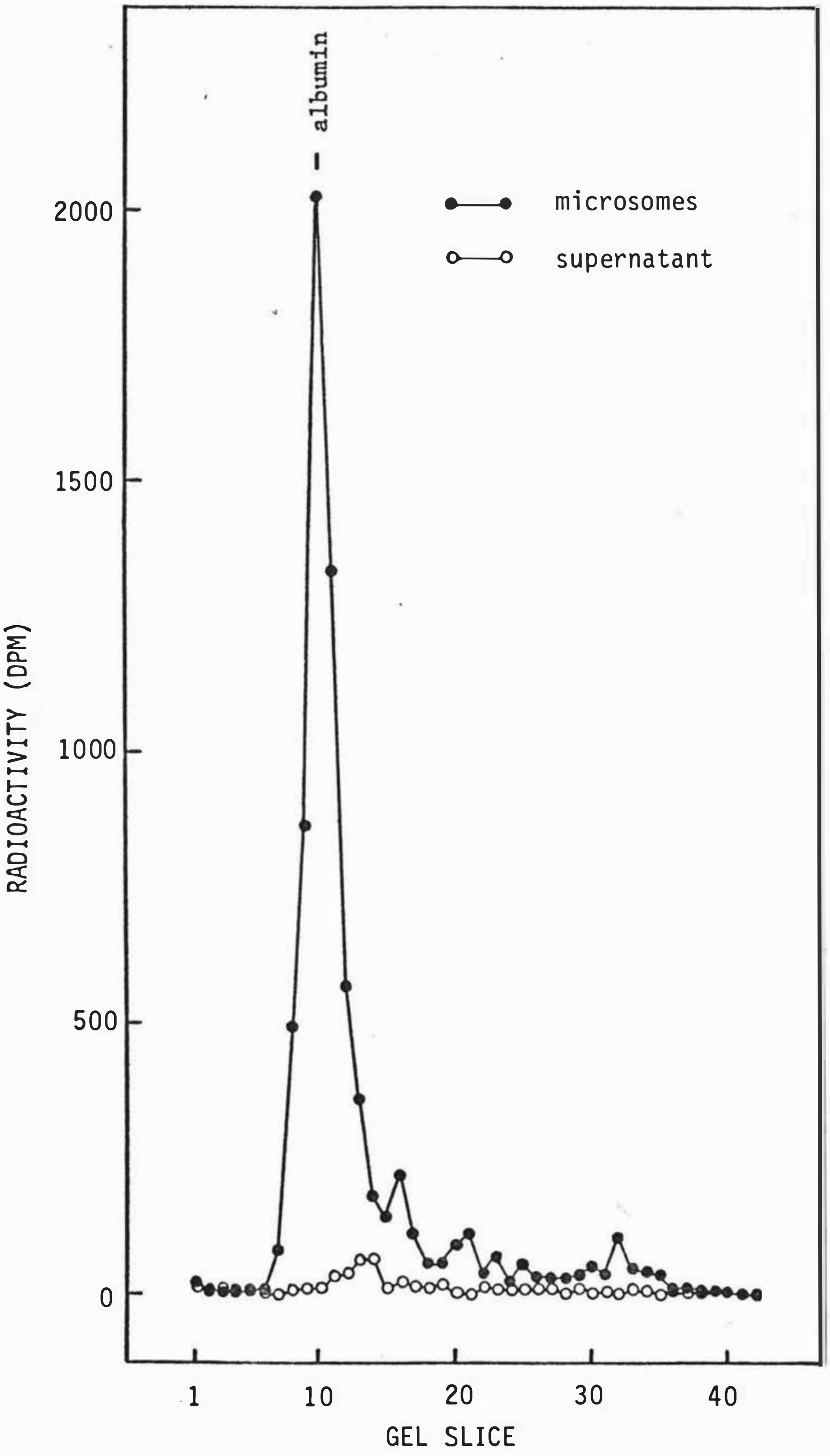


Figure 6.8

SDS GEL (7%) ANLAYSIS OF ALBUMIN IMMUNOPRECIPITATED FROM LIVER SUPERNATANT AND MICROSOME FRACTIONS AFTER A 10 MINUTE PULSE LABEL OF [^{35}S]METHIONINE VIA THE PORTAL VEIN

Albumin was precipitated from 48 260 dpm of total protein in each fraction



CHAPTER SEVEN

POLYSOME ISOLATION

7.1 Introduction

Membrane-bound and free polysomes can be separated by exploiting the difference in density or sedimentation rate of free polysomes and rough microsomes. Methods based on both these approaches have been developed to separate and quantitate rat liver polysomes (Bloemendahl et al., 1964; Webb et al., 1964; Howell et al., 1964; Lawford et al., 1966; Blobel and Potter, 1967; Venkatesan and Steele, 1972; Ramsey and Steele, 1976; Ramsey and Steele, 1977).

Conventionally rat liver polysomes separated by isopycnic centrifugation have been prepared from a postmitochondrial fraction, which is relatively free of nuclease-rich subcellular organelles (Roth, 1967), and which contains a natural nuclease inhibitor (Roth, 1956; Bont et al., 1965). Postmitochondrial fractions however contain about 90% of the free and only 30% of the bound polysomes. This approach is subject to the criticism that the postmitochondrial supernatant contains only a small, possibly nonrepresentative portion of the bound polysomes (Palade and Siekevitz, 1956; Blobel and Potter, 1967; Vankatesan and Steele, 1972). It is thus impossible to estimate the total amounts of free and bound polysomes in such a supernatant. The completeness of separation of polysomes by isopycnic centrifugation has also not been verified.

The alternative approach for polysome separation, based on size differences, has been investigated extensively by Steele and coworkers (Venkatesan and Steele, 1972; Ramsey and Steele, 1976; Ramsey and Steele, 1977). Free and bound polysomes were prepared from whole liver homogenates by salt extraction and differential centrifugation. Polysomes prepared in this manner were reported to be undegraded, both types equally active in cell-free protein synthesis, and virtually free of ribonuclease, membraneous material, glycogen, deoxycholate, completed protein and cross-contamination.

The following procedure for the preparation of free and bound polysomes was based as closely as possible on the methods of Steele and coworkers.

7.2 Materials

Sucrose (density-gradient grade, RNase-free) was obtained from Schwartz/Mann, Orangeburg, New York, and glutathione (reduced) from Sigma.

7.3 Methods

7.3.1 Preparation of free and bound polysomes

Male rats, 150-200 g were starved overnight (16 hours), stunned and killed by cervical dislocation, Ice-cold 250 mM sucrose, 1 mM $MgCl_2$ was perfused via the portal vein until the liver bleached. Livers were excised and pooled in the same medium. All further operations were performed at 4^oC.

Livers were trimmed, blotted dry and minced in a chilled stainless-steel tissue press to remove fibrous tissue. The minced liver was homogenized with 3 volumes (w/v) of 250 mM sucrose, 3 mM glutathione in 50 mM Hepes, 250 mM KCl, 5 mM $MgCl_2$, pH 7.2 (HK(250)M), with 5 strokes of a Potter-Elvehjem homogenizer (clearance 0.23 mm, rotating at 2700 rpm).

Aliquots of the homogenate (13 cm^3) were centrifuged at 740 g for 2 minutes (Beckman S.W. 41Ti rotor, 2000 rpm) and then the centrifugal force was increased and maintained at 135 000 g for 12 minutes (28 000 rpm). The supernatant was decanted and used for the purification of free polysomes. The pellet was suspended in cell sap modified to contain 250 mM KCl (see below) to a total volume of 11 cm^3. A one-ninth volume of 10% (w/w) Triton X-100 was added, the mixture homogenized with 3 strokes of a Potter-Elvehjem homogenizer (clearance 0.36 mm, rotating at 1000 rpm), and then centrifuged at 1470 g for 5 minutes (Sorvall HL-8 rotor, 2500 rpm) to sediment nuclei. Triton X-100 partially solubilizes rough membranes, but nuclei retain their structural integrity and loose their outer membrane and attached ribosomes (Hymer and Kuff, 1964; Blobel and Potter, 1966). The supernatant was decanted and mixed with a one-ninth volume of 13% (w/w) sodium dooxycholate to free bound polysomes from membraneous material.

Aliquots of the supernatants (7.0 cm^3) containing either free or bound polysomes were layered over discontinuous gradients comprised of 3.0 cm^3 each of 1.38 M and 2.00 M sucrose (modified to contain cell sap, see below) and centrifuged at 174 000 g for 20 hours (Beckman 50Ti rotor, 44 000 rpm) to pellet polysomes. After centrifugation the supernatant layers were aspirated, the tubes inverted and drained for 10 minutes. The tube walls were washed with swabs moistened with HK(75)M. Two cm^3 of HK(75)M were carefully run down the side of the tube opposite the pellet, the tube gently rotated and then inverted to drain. The polysome pellets were suspended in HK(75)M and homogenized by hand in an all glass homogenizer. For sedimentation analysis, the suspensions were clarified by centrifugation at 4000 g (Sorvall HB-4 rotor, 5000 rpm) for 5 minutes.

Polysomes prepared in this manner were found to have the same size distribution when analysed either immediately after isolation or after storage (48 hours at 4°C, 8 weeks at -20°C, or 6 months at -196°C).

Cell sap preparation

Cell sap was used as a natural source of RNase inhibitor. A 50% homogenate was prepared in HK(75)M from fasted rats, centrifuged first at 35 000 g for 10 minutes (Sorvall SS-34 rotor, 17 000 rpm) and the resulting supernatant at 361 000 g for 100 minutes (Beckman 60Ti rotor, 60 000 rpm). The supernatant (upper three-quarters, excluding the lipid layer) was removed and stored at -20°C for no longer than two months. Before use, thawed cell sap was clarified by centrifugation at 35 000 g for 10 minutes.

Discontinuous gradient preparation

Discontinuous gradients were perpared by the addition of sufficient 50% cell sap to 2.3M sucrose solutions in HK(75)M to give the desired sucrose concentration. KCl was added to give a final concentration of 250 mM.

7.3.2 Preparation of total polysomes

Liver tissue was prepared and homogenized as described. The separation of free polysomes from rough microsomes by differential centrifugation was omitted and the total homogenate processed by the detergent treatment outlined for bound polysomes. Further purification by sedimentation through cell sap-containing discontinuous sucrose gradients was as described (section 7.3.1).

7.3.3 Polysome sedimentation analysis

Five $A_{260\ nm}$ units of polysome suspension in a volume of not less than 50 mm^3 were layered over 15% isokinetic sucrose gradients containing 10 mM Hepes, 75 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, pH 7.2 and centrifuged at 286 000 g for 50 minutes (Beckman S.W. 41Ti rotor, 41 000 rpm) (Ramsey and Steele, 1976; McCarty et al., 1974). After centrifugation, gradients were monitored at 254 nm on an automatic density gradient analyser (ISCO Model 640).

7.4 Results and Discussion

The method adopted for the separation of free and bound polysomes is summarized in Figure 7.1, and produced essentially undegraded polysomes in each class (Figure 7.2). A noticeable feature of polysomes purified through 250 mM KCl was the presence of peaks with intermediate sedimentation coefficients, which have been ascribed to initiation complexes (Hoerz and McCarty, 1969). The size distribution of both classes of polysomes is given in Table 7.1. Compared to the data presented by Ramsey and Steele (1976), it can be seen that the preparations described here gave an even greater proportion of ribosomes sedimenting in the polysome region of the gradient (>2-somes). For free polysomes this represented 87% and bound 94% of the total ribosome population.

The $A_{260\ nm}/A_{280\ nm}$ ratios of both free and bound polysomes were 1.78-1.82. A value of 1.7 is generally considered to be indicative of a highly purified polysome preparation (Noll, 1969). The $A_{260\ nm}/A_{320\ nm}$ ratios indicated very little ferritin contamination. For bound polysomes the ratio was always greater than 24, and for free polysomes greater than 15. This is presumably due to the use of male rats (Helgeland, 1968).

The recovery of bound polysomes compared to free varied from 1.85 to 2.10, compared to an average value of 2.10 reported by Ramsey and Steele (1977). About 90 $A_{260\ nm}$ units of total polysomes were routinely obtained per gram wet weight of liver.

An estimate of the contamination of bound polysomes by free was obtained by resuspending the microsome-containing pellet in cell sap containing 250 mM KCl to the original homogenate volume. After resedimentation at 135 000 g for 12 minutes, the supernatant was decanted and treated as for free polysomes. From the polysomes pelleted from this intermediate supernatant, it was concluded that bound polysomes were contaminated by at most 12% free polysomes. The contamination of free polysomes by bound should be negligible, since any contaminating microsomes present in the free polysomal supernatant would not be sedimented through the 2.00 M sucrose layer, but would band at the 1.38-2.00 M sucrose interface. Ramsey and Steele (1976) obtained a more accurate estimate of cross-contamination by measuring the distribution of purified, radioactive free polysomes and microsomes. They concluded that there was about 3% contamination of bound polysomes with free and less than 1% contamination of free polysomes with bound.

Figure 7.1

ISOLATION SCHEME FOR FREE AND BOUND POLYSOMES FROM RAT LIVER

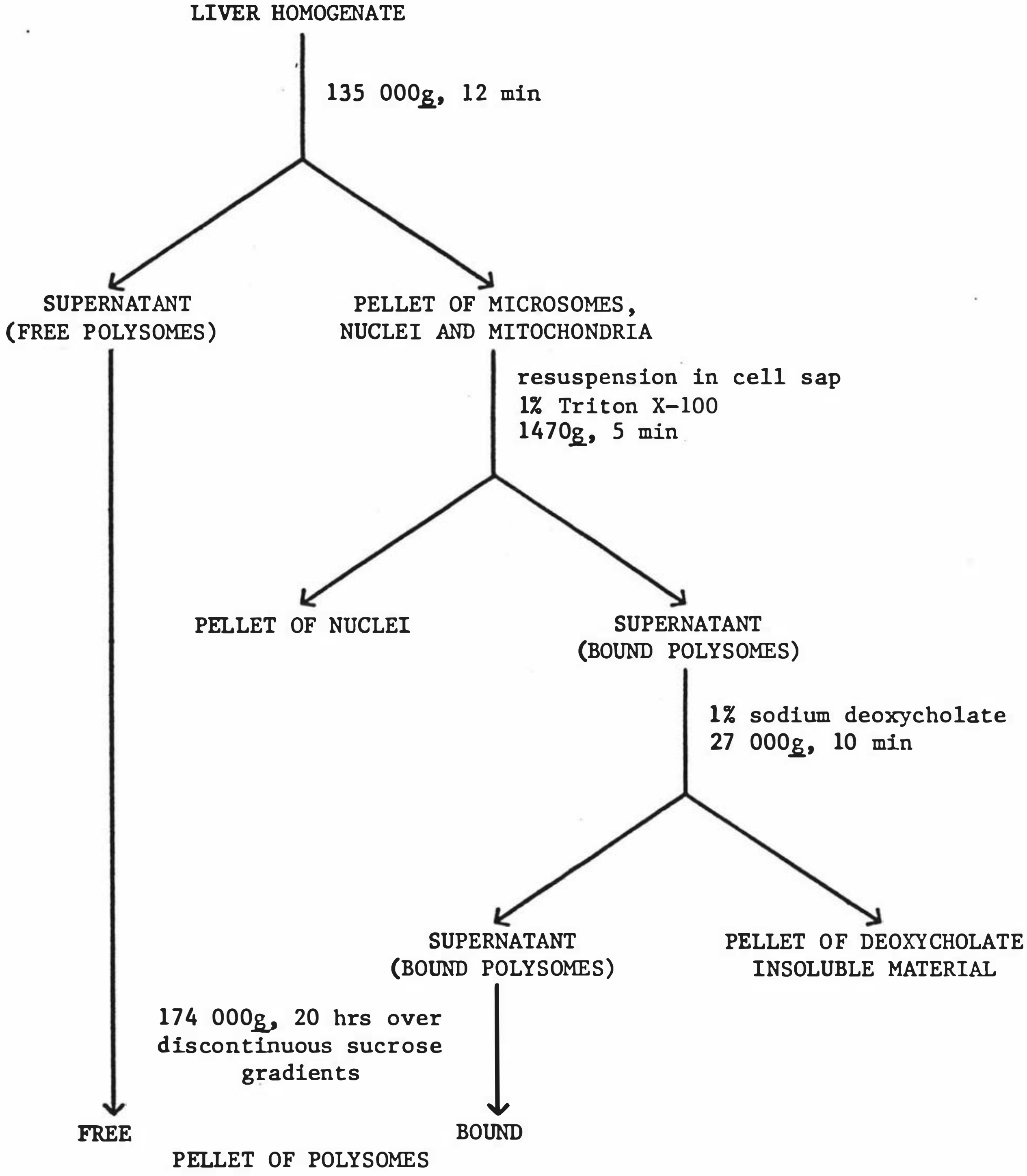


Figure 7.2

SEDIMENTATION PROFILES OF FREE AND BOUND POLYSOMES ON ISOKINETIC SUCROSE GRADIENTS

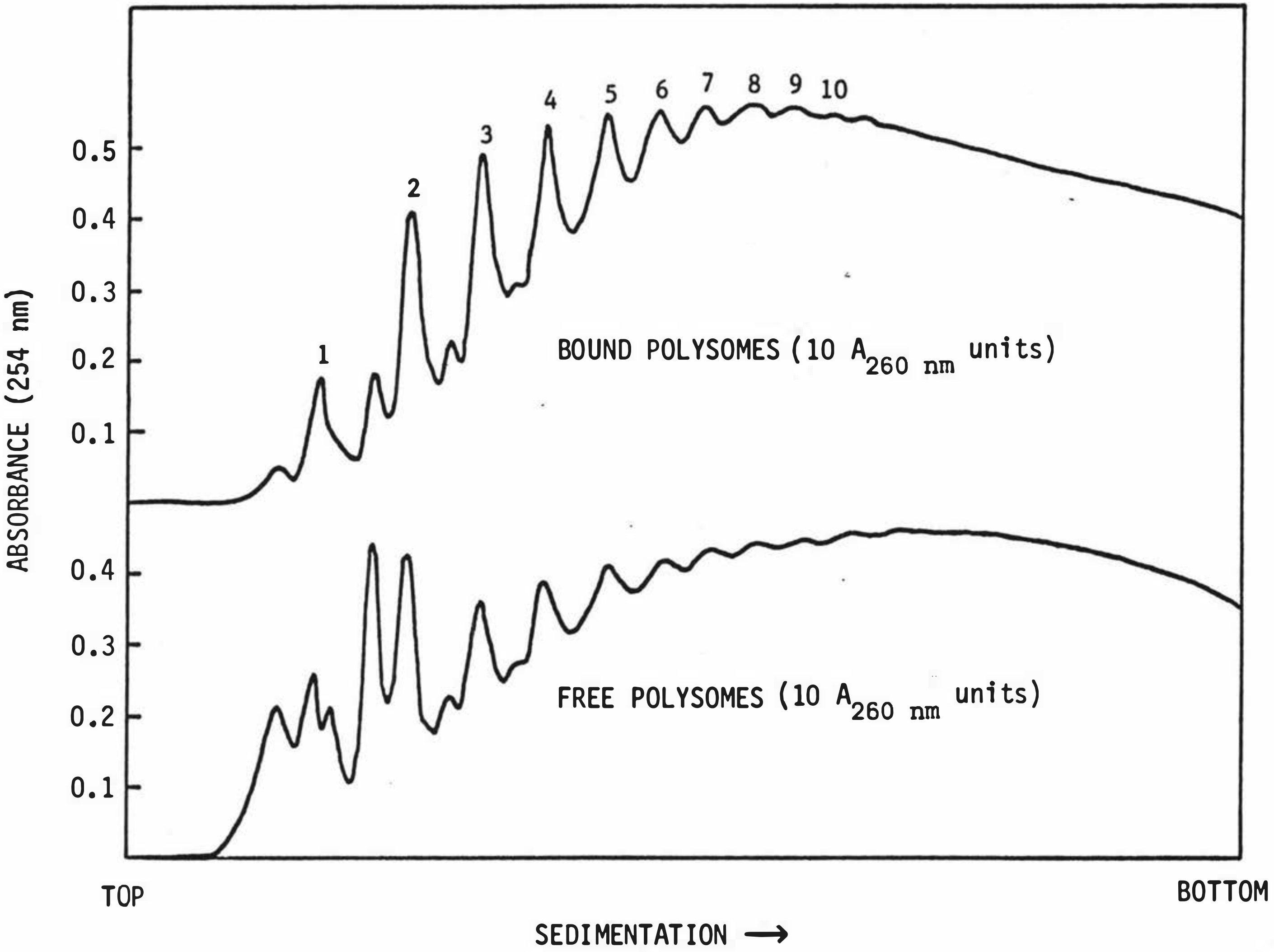


Table 7.1

SEDIMENTATION DISTRIBUTION OF POLYSOME SIZE CLASSES

Polysome class	% of total absorbance encompassed by		
	½ to 2-somes	3- to 8-somes	>8-somes
Free polysomes	13 [20]	35 [30]	52 [50]
Bound polysomes	6 [12]	37 [38]	57 [50]

[Data of Ramsey and Steele (1976)]

CHAPTER EIGHT

^{125}I-LABELLED ANTIBODY BINDING TO POLYSOMES

8.1 Introduction

The identification, localization and quantitation of specific mRNA templates can be achieved in part through the use of immunochemical techniques which permit indirect recognition of the nascent protein chains of polysome-mRNA complexes.

It has been shown that antibodies raised against completed proteins will bind to the nascent chains of polysomes synthesizing those proteins: rat albumin (Warren and Peters, 1965); immunoglobulin light and heavy chains (Holme et al., 1971a,b; Alexander et al., 1978); chicken ovalbumin, muscle myosin, neural retinal glutamine synthetase and reticulocyte histone H5 (Palacios et al., 1972; Allen and Terrence, 1968; Sarkar and Moscona, 1973; Scott and Wells, 1975, 1976); bovine thyroglobulin (Vassart and Dumont, 1973); and sheep caseins (Houdebine and Gaye, 1976).

The binding of radiolabelled antibody molecules to polysomes has been used to study both the qualitative and quantitative aspects of the synthesis of specific proteins. In particular, binding to polysomes from animals under various hormonal and dietary regimes and the relative binding to free and bound polysomes have been studied: chicken oviduct [ovalbumin, conalbumin, ovomucoid and lysozyme] (Palmiter et al., 1972; Palmiter and Haines, 1973; González et al., 1974; Groner et al., 1977; Schütz et al., 1977); rat liver [albumin, ferritin, serine dehydratase, fibrinogen, fatty acid synthetase, α-fetoprotein, catalase and acetyl CoA carboxylase](Higashi et al., 1972; Ikehara and Pitot, 1973; Konijin et al., 1973; Taylor and Schimke, 1974; Bouma et al., 1975; Alberts et al., 1975; Keller and Taylor, 1976; McLaughlin and Pitot, 1976a,b; Nakanishi et al., 1976; Tanabe et al., 1976; Innis and Miller, 1977); mouse myeloma [immunoglobulin light chain](Schecter, 1974); and chicken liver [vitellogenin](Jost and Pehling, 1976).

The extent of antibody binding to isolated polysomes has been found to correlate well with flucuations in the level of synthesis of particular proteins. Antibodies to exported proteins bound predominantly to bound polysomes, whereas intracellular proteins bound to both classes of polysome.

A study of the binding of ^{125}I-labelled anti-FBPase to free and bound polysomes was undertaken in order to estimate their relative synthetic capacity for FBPase. Labelled anti-albumin binding to free and bound polysomes was studied in conjunction, since the binding characteristics for this protein have already been extensively studied and have confirmed that bound polysomes are the sole site of albumin synthesis (Warren and Peters, 1965; Ikehara and Pitot, 1973; Taylor and Schimke, 1974; Keller and Taylor, 1976; McLauglin and Pitot, 1976a,b). Labelled anti-ovalbumin was used to assess non-specific interactions, since it does not cross-react with mammalian proteins (Palmiter et al., 1971). In general the reaction conditions of Taylor and Schimke (1974) were followed. Non-specific interactions reported by Holme et al., (1971a,b) and Ikehara and Pitot (1973) were minimised by the use of antibody preparations that had been purified by affinity chromography.

The basic principle involved in the quantitation of the specific polysomes synthesizing a particular protein is that the polysomes are added at levels such that they are the limiting reactant. Estimates of the relative amount of actively translating polysomal units synthesizing a single protein can be made with the proviso that changes in antibody binding do not necessarily reflect changes in the quantity of translatable RNA for the protein being studied. However, the binding would not be affected by variations in the rate of translation, provided that such a variation did not result in changes in the number of ribosomes per unit length of polysome.

8.2 Materials

^{125}Iodine (100 mCi per cm^3, carrier free in NaOH solution, pH 8-11) was obtained from the Radiochemical Centre. Lactoperoxidase (E.C. 1.11.1.7, bovine milk) was from Sigma and was dialysed against PB before use. Sucrose (density gradient grade, RNase-free) was from Schwartz/Mann and Biogel P150 (100-200 mesh) was from Biorad Laboratories.

Ribonuclease I (E.C. 3.1.4.22, bovine pancreas) was from BDH. DEAE- and CM-cellulose (DE-23 and CM-23) were obtained from Whatman, precycled according to the manufacturer's instructions and equilibrated in PB. Cellulose slurries were treated with 0.1% diethylpyrocarbonate 24 hours before use to inactivate any RNase.

8.3 Methods

8.3.1 Polysome preparation

Free and bound polysomes were prepared as described (section 7.3.1) with one modification: cell sap was omitted from the 1.38 M and 2.00 M sucrose solutions. This was necessary to ensure that cytoplasmic FBPase and serum albumin did not contaminate the polysome pellet and interfere with subsequent antibody-polysome binding. Such contamination was evidenced by a pellet of antigen-^{125}I-antibody sedimenting to the bottom of sucrose gradients in antibody-polysome binding experiments.

8.3.2 Antibody iodination

Antibodies were iodinated by the lactoperoxidase method of Taylor and Schimke (1973). To 10 mg of affinity-purified antibody in 3 cm^3 of PB were added 100 mm^3 of lactoperoxidase (2.5 mg per cm^3), 90 mm^3 of 0.1 M NaOH, 300 mm^3 of freshly-prepared 100 μM H_2O_2, and 1 mCi of ^{125}iodine. The mixture was allowed to stand at room temperature for 30 minutes, followed by the addition of an equal volume of saturated ammonium sulphate (pH 7.0). After an additional 30 minutes at 4°C, the precipitate (containing the ^{125}I-antibody) was collected by centrifugation and dissolved in the minimum volume of PB. This material was applied to a 1.6 x 40 cm column of Biogel P150 equilibrated in PB, and eluted at 10 cm^3 per hour. Fractions of 1 cm^3 were collected and monitored for protein and radioactivity. The four fractions centered around the antibody protein peak were combined and made RNase-free by application to a column of 1 cm^3 of both DEAE- and CM-cellulose and eluted with PB.

8.3.3 Polysome binding experiments

Antibody was mixed with polysomes and the reaction mixture brought to 150 mm^3 with HK(75)M. After standing on ice for 30 minutes, the mixtures were layered over 15% isokinetic sucrose gradients (section 7.3.3) and centrifuged at 286 000 g for 50 minutes (Beckman S.W. 41Ti rotor, 41 000 rpm). In experiments involving RNase treatment of polysome-antibody complexes, reaction mixtures were incubated with 10 μg of RNase for 30 minutes at 0^oC after the initial 30 minute binding step. Reaction mixtures were then centrifuged at 286 000 g for 65 minutes. After centrifugation, tubes were pierced and gradients pumped out at 0.6 cm^3 per minute on a modified density gradient fractionator (ISCO Model 640). Fractions of 0.4 cm^3 were monitored for radioactivity and RNA at 254 nm.

8.4 Results and Discussion

Figures 8.1 and 8.2 show the binding of 10 μg each of anti-albumin, anti-FBPase and anti-ovalbumin to 10 $A_{260\ nm}$ units of free and bound polysomes. The specificity of binding was demonstrated by the lack of binding to liver polysomes of anti-ovalbumin, a non-mammalian protein. An important characteristic of the binding curves was that radioactivity was not uniformly distributed along the absorbance profile but was associated with the large polysome fraction. The radioactivity in the supernatant at the top of the gradient represented unreacted gamma globulin molecules. That the binding sites were specific was further indicated by the fact that the extent of binding of both anti-albumin and anti-FBPase was unaffected by the inclusion of 250 μg of unlabelled ovalbumin into the reaction mixture. Binding sites were apparently limited under the experimental conditions, since when polysomes were incubated for 30 minutes with 500 μg of specific unlabelled antibody prior to addition of ^{125}I-antibody, no binding to polysomes was found.

The sucrose density gradient distribution of antibody indicated that both antibodies are bound to polysomes of large size, although the peak of anti-albumin radioactivity did not correspond to the expected 19 to 20-some size class that would have been postulated on the basis of antibody binding to other nascent protein. A general relationship relating polysome size class and the size of the protein being synthesized has been established for ovalbumin (Palmiter et al., 1972; González et al., 1974; Schütz et al., 1977; Groner et al., 1977), ovomucoid and lysozyme (Groner et al., 1977), globin (Warner et al., 1962), casein (Houdebine and Gaye, 1976) and proinsulin (Tjioe and Kroon, 1973). The size class is roughly the protein molecular weight divided by 3300 (i.e. a spacing of about 90 bases between ribosomes).

Electron microscopic analysis of chick oviduct confirmed the in vivo polysome size class synthesizing ovalbumin to be 12-13 ribosomes (Palmiter et al., 1970). The binding of anti-albumin (Ikehara and Pitot, 1973; Taylor and Schimke, 1974; Keller and Taylor, 1976), anti-fibrinogen (Bouma et al., 1975) and anti-fatty acid synthetase (Alberts et al., 1975) to rat liver polysomes gave a heterodisperse distribution of label, with a peak associated with a smaller polysome size class than would have been postulated by the relationship above. Thyroglobulin synthesis on thyroid polysomes has also been shown to be predominantly associated with a much smaller size class than expected (Vassart and Dumont, 1973). In contrast, ferritin isoenzyme synthesis on rat liver free and bound polysomes correlated well with the molecular weight of the synthetized protein (Konijn et al., 1973). This may indicate a breakdown of the relationship between mRNA size and the number of ribosomes associated with it as the size of the messenger increases. This could be attributed to a higher ratio of elongation to initiation for such messengers. The binding of anti-FBPase to both free and bound polysomes did not show any pronounced peak but was rather broad and heterodisperse. However the position of maximum binding for anti-FBPase also corresponded to a smaller polysome size class than would have been expected from the molecular weight of the enzyme subunits. The binding of antibody throughout the polysome profile may also indicate extensive immunological cross-linking between nascent protein chains on adjacent polysomes. The binding of antibody to individual ribosomes should not alter the sedimentation characteristics of the polysome, since the change in molecular weight upon binding is less than 1%.

To demonstrate that antibody was bound to nascent protein chains on polysomes, antibody-polysome complexes were treated with RNase. Bound polysomes were incubated with ^{125}I-antibody followed by 10 μg of RNase before sucrose gradient centrifugation (Figure 8.3). The absorbance profile indicated extensive enzymatic breakdown to ribosome monomers and small polysome size classes. The peak of radioactivity for anti-albumin was primarily in the 2-some region, with a considerable amount of radioactivity bound to larger aggregates, as was the case for anti-FBPase. This was probably indicative of immunological cross-linking between nascent protein chains. Treatment of polysomes with RNase caused an enhancement of antibody binding. It is conceivable that formation of monomeric ribosomal units reduced steric hindrance which might affect the interaction of the antibody with nascent protein chains attached to large polymeric ribosomal complexes (McLaughlin and Pitot, 1976a,b). It would also appear that the potentially RNase sensitive phosphodiester bond in the tRNA portion of the peptidyl-tRNA molecule is protected from attack, presumably by steric hindrance resulting from antibody binding.

The specificity of antibody binding to nascent protein chains should allow a quantitative estimate of the synthesis of both albumin and FBPase on both free and bound polysomes (Taylor and Schimke, 1974; Keller and Taylor, 1976). Constant amounts of polysome were incubated with varying amounts of antibody and the mixtures examined by sucrose density gradient centrifugation. The radioactivity bound to polysomes larger than 2-somes was summed, and the results are summarized in Figure 8.4. Increasing amounts of antibody added resulted in an increase in the amount of antibody bound. The binding of anti-albumin predominantly to bound polysomes was preserved over the range of antibody added (bound to free ratio of 19.2-25.3) as was the binding of anti-FBPase to both classes of polysome (bound to free ratio of 0.85-1.32). When the amount of binding was expressed as a percentage of the quantity of antibody added (Figures 8.5 and 8.6), relatively less antibody was bound at higher concentrations, suggesting that binding to polysomes was approaching saturation. Taylor and Schimke (1974) reported that for anti-albumin, saturation was not readily achieved in such short term incubations, and longer incubations resulted in polysome degradation. This non-achievement of saturation could be a function of the heterogeneity of binding sites on both polysomes and antibody.

The alternative approach to quantitating binding, namely examining the binding to varying amounts of polysomes at a fixed antibody concentration was also investigated. Again a much greater degree of binding of anti-albumin to bound polysomes and an almost equal degree of binding of anti-FBPase to both classes of polysome was demonstrated (Figure 8.7). A greater variation in the bound to free binding ratio for anti-albumin (5.1-18.8) was a reflection of the increased binding of antibody to free, albumin-synthesizing polysomes at high antibody to polysome ratios.

Figure 8.1

BINDING OF ^{125}I-LABELLED ANTIBODIES TO FREE POLYSOMES

10 μg of affinity-purified antibody were incubated with 10 $A_{260\ nm}$ units of polysomes

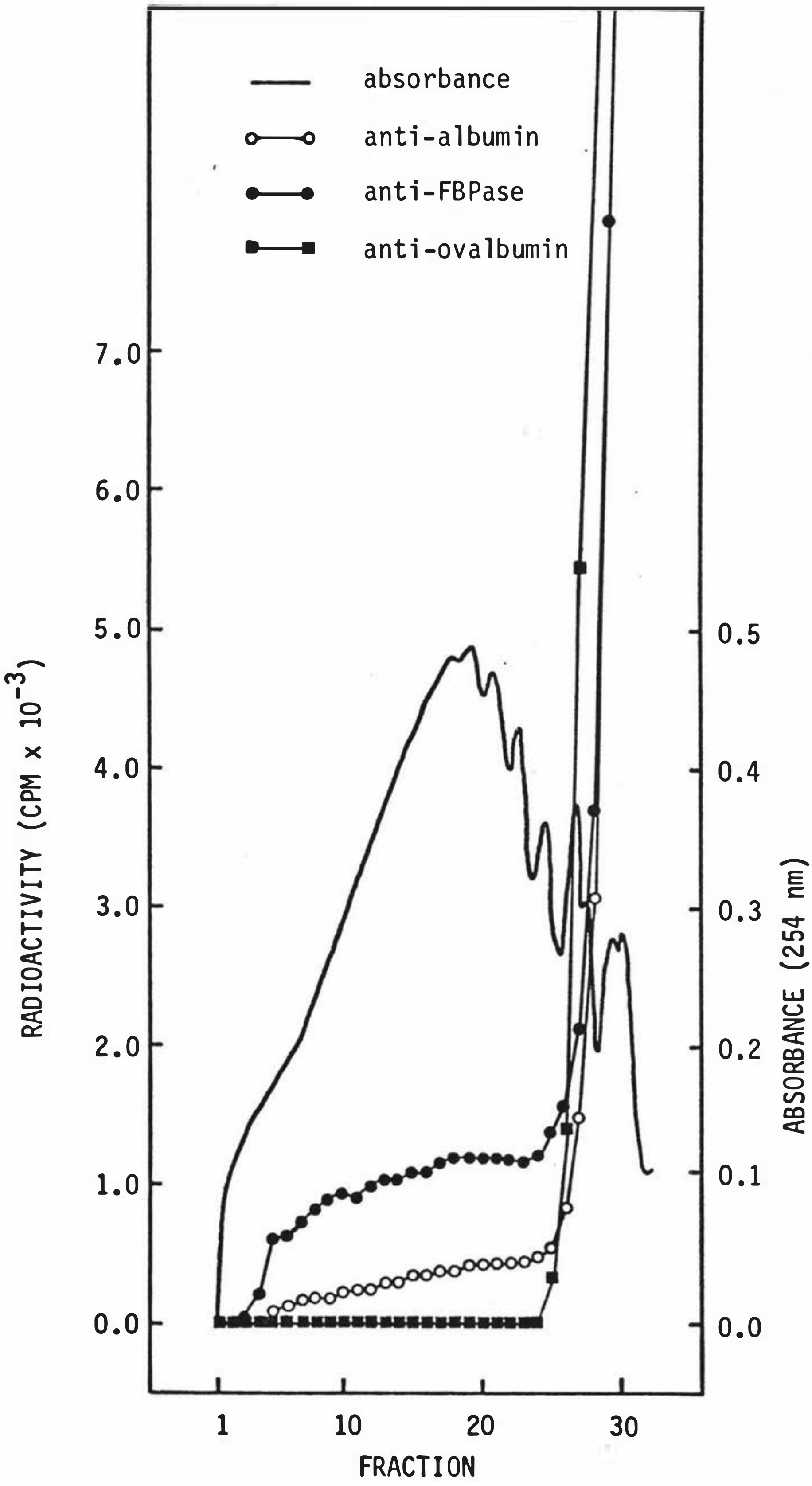


Figure 8.2

BINDING OF ^{125}I-LABELLED ANTIBODIES TO BOUND POLYSOMES

10 μg of affinity-purified antibody were incubated with 10 $A_{260\ nm}$ units of polysomes

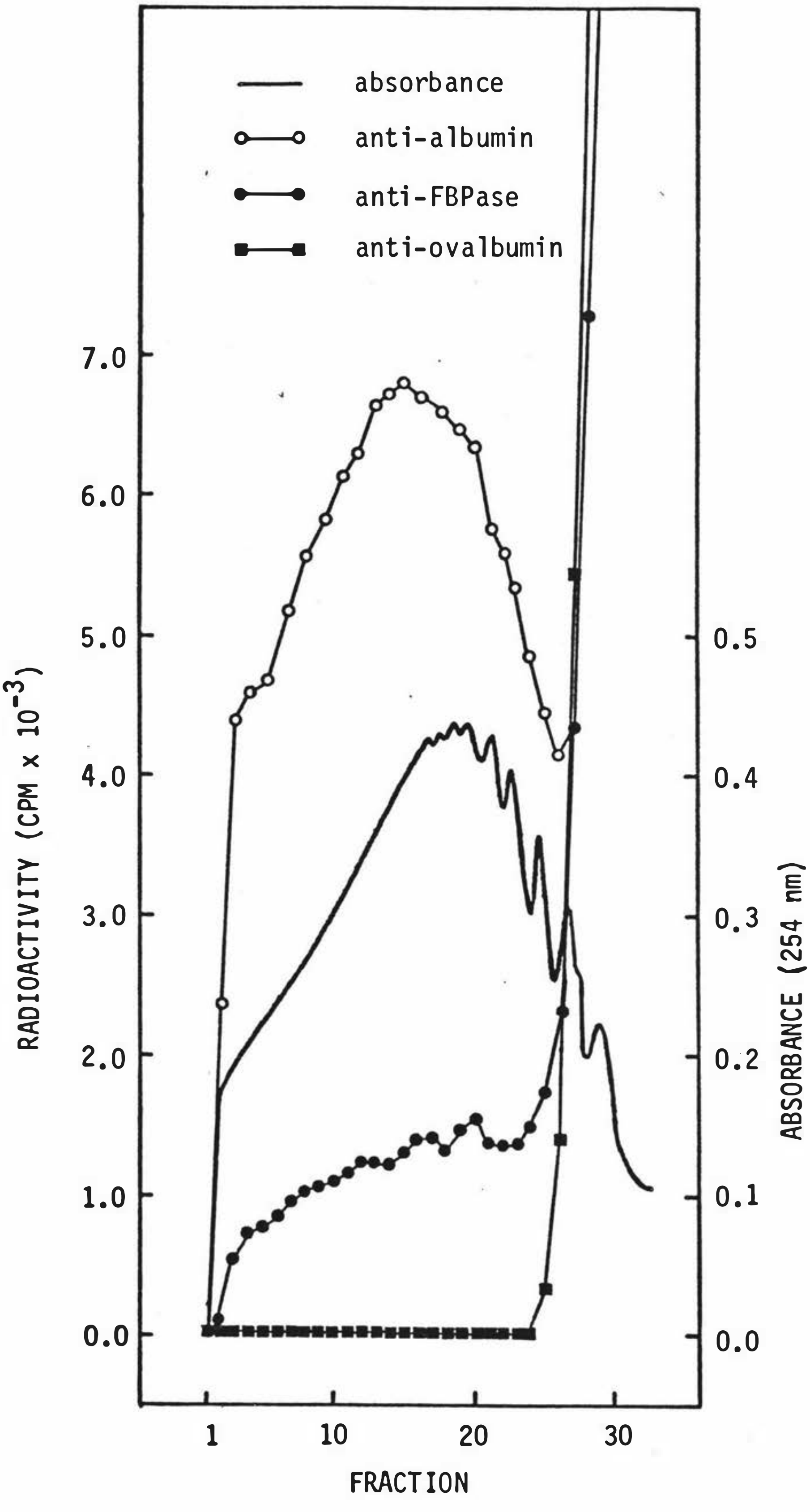


Figure 8.3

EFFECT OF RNase ON THE BINDING OF ^{125}I-LABELLED ANTIBODIES TO BOUND POLYSOMES

10 µg of affinity-purified antibody were incubated with 10 $A_{260\ nm}$ units of polysomes followed by incubation with 10 µg of RNase

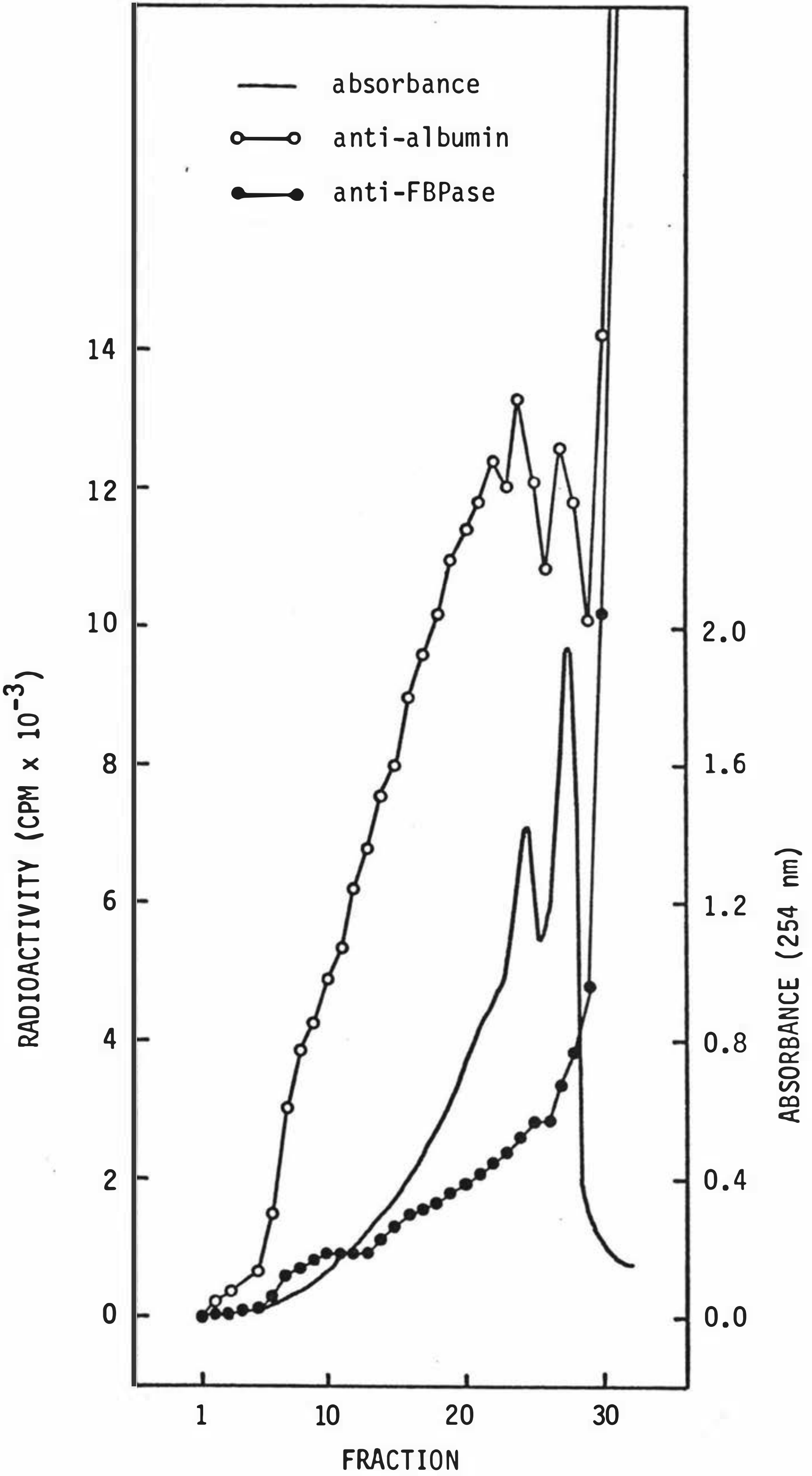


Figure 8.4

BINDING OF DIFFERENT CONCENTRATIONS OF ^{125}I-LABELLED ANTI-ALBUMIN AND ANTI-FRUCTOSE-1,6-BISPHOSPHATASE TO FREE AND BOUND POLYSOMES

Affinity-purified antibody was incubated with 10 $A_{260\ nm}$ units of polysomes

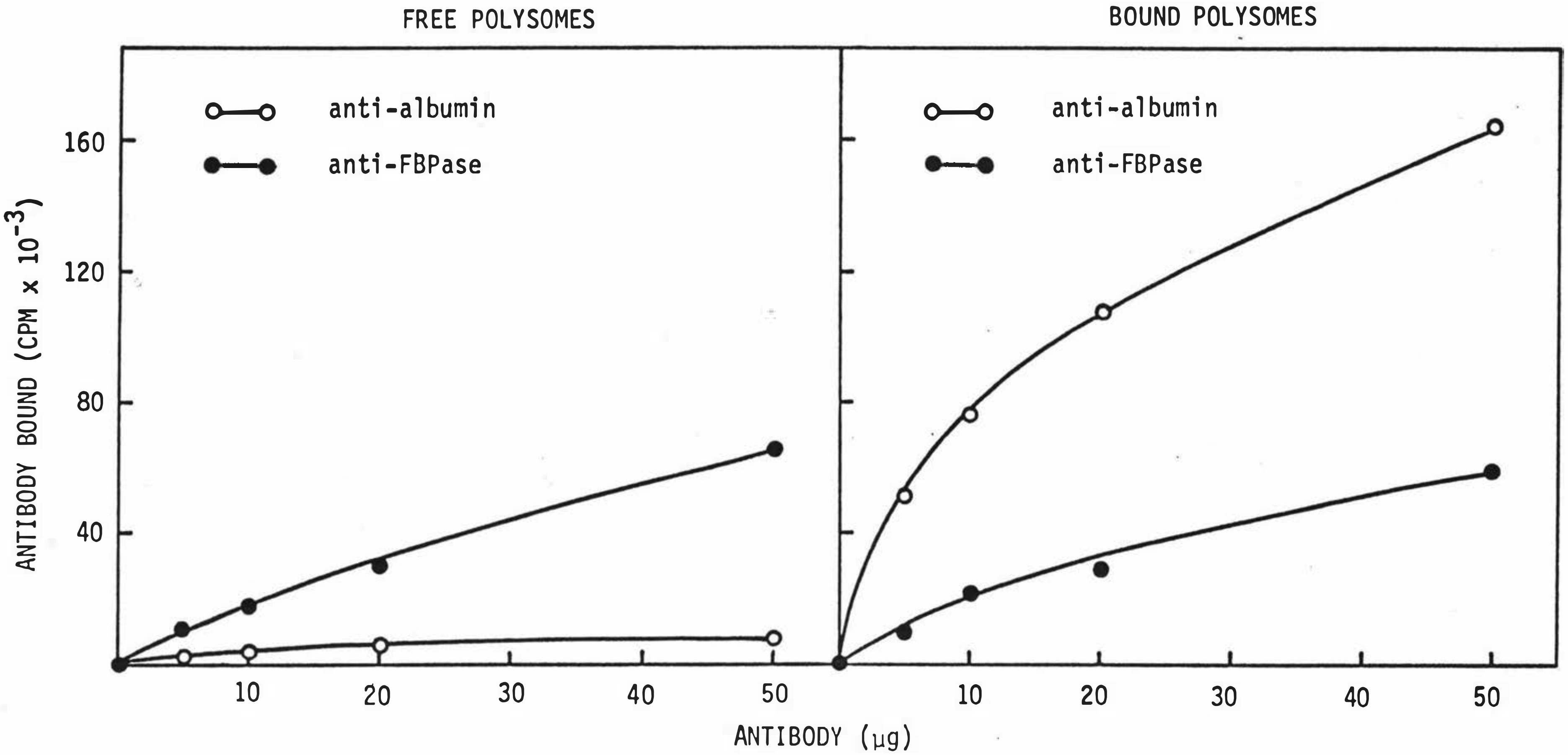


Figure 8.5

RELATIVE AMOUNT OF ^{125}I-LABELLED ANTI-FRUCTOSE-1,6-BISPHOSPHATASE BINDING TO FREE AND BOUND POLYSOMES

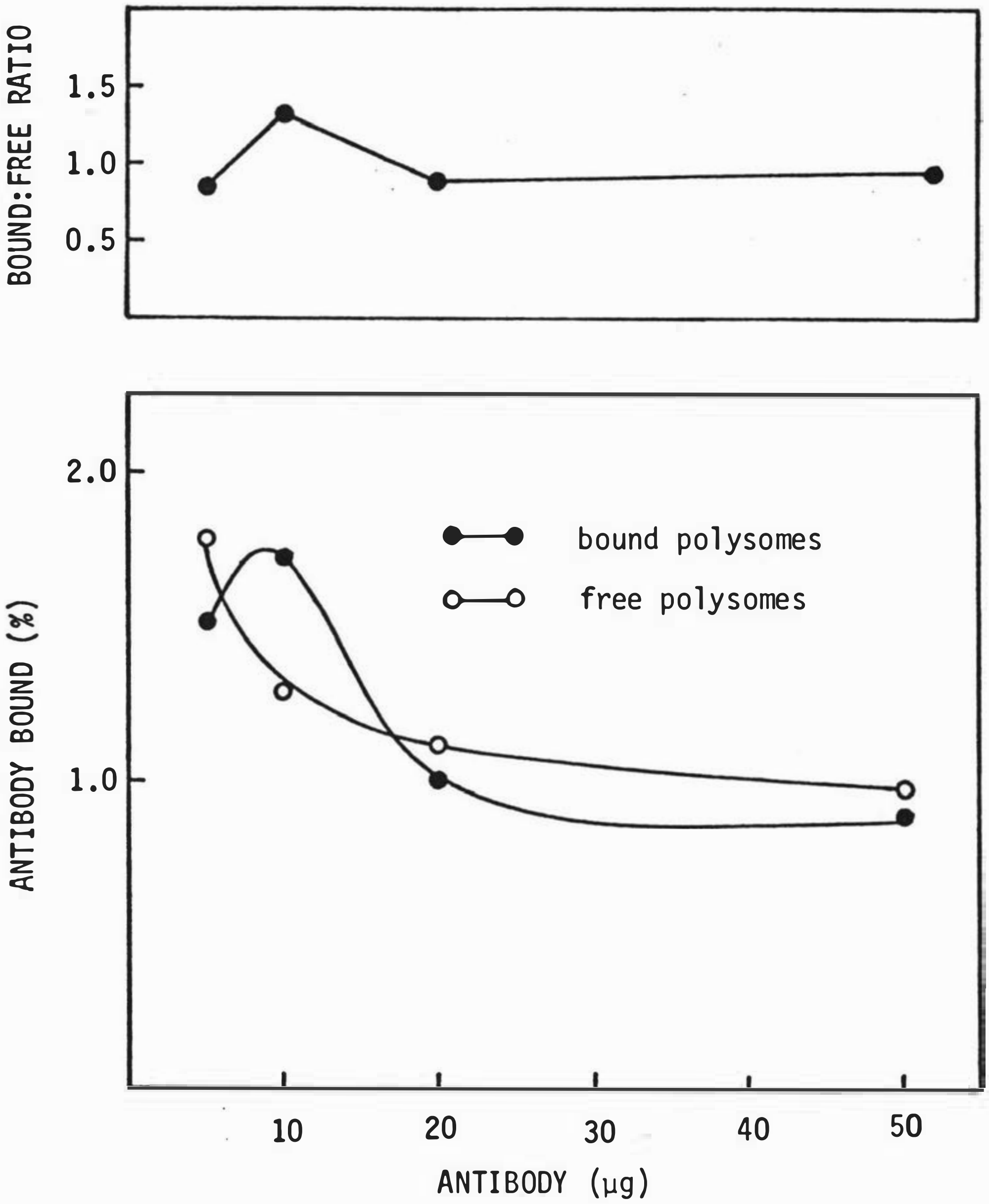


Figure 8.6

RELATIVE AMOUNT OF ^{125}I-LABELLED ANTI-ALBUMIN BINDING TO FREE AND BOUND POLYSOMES

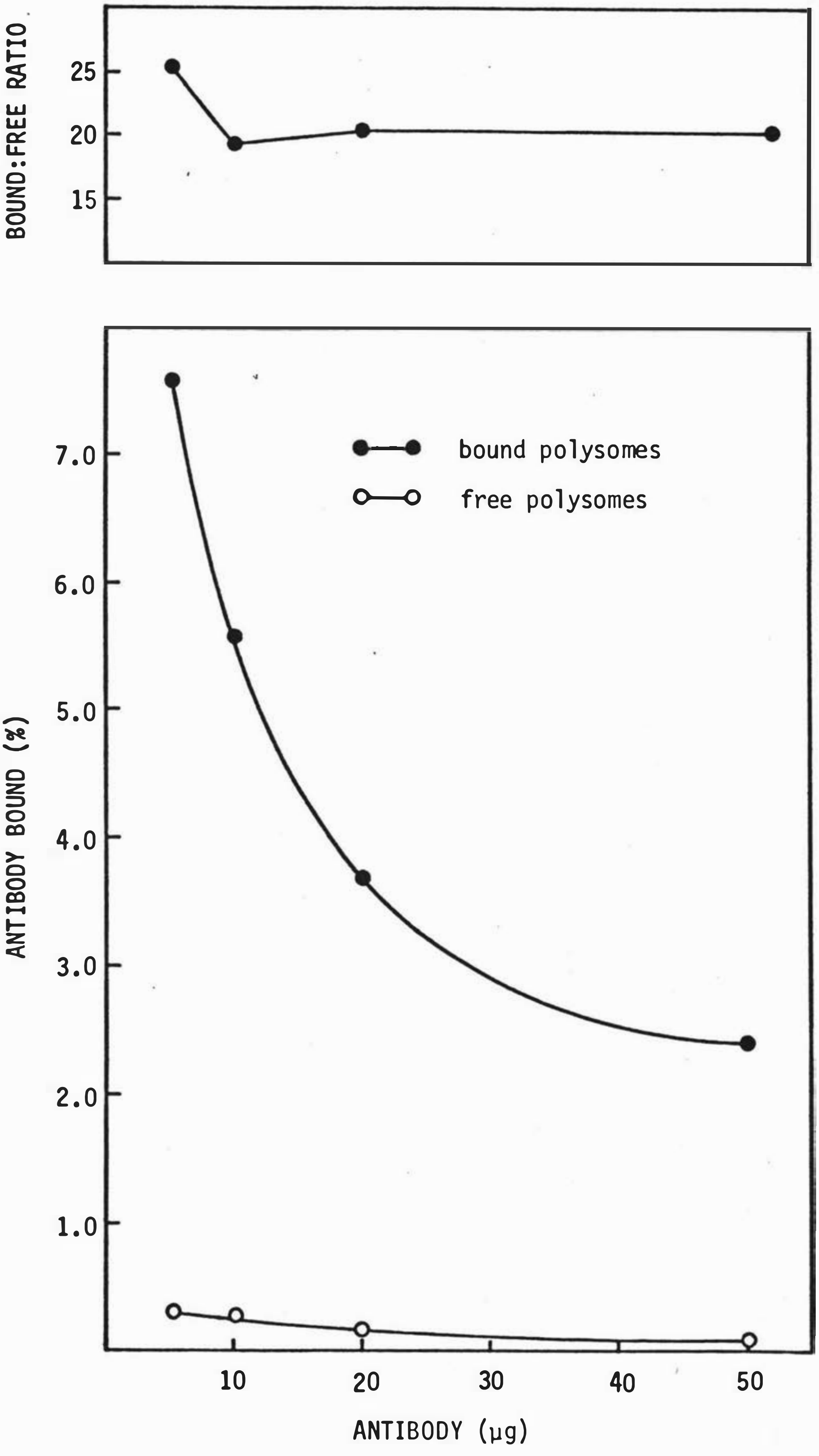
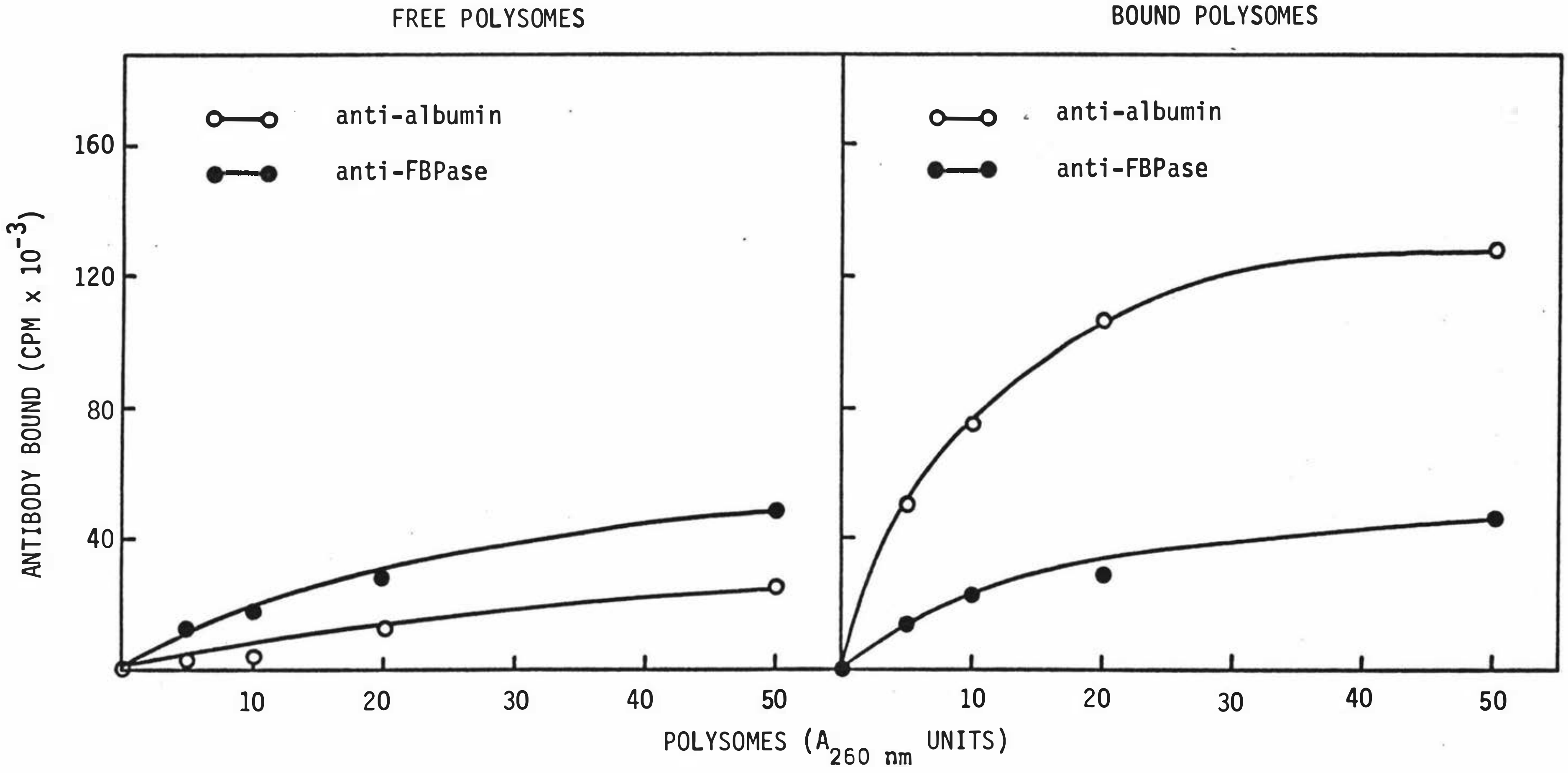


Figure 8.7

BINDING OF ^{125}I-LABELLED ANTI-ALBUMIN AND ANTI-FRUCTOSE-1,6-BISPHOSPHATASE TO DIFFERENT CONCENTRATIONS OF FREE AND BOUND POLYSOMES

Polysomes were incubated with 10 µg of affinity-purified antibodies



CHAPTER NINE

PROTEIN SYNTHESIS BY ISOLATED POLYSOMES *IN VITRO*

9.1 Introduction

The isolation of free and bound polysomes, with intact mRNA and nascent peptide chains, should allow such peptide chains to be completed *in vitro* to give full-length protein transcripts. The subsequent immunochemical isolation of specific proteins from such transcripts should provide an estimate of the relative synthetic capacity of free and bound polysomes for a particular protein. This should reflect the *in vivo* situation at the time of polysome isolation.

The ability of isolated free and bound polysomes from rat liver to incorporate amino acids into protein has been found to differ in many studies. Depending on the polysome isolation procedure and incubation conditions, bound polysomes have been reported to be more active (Campbell et al., 1964; Hallinan and Munro, 1964; Takagi and Ogata, 1968; Tanaka and Ogata, 1971), equally active (Manganiello and Phillips, 1965; Kwan et al., 1968), and less active (Ganoza and Williams, 1969; Sabatini and Blobel, 1970; Uenoyama and Ono, 1972; Venkatesan and Steele, 1972) than free polysomes.

Of all the data distinguishing free and bound polysomes, the most compelling in significance has been the demonstration of special template activities displayed by these two classes. A number of exported proteins including albumin (Redman, 1968; Hicks et al., 1969; Takagi and Ogata, 1968,1969; Ganoza and Williams, 1969; Takagi et al., 1969, 1970; Glaumann, 1970; Tanaka and Ogata, 1972; Ueoyama and Ono, 1972; Zähringer et al., 1977), and various glycoproteins (Hallinan et al., 1968) have been reported to primarily synthesized on bound polysomes. The intracellular proteins ferritin (Redman, 1969; Zähringer et al., 1977) and arginase (Tanaka and Ogata, 1971) have been reported to by synthesized predominantly on free polysomes. However the intracellular proteins serine dehydratase (Pitot and Jost, 1968), catalase (Ono, 1970; Takagi et al., 1970; Kashiwagi et al., 1971), NADPH-cytochrome c reductase (Ragnotti et al., 1969) and cytochrome c (González-Cadavid and Sáez de Córdova, 1974) were shown to be synthesized on both free and bound polysomes, but not exported from the liver.

In addition, the regulation of synthesis of hepatic acetyl CoA carboxylase (Horikawa et al., 1977), phosphoenolpyruvate carboxykinase (Iynedjian and Hanson, 1977) and fatty acid synthetase (Strauss et al., 1975) have been studied in unfractionated polysomal preparations.

Several reports dating back to 1954 (Zamecnik and Keller) have described conditions for assaying amino acid incorporation by rat liver polysome preparations in vitro, in the presence of cell sap or 'soluble factors'. Huston et al. (1970) have extensively investigated the rat liver system and have reported optimal conditions for protein synthesis. Cell-free protein synthesis was performed according to Huston et al. (1970) with modifications described by Ramsey and Steele (1976). Total released products, as well as immunoprecipitable albumin and FBPase from free and bound polysome-containing incubation mixtures were analysed by SDS gel elctrophoresis.

9.2 Materials

Hepes, DTT, GTP (diNa salt), glutathione (reduced), creatine phosphate (diNa salt) and creatine phosphokinase (E.C. 2.7.3.2, rabbit muscle) were obtained from Sigma. ATP (diNa salt) was from Fluka A.G., Buchs, and unlabelled amino acids from Schwartz/Mann. Sephadex G10 was from Pharmacia, Uppsala, and L-[^{35}S]methionine was from the Radiochemical Centre.

9.3 Methods

9.3.1 Cell sap preparation

Cell sap was prepared just before use as described (section 7.3.1). Cell sap (15 cm^3 was loaded onto a 2.0 x 40 cm column of Sephadex G10 equilibrated in HK(75)M, containing 250 mM sucrose and 3 mM glutathione. The column of developed at 1.0 cm^3 per minute with the same buffer. The first 10 cm^3 of protein-containing eluate was collected, which was essentially free of amino acids (Huston et al., 1970)

9.3.2 Translation assays

A reaction mixture of 1.0 cm^3 with a final pH of 7.3 (30^oC) contained: 50 mM Hepes, 250 mM sucrose, 80 mM KCl, 5 mM magnesium acetate, 1 mM ATP, 0.1 mM GTP, 20 mM creatine phosphate, 20 µg of creatine phosphokinase, 20 µM each of the 19 amino acids required for protein synthesis (no methionine), 10 mg of Sephadex-treated cell sap, and about 5 µCi of L-[^{35}S]methionine. Polysomes (7.5 $A_{260\ nm}$ units) were added as indicated in the text.

The reaction mixtures were incubated at 30^oC for 40 minutes. Duplicate aliquots of 10 mm^3 were spotted onto 2 x 2 cm squares of Whatman 3MM paper at the times indicated and processed as described (section 2.10.2).

9.3.3 Analysis of translation products

Ribosomes were removed from the final reaction mixture by centrifugation at 200 000 g for 1 hour at 4^oC (Beckman S.W. 41 Ti rotor, 41 000 rpm). Centrifugation was performed in 500 mm^3 conical plastic tubes, floating in water-filled ultracentrifuge tubes.

The postribosomal supernatant, containing the released radioactive peptides was carefully removed. Duplicate aliquots were spotted on 3MM paper as above. Protein was precipitated from duplicate aliquots (90 mm^3) by the addition of a 20 volume excess of 10% TCA, 10 mM methionine, heating to 90^oC for 10 minutes to hydrolyse methionine-tRNA, and standing overnight at 4^oC. Precipitated protein was collected by centrifugation and the pellet washed by suspension and recentrifugation twice in TCA-methionine and once in ice-cold water. Pellets were dissolved in 1.0 cm^3 of 0.1 M NaOH in preparation for liquid scintillation counting (section 2.10), or analysed by SDS gel electrophoresis (section 2.7.2) and the radioactivity incorporated into individual gel slices was determined (section 2.10.1).

9.3.4 Immunoprecipitation assay

Triton X-100 and sodium deoxycholate were added to the postribosomal supernatant to give a final concentration of 1% in each. Sufficient antibody was added to duplicate aliquots (0.25-1.0 cm^3) of detergent-treated supernatant to give an immunoprecipitate containing about

70 μg of antigen. After incubation at 37°C for 30 minutes and overnight at 4°C, immunoprecipitates were collected by centrifugation through discontinuous sucrose gradients (section 6.2.4). Immunoprecipitates were analysed by SDS gel electrophoresis or the radioactivity determined by liquid scintillation counting after dissolution in 1.0 cm^3 of 0.1 M NaOH. Immunoprecipitated FBPase and albumin from labelled rat liver homogenate were used to identify FBPase and albumin synthesized *in vitro*.

9.4 Results and Discussion

Both free and bound polysomes were active in protein synthesis (Figure 9.1). Isotope was incorporated into protein at a linear rate for 20 minutes, and incorporation was complete at about 40 minutes. Free and bound polysome preparations showed similar initial rates of incorporation, however the incorporating ability of bound polysomes decreased more rapidly than that of free polysomes. These results are in line with the observations of other workers (MacDond and Korner, 1971; Sabatini and Blobel, 1970; Chu et al., 1978). The lower incorporation exhibited by bound polysomes was not due to detergent treatment during isolation, since isolated total polysomes incorporated isotope at levels intermediate between free and bound polysomes. The maintenance of active protein synthesis for as long as 20 minutes probably reflects a rate of peptide elongation slower than *in vivo*.

Approximately 70% of radioactivity incorporated into TCA-precipitable protein was released from free polysomes and 65% from bound polysomes. Released polypeptides were analysed by SDS gel electrophoresis (Figure 9.2), and were shown to migrate over a wide range of molecular weights. The profiles for free and bound polysomes were clearly different. However, the molecular weight distribution of peptides synthesized did not correspond to the distribution of pulse labelled cytoplasmic and microsomal proteins (section 6.4), particularly at higher molecular weights. This phenomenon has been reported to be caused by a decline in the rate of elongation and release of proteins with increasing polysome size (Ramsey and Steele, 1976). Another noticeable feature was the large amount of radioactivity associated with very short peptides that migrated near the bromophenol blue dye front. This seems to be a common feature of *in vitro* polysomal protein-synthesizing systems (Ramsey and Steele, 1976), and probably represents prematurely terminated peptidyl tRNAs. The low molecular weight and high radioactivity of these peptides may be due to reinitiation of mRNA followed by only limited elongation before release.

Addition of anti-albumin or anti-FBPase to postribosomal supernatants immunoprecipitated substantial amounts of radioactivity which was not quantitatively recovered on SDS gels. Addition of carrier ovalbumin to supernatants followed by anti-ovalbumin also gave rise to substantial radioactivity in immunoprecipitates, although subsequent gel electrophoretic analysis revealed no radioactivity. This was most probably due to a non-specific interaction between methionine tRNA or free methionine and immunoprecipitates. Because of this, quantitation of immunoprecipitated peptides was performed after gel electrophoresis.

SDS gel analysis of immunoprecipitates from free and bound polysome incubations are shown for FBPase (Figure 9.3) and albumin (Figure 9.4). Radioactivity was associated with FBPase in immunoprecipitates from both free and bound polysome incubations, whereas radioactivity associated with albumin in immunoprecipitates was almost totally confined to bound polysome incubations. Although a peak of radioactivity migrating with authentic protein was seen, a considerable amount of lower molecular weight meterial was specifically immunoprecipitated. These were probably fragments of authentic protein that were released during premature termination, as has been reported previously (Strauss et al., 1975; Horikawa et al., 1977).

Quantitation of both total immunoprecipitates, and material migrating with authentic protein is summarized in Table 9.1. On a relative synthetic basis, FBPase synthesis was associated 1.23 times more with free than with bound polysomes when total immunoprecipitated material was taken into account. Albumin synthesis was associated 20.9 times more with bound polysomes when calculated on a similar basis. When only material migrating with authentic protein was used in the calculation, FBPase synthesis was associated 1.5 times more with free polysomes and albumin synthesis 18.0 times more with bound polysomes. The relative synthesis of FBPase and albumin peptides was lower than the in vivo values obtained (section 6.4), especially for the latter. This may be the result of the decline in elongation and release of peptides from larger polysomes, as noted earlier.

A further consideration was the non-immunoprecipitability of some specific peptide material which may lack any antigenic determinants. An _in vitro_ synthetic rate 5 to 10 times lower than the corresponding _in vivo_ rate has been reported by other workers (Iynedjian and Hanson, 1977; Zähringer et al., 1977).

Figure 9.1

TIME COURSE OF PROTEIN SYNTHESIS ON ISOLATED FREE AND BOUND POLYSOMES INCUBATED IN VITRO

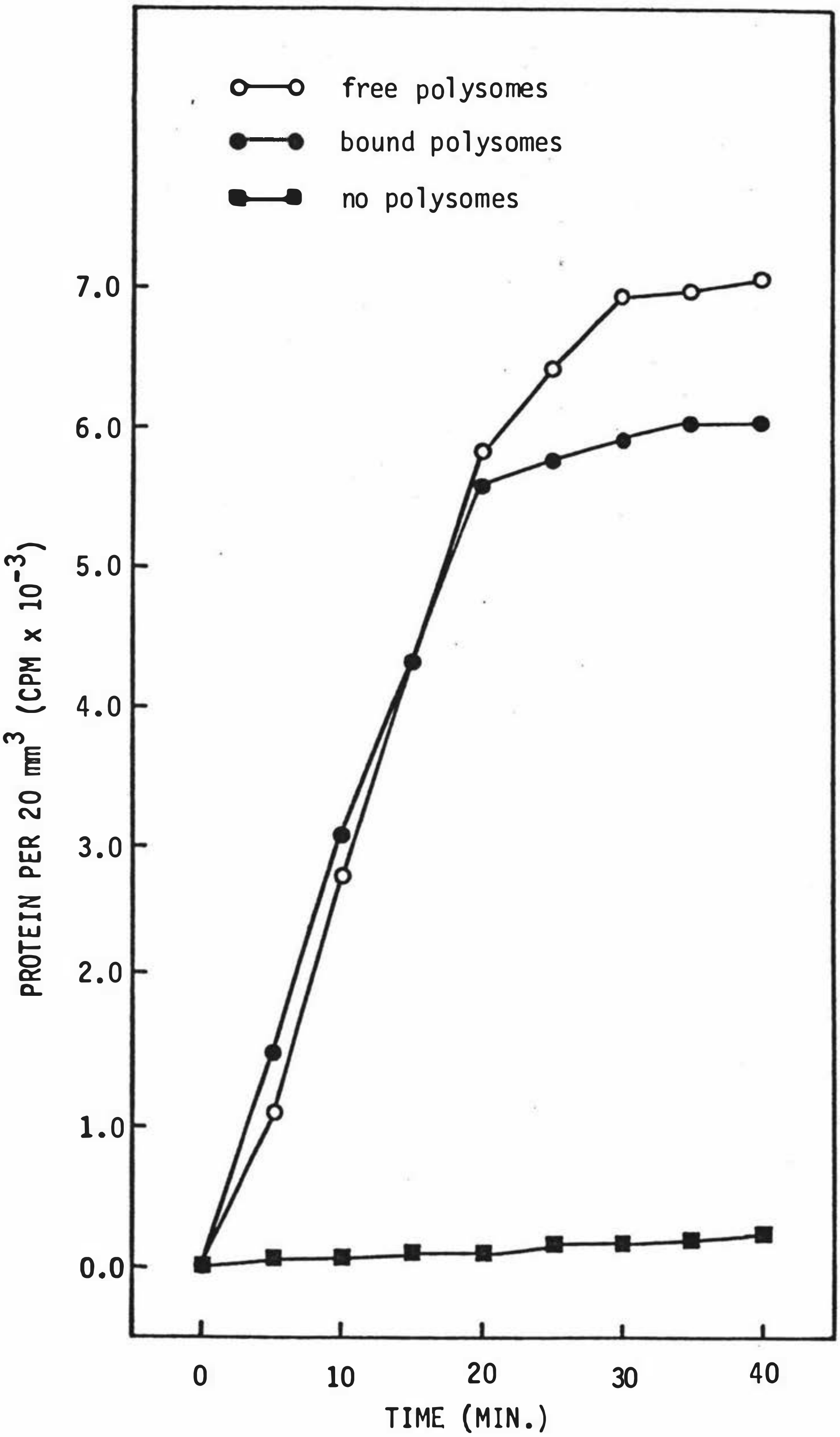


Figure 9.2

SDS GEL (10%) ANALYSIS OF TCA-PRECIPITABLE PROTEIN PRODUCED IN FREE AND BOUND POLYSOME INCUBATIONS

Protein was derived from 90 mm^3 of postribosomal supernatant from each incubation mixture

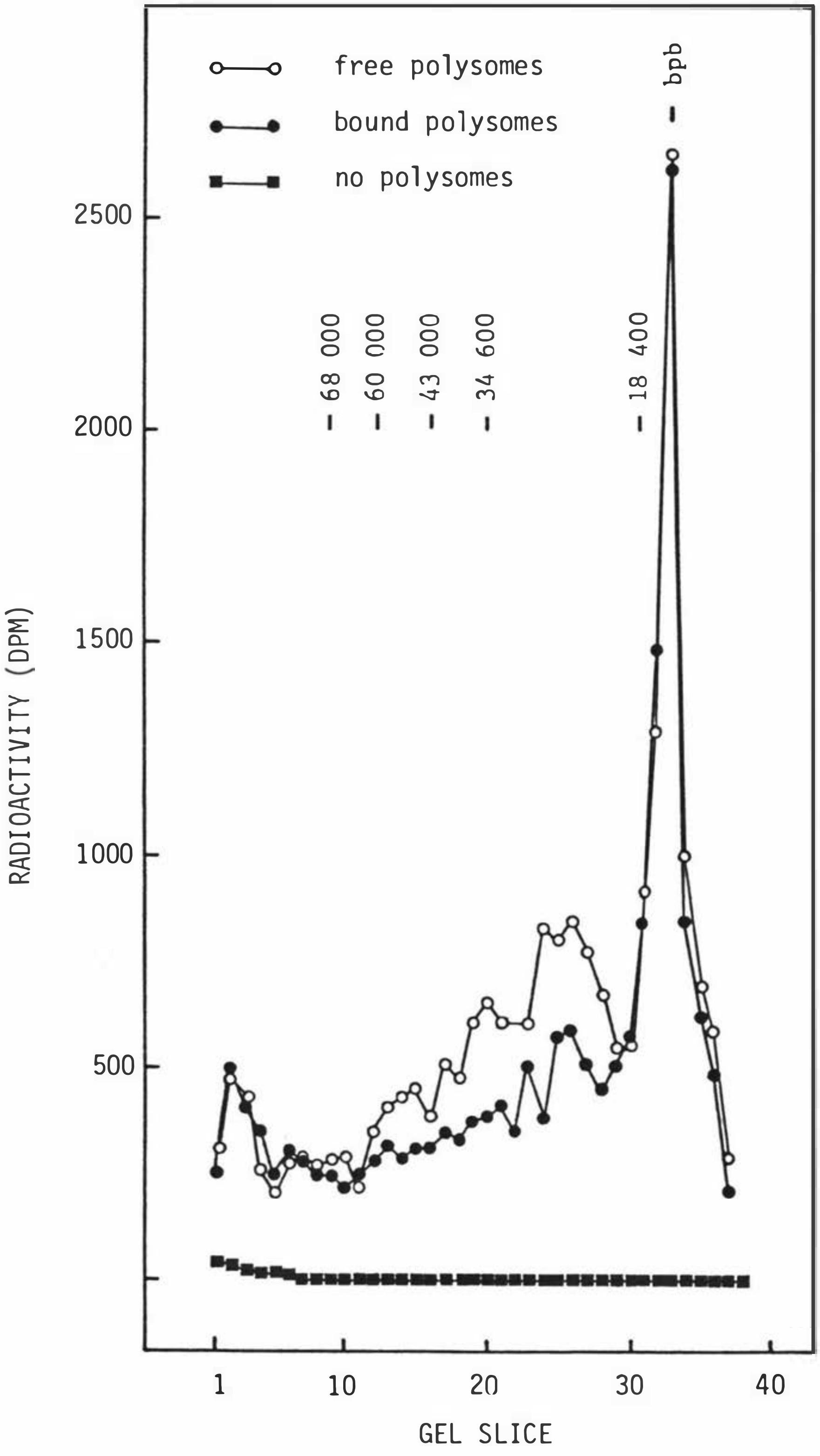


Figure 9.3

SDS GEL (10%) ANALYSIS OF FRUCTOSE-1,6-BISPHOSPHATASE IMMUNOPRECIPITATED FROM FREE AND BOUND POLYSOMES INCUBATIONS

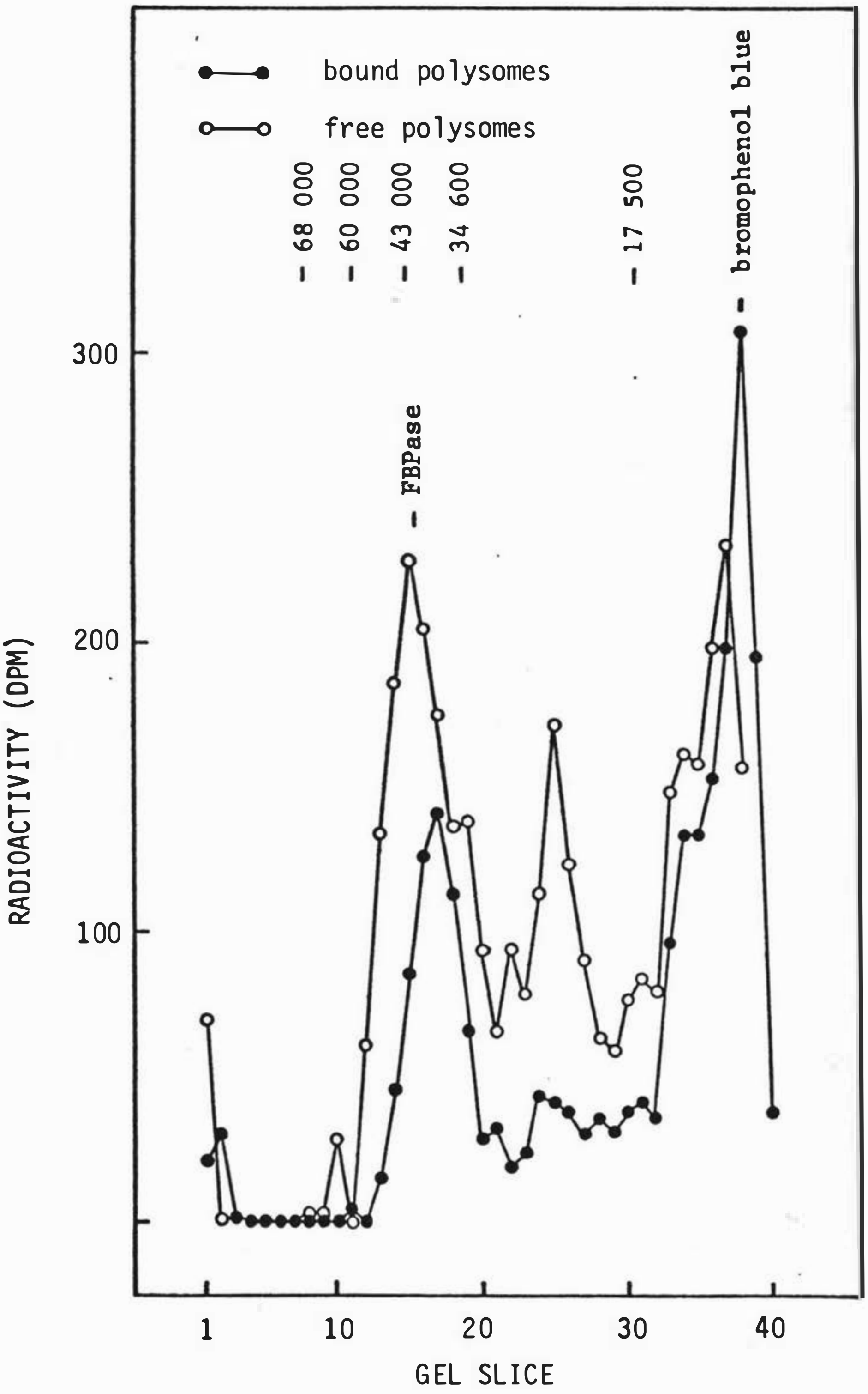


Figure 9.4

SDS GEL (10%) ANALYSIS OF ALBUMIN IMMUNOPRECIPITATED FROM FREE AND BOUND POLYSOME INCUBATIONS

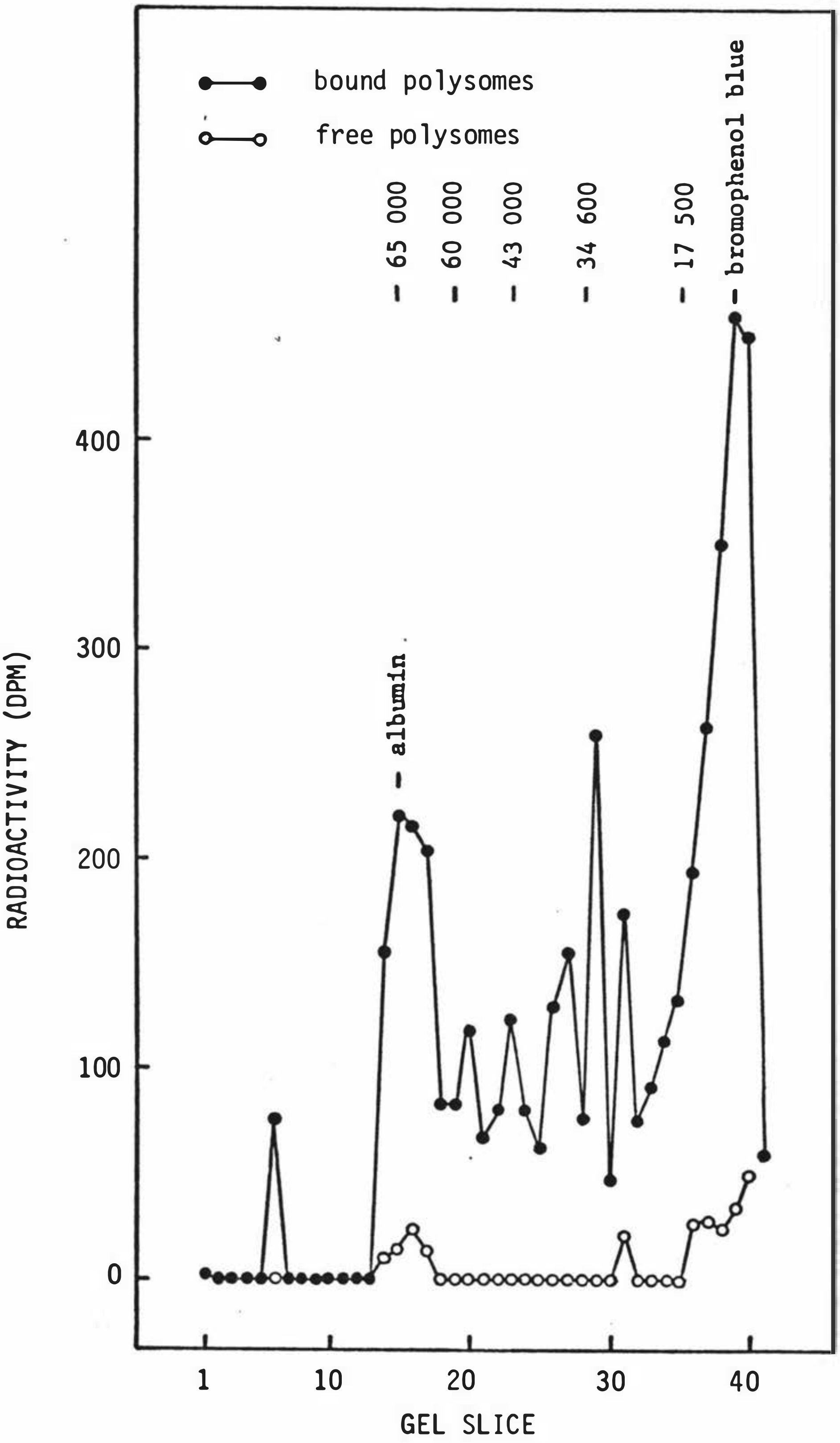


Table 9.1

DISTRIBUTION OF IMMUNOPRECIPITABLE ALBUMIN AND FRUCTOSE-1,6-BISPHOSPHATASE PEPTIDES SYNTHESIZED BY ISOLATED FREE AND BOUND POLYSOMES *IN VITRO*

Polysome type	Total protein	Radioactivity in Albumin (DPM/cm^3)	FBPase
Free	251 720	588 (0.22%)	1 772 (0.70%)
Bound	201 080	9 240 (4.6%)	1 153 (0.57%)

Distribution of immunoprecipitable albumin and FBPase migrating with authentic protein on the above SDS gels

		122 (0.05%)	591 (0.23%)
		1 760 (0.88%)	306 (0.15%)

CHAPTER TEN

PROTEIN SYNTHESIS IN WHEAT GERM EXTRACTS *IN VITRO*

10.1 Introduction

The translation of eukaryotic mRNAs in cell-free mRNA-dependent protein-synthesizing systems has been an important technique for examining certain regulatory events in protein synthesis. In such systems the mechanisms of translational control of specific mRNAs and post-translational processing can be studied in detail. In particular, any functional differences between free and bound polysomes which could affect antibody binding to nascent protein chains, or protein synthesis *in vitro*, should be eliminated by mRNA isolation and translation in an exogenous cell-free system.

Cell-free protein-synthesizing extracts prepared from untoasted commercial wheat germ have been developed in several laboratories (Efron and Marcus, 1973; Roberts and Paterson, 1973; Marcu and Ducock, 1974). These extracts have been found to dependent upon the addition of exogenous mRNA for protein synthetic activity, and have been applied to a wide variety of translational problems. Such extracts faithfully translate crude and purified mRNA preparations from animal, plant and viral sources into intact functional proteins. Wheat germ systems typically lack some post-synthetic processes (Campbell and Blobel, 1976), and this lack has been an important factor in the isolation of primary transcripts of secreted proteins containing 'signal peptides'. However cleavage of N-terminal initiator methionine and N-terminal acetylation have been observed in such extracts (Bloemendal, 1977).

Wheat germ extracts have been used to translate several mRNAs coding for specific proteins from rat liver and hepatoma. Albumin has been specifically immunoprecipitated from extracts programmed with poly(A)$^+$ RNA (Astel and Ganoza, 1974; Peterson, 1976; Sonnenshein and Brawerman, 1977; Striar et al., 1977; Kioussis et al., 1978), and programmed with purified albumin mRNA (Tse and Taylor, 1977). Albumin synthesis has been found to be associated only with RNA from bound polysomes (Zähringer et al., 1977). The 'signal peptide' for albumin has also been elucidated by sequence analysis of the protein synthesized by wheat germ (Strauss et al., 1977a,b).

The induction of phosphoenolpyruvate carboxykinase has been correlated with an increase in translatable messenger (Kioussis et al., 1978; Iynedjian and Hanson, 1977) as has the induction of tyrosine aminotransferase (Rether et al., 1978). Ferritin subunits have been immunoprecipitated from extracts programmed with total poly(A)$^+$ RNA (Arosio et al., 1978), and ferritin mRNA activity was found to be equally associated with RNA from free and bound polysomes (Zähringer et al., 1977). The induction of ferritin synthesis was associated only with an increase in mRNA activity from bound polysomal RNA. The intracellular site of synthesis of two rat liver peroxisomal enzymes, catalase and uricase has been determined to be on free polysomes by translation of isolated polysomal RNA in a cell-free protein-synthesizing system derived from rabbit reticulocytes (Goldman and Blobel, 1978).

Free and bound polysomal poly(A)$^+$ RNA from rat liver was isolated and translated _in vitro_ in wheat germ extracts to further investigate the intracellular polysomal distribution of FBPase mRNA. Albumin synthesis was used as an internal control to show that its mRNA activity was associated with bound polysomes. The translation of isolated mRNA in wheat-germ extracts should eliminate any effect due to differences in translational efficiency between free and bound polysomes.

10.2 Materials

Tobacco leaves (_Nicotiana tabacum_ L.) systemically infected with Tobacco Mosaic Virus (TMV) were kindly provided by Mr H. Nielson of the Plant Health Department, Massey University. Rolled wheat germ was obtained from the Manawatu Mills Ltd., Palmerston North, and was stored desiccated at 4°C. Hepes, DTT, GTP (diNa salt), creatine phosphate (diNa salt), spermine and creatine phosphokinase (E.C. 2.7.3.2, rabbit muscle were obtained from Sigma. ATP (diNa salt) was from Fluka, unlabelled amino acids from Schwartz/Mann, and ribonuclease I (E.C. 3.1.4.22, bovine pancreas) from BDH. Biogel P6 was obtained from Biorad, and cellulose-coated thin layer plates from Eastman Kodak.

L-[^{35}S]methionine (775 Ci per mmole) was from the Radiochemical Centre and was stored at -196°C. Poly(U)-Sepharose 4B was from Pharmacia.

EDTA-washed phenol was prepared as follows: to 500 g of phenol were added 300 cm^3 of water, and the mixture melted at 30°C. Twelve cm^3 of 0.5 M EDTA, pH 7.4 and 8 cm^3 of 1.0 M KOH were added and the suspension stirred for 10 minutes at room temperature. The emulsion was allowed to separate and the top layer was removed and discarded. Four hundred cm^3 of water were added to the bottom layer and the suspension stirred for 2 minutes. After standing in the dark for 30 minutes, the top layer was removed and discarded, and the bottom layer stored in a brown bottle at 4°C.

10.3 Methods

10.3.1 Radiochemical purity of L-[^{35}S]methionine

The radiochemical purity of L-[^{35}S]methionine was verified according to the manufacturer's instructions. Cellulose thin layer plates (20 x 5 cm) were pre-run in n-butanol-pyridine-water (1:1:1, v/v/v), spotted with 25 nmoles each of L-methionine, L-methionine sulphone and L-methionine DL-sulphoxide, and about 1 μCi of isotope. Plates were developed in the same solvent, dried and sprayed with a solution of 17 parts 1% ninhydrin in acetone - 3 parts 1.5% cadmium acetate in 67% acetic acid. After the spots had developed, the plates were scanned on a Packard Model 7200 Radiochromatogram scanner (slit width 2.5 mm, scan speed 1 cm per minute, range 3 x 10^4-10^5 cpm, time constant of 1)(Figure 9.1). The areas under the radioactive peaks were determined by cutting out the appropriate sections and weighing. Isotope was used in translation experiments when the radiochemical purity was greater than 90%, since radioactive decomposition products have been shown to inhibit translation in wheat germ systems (Cashmore, 1978).

10.3.2 Preparation of Tobacco Mosaic Virus RNA

TMV was prepared from systemically infected tobacco leaves according to Gooding and Herbert (1967). RNA was extracted from purified TMV by the method of Marcus et al. (1974).

The following procedure was carried out at room temperature. Frozen tobacco leaves were homogenized in 1 volume (w/v) of 0.5 M Na_2HPO_4 - KH_2PO_4, 1% 2-mercaptoethanol, pH 7.2 in a Waring blendor for 5 minutes. The homogenate was strained through muslin, and the filtrate stirred during the addition of 8 cm^3 of _n_-butanol per 100 cm^3. After the chloroplasts had coagulated, the extract was stirred for an additional 15 minutes and then centrifuged at 10 000 _g_ for 30 minutes (Sorvall SS-34 rotor, 12 000 rpm). The supernatant was decanted and 4.0 g of finely ground polyethylene glycol (PEG) $\overline{MW}$ 6000 per 100 cm^3 added over 15 minutes while stirring. After centrifugation at 10 000 _g_ for 15 minutes, the pellet was resuspended by stirring for 40 minutes in 20 cm^3 of 0.01 M phosphate buffer, pH 7.2 per 100 cm^3 of initial extract. The solution was clarified by centrifugation at 10 000 _g_ for 15 minutes. The supernatant was decanted and 0.4 g each of NaCl and PEG per 10 cm^3 added over 15 minutes while stirring. After centrifugation at 10 000 _g_ for 15 minutes, the pellet was resuspended in 2 cm^3 of 0.01 M phosphate buffer, pH 7.2 per 100 cm^3 of initial extract. After standing overnight, the solution was clarified by centrifugation and stored at -20°C. Approximately 1 mg of TMV, having an $A_{260\ nm}/A_{280\ nm}$ ratio of 1.16-1.24 was obtained per gram of tissue.

A suspension of TMV at 20 mg per cm^3 was brought to 5.0 mM EDTA and 0.5% SDS. An equal volume of EDTA-washed phenol was added and the mixture stirred for 10 minutes. The emulsion was separated by centrifugation at 27 000 _g_ for 10 minutes (Sorvall HB-4 rotor, 13 000 rpm), and the upper layer reextracted twice with EDTA-washed phenol. The RNA was precipitated from the aqueous phase by the addition of 2.5 volumes of 0.1 M potassium acetate, pH 5.5 in ethanol, and standing overnight at -20°C. The RNA was collected by centrifugation and dissolved in 0.5 volumes of 0.1 M potassium acetate, pH 5.5 and reprecipitated overnight at - 20°C, following the addition of 2.5 volumes of ethanol. From 150 mg of TMV, 7.2 mg of RNA was obtained with an $A_{260\ nm}/A_{280\ nm}$ ratio of 1.99.

10.3.3 Extraction of poly(A)$^+$ RNA from polysomes

Polysomes were deproteinized by the phenol-chloroform method and poly(A)$^+$ RNA isolated by chromatography on poly(U)-Sepharose (Keller and Taylor, 1976; Taylor and Tse, 1976).

Free, bound or total polysomes were dissolved in 50 mM Hepes, 25 mM NaCl, 5 mM $MgCl_2$, pH 7.2 (HNa(25)M), to give a solution containing not more than 25 $A_{260\ nm}$ units per cm^3. A one-ninth volume of 10% (w/v) SDS was added and the solution mixed for 1 minute at room temperature. A one-half volume of freshly-distilled, buffer saturated phenol was added, and the mixture shaken for 1 minute, followed by the addition of a volume of freshly-distilled chlorofrom equal to that of the phenol. The prior addition of phenol was found to be necessary to prevent protein-RNA aggregation and loss of the RNA into the phenol phase (Brawerman et al., 1977). After vigorous shaking for 10 minutes at room temperature, the emulsion was centrifuged at 13 000 g for 10 minutes (Sorvall HB-4 rotor, 9000 rpm). The aqueous phase was carefully removed, adjusted to 0.2 M NaCl, and the RNA precipitated overnight at -20°C by the addition of 2.5 volumes of ethanol. The precipitated RNA was collected by centrifugation at 13 000 g for 10 minutes, washed twice with 0.2 M NaCl in 70% ethanol (at -20°C), redissolved in 0.2 M NaCl and precipitated again with ethanol.

Deproteinized RNA was dissolved in 20 mM Hepes, 10 mM EDTA, 1% SDS pH 7.2 to give a solution of not more than 50 $A_{260\ nm}$ units per cm^3. The solution was heated at 65°C for 10 minutes, then quickly cooled to room temperature. The RNA solution was then diluted with an equal volume of water and adjusted to 300 mM in NaCl. Samples containing up to 3000 $A_{260\ nm}$ units of RNA were applied to a 10 cm^3 column of poly(U)-Sepharose, equilibrated in the above final buffer, at a flow rate of 1 cm^3 per minute. The column was washed with 10 cm^3 of the above final buffer, followed by 15 cm^3 pf the same buffer minus the NaCl. The poly(A)$^+$ RNA was eluted with 20 cm^3 of 70% formamide (freshly deionized, section 2.9.2), containing 1 mM Hepes, 2 mM EDTA, pH 7.2. The eluate was adjusted to 0.2 M NaCl and the RNA precipitated overnight at -20°C after the addition of 2.5 volumes of ethanol. RNA was collected by centrifugation, dissolved in 0.2 M NaCl, and reprecipitated with ethanol. Precipitates of poly(A)$^+$ RNA were dissolved in water at about 20 $A_{260\ nm}$ units per cm^3, and stored at -20°C. RNA isolated in this manner had a typical $A_{260\ nm}/A_{280\ nm}$ ratio of 2.0-2.1.

10.3.4 Preparation of wheat germ extracts

Cell-free extracts from untoasted commercial wheat germ were prepared according to Roberts and Paterson (1973).

Six g of wheat germ (Triticum vulgaris) were ground in a chilled mortar with an equal weight of acid-washed sand and 28 cm^3 of a solution containing 20 mM Hepes (pH 7.6, adjusted with KOH), 100 mM potassium acetate, 1 mM magnesium acetate, 2 mM $CaCl_2$, and 6 mM 2-mercaptoethanol. The homogenate was centrifuged at 30 000 g for 10 minutes at 4°C (Sorvall SS-34 rotor, 16 000 rpm), and the supernatant was removed, avoiding both the surface lipid layer and the pellet. The supernatant was made up to 3.5 mM magnesium acetate, 1 mM ATP, 25 μM GTP, 2 mM DTT, 8 mM creatine phosphate and 40 μg of creatine phosphokinase per cm^3, and incubated for 15 minutes at 30°C. Finally, 10 cm^3 of the pre-incubated extract was passed through a column (50 x 2 cm) of Biogel P6 equilibrated in 20 mM Hepes, 120 mM potassium acetate, 5 mM magnesium acetate and 6 mM 2-mercaptoethanol, pH 7.6, at a flow rate of 1.3-1.4 cm^3 per minute. The peak of the turbid fraction eluting from the column was collected and stored in 0.5 cm^3 aliquots at -196°C. No loss of activity was evident in these extracts after several months storage. The final wheat germ extract had an $A_{260\ nm}/A_{280\ nm}$ ratio of 1.5 and a concentration of about 70 $A_{260\ nm}$ units per cm^3.

10.3.5 Translation assays

Translation assays were based on those of Roberts and Paterson (1973) with modifications described by Tse and Taylor (1977).

A reaction mixture of 100 mm^3 with a final pH of 7.5 contained: 20 mm^3 of preincubated wheat germ extract, 28 mM Hepes, 2 mM DTT, 1 mM ATP, 25 μM GTP, 8 mM creatine phosphate, 25 μM of each of the 19 amino acids required for protein synthesis (no methionine), 0.5-5.0 μCi of L-[^{35}S]methionine, magnesium acetate, potassium acetate, spermine and RNA as indicated in the text. Potassium acetate was used to replace the usual potassium chloride as it has been shown to enhance amino acid incorporation (Gozes et al., 1975).

Reaction mixtures were incubated at 25°C for 120 minutes. Aliquots of 10 mm^3 were spotted onto 2 x 2 cm squares of Whatman 3MM paper at the times indicated, and processed as described (section 2.10.2). The remaining reaction material was used immediately or stored at -20°C.

10.3.6 Analysis of translation products

Ribosomes were removed from the final reaction mixture by centrifugation at 200 000 g for 1 hour at 4°C (Beckman S.W. 41Ti rotor, 41 000 rpm). Centrifugation was performed in 500 mm^3 conical centrifuge tubes floating in water-filled ultracentrifuge tubes.

The postribosomal supernatant, containing the released radioactive peptides was carefully removed. Duplicate aliquots of 10 mm^3 were spotted onto 3MM paper squares as above and the remainder adjusted to 25 mM EDTA, 25 mM methionine, pH 7.4 and incubated with 1 µg of RNase for 15 minutes at 37°C. Protein was precipitated from duplicate aliquots (100 mm^3) by the addition of a 20 volume excess of 10% TCA, 10 mM methionine, and standing overnight at 4°C. Precipitated protein was collected by centrifugation and the pellet washed by suspension and recentrifugation twice in TCA-methionine and once in ice-cold water. Pellets were dissolved in 1.0 cm^3 of 0.1 M NaOH in preparation for liquid scintillation couting (section 2.10), or analysed by SDS gel electrophoresis (section 2.7.2) and the radioactivity incorporated into individual gel slices determined (section 2.10.1).

10.3.7 Immunoprecipitation assay

Postribosomal supernatants (50-100 mm^3) were treated with EDTA, methionine and RNase as described above. Ten µg of the appropriate carrier antigen were added followed by Triton X-100 and sodium deoxycholate to give a concentration of 1% in each. Sufficient antibody to assure quantitative precipitation of the translated products and carrier was added. After incubation at 37°C for 30 minutes and overnight at 4°C, immunoprecipitates were collected by centrifugation through small discontinuous sucrose gradients (section 5.3.6). Precipitates were analysed by SDS gel electrophoresis or the radioactivity determined by liquid scintillation counting after dissolution in 1.0 cm^3 of 1.0 M NaOH. Immunoprecipitated FBPase and albumin from labelled rat liver homogenate were used to identify FBPase and albumin synthesized in wheat germ extracts.

10.4 Results and Discussion

In developing the wheat germ in vitro translation system, three major problems were encountered concerning the source of various materials. TMV RNA was used to verify the fidelity of translation, as this messenger is easy to purify and is translated efficiently into peptides in wheat germ extracts (Roberts et al., 1973).

One particular batch of wheat germ which was obtained fresh and unrolled from the mill and sorted by hand, produced extracts having a very high endogenous background. Addition of either TMV or poly(A)$^+$ RNA gave only a 2-fold increase in incorporation over background. The level of incorporation found on addition of RNA was found to be comparable with later results using a different batch of rolled, unsorted wheat germ which had been stored desiccated at 4°C for several years. This batch of wheat germ which had been used for the successful translation of several plant mRNAs, had a low endogenous background, and was used in all subsequent work.

Different batches of creatine phosphokinase from Sigma were found to cause major variations in the stimulation of incorporation by TMV RNA. A systematic investigation revealed that at 4 μg of enzyme per 100 mm^3 assay, the stimulation under optimal translation conditions varied between 1.4 and 53-fold over background, depending on the batch used. The low level of incorporation supported by some batches was correlated with the time of cessation of translation during the incubation. With all batches of enzyme however, as the concentration of creatine phosphokinase was increased to 40 μg per 100 mm^3 assay, the level of incorporation dropped to about one-quarter that at 4 μg per 100 mm^3 assay. This problem is most probably caused by nucleases contaminating the commerical enzyme. This view is supported by the recent findings of Hickey et al. (1978), who found that the level of nuclease in commercial creatine phosphokinase preparations correlated with their ability to support protein synthesis in cell-free systems prepared from HeLa cells, and Gozes et al. (1975) who reported that high levels of creatine phosphokinase to be inhibitory in wheat germ systems.

Similar problems were encountered with batches of creatine phosphate from Sigma. With some samples, increasing the concentration of creatine phosphate from 0 to 8 mM was accompanied by a depression in incorporation. Gel electrophoretic analysis of the translation products revealed that more low molecular weight products were produced by batches that poorly supported translation.

Possible ribonuclease contamination of reagents in a wheat germ system was reported to render it unsuitable for mRNA translation (Hutchison and Holten, 1978).

Protein synthesis directed by TMV RNA

TMV RNA was purified to homogeneity as shown by the single high molecular weight band on electrophoresis in the presence of formamide (Figure 10.2)

The incorporation of isotope by the preincubated wheat germ extract was greatly enhanced by the addition of TMV RNA. The time course of the reaction showed linear incorporation for 60 minutes and no further increase in radioactive products after about 120 minutes (Figure 10.3). The system was saturated by 5 μg of TMV RNA; further addition led to a decrease in incorporation (Figure 10.3). Under optimal conditions, incorporation of isotope directed by TMV RNA was more than 40-times that of reactions incubated in the absence of added RNA. Stimulation of incorporation was optimal at a potassium concentration of 140 mM and a magnesium concentration of 2.5 mM (Figure 10.4). The potassium optimum is somewhat different from the optima given in the literature for wheat germ systems programmed with the same messanger (Shih and Kaesberg, 1973; Roberts and Paterson, 1973; Marcu and Dudock, 1974). This may be a result of the substitution of potassium acetate for the chloride salt, which has been shown to give a broader potassium optimum for some messengers (Gozes et al., 1975). About 40% of the isotope was incorporated into protein, of which 38% was released from the ribosomes. This compares with a 56.6% release with endogenous messenger. Sala-Trepat et al. (1978) reported a 36.5% release with TMV RNA in a rye embryo translation system, and a much higher release with endogenous messanger. The polypeptides synthesized *in vitro* in response to TMV RNA are shown in Figure 10.5. The presence of eight discrete radioactive bands was confirmed by radioautography of dried slab gels. A peak of radioactivity was also found migrating with the bromophenol blue dye front. The decreasing incorporation at high RNA concentration was reflected in a uniform decrease in incorporation into all the radioactive polypeptide bands. The absence of any radioactive material co-migrating with authentic TMV coat protein was due to the absence of methionine in this protein (Wittman-Liebold and Wittman, 1975). The spectrum of polypeptides produced contrasts sharply with that of Roberts et al. (1973), who obtained a very large number of polypeptides ranging in molecular weight from 10 000 to 140 000 daltons. Davies and Kaesberg (1974) also reported a disperse, heterogeneous mixture of products of mostly less than 50 000 daltons, with a considerable amount of material migrating with the bromophenol blue dye front. These differing results may be due in part to the differing potassium concentrations used (see below).

Protein synthesis directed by rat liver polysomal RNA

Polysomal RNA from rat liver was fractionated by chromatography on poly(U)-Sepharose (Figure 10.6). Poly(A)$^+$ RNA eluted with the formamide buffer front, and represented approximately 1.3% of total polysomal RNA for both free and bound polysomes. The percentage of poly(A)$^+$ RNA reported isolatable from rat liver polysomal RNA varies from about 0.7 to 2.0% (Sonnenshein and Brawerman, 1977; Zähringer et al., 1977). Examination of both poly(A)$^+$ and poly(A)$^-$ RNA from free and bound polysomes by nondenaturing gel electrophoresis is shown in Figures 10.7 and 10.8. The major peaks for poly(A)$^-$ RNA were identified as 28s, 18s and 5s ribosomal RNAs, and a broader 4s peak of aminoacyl tRNAs. The poly(A)$^+$ RNA was also contaminated with ribosomal RNAs, which is typical for a single purification of poly(A)$^+$ RNA by affinity chromatography (Tse and Taylor, 1977; Sala-Trepat et al., 1978). An altered distribution between 28s and 18s RNA was also noted between poly(A)$^+$ and poly(A)$^-$ RNAs, and probably represented the association between 18s RNA and mRNA noted by other workers in partially purified mRNA preparations (Haines et al., 1972; Palmiter, 1973; Rosen et al., 1975; Groner et al., 1977). The size distribution of the poly(A)$^+$ RNA was heterodisperse and showed a broad distribution ranging from about 5s to 30s, similar to that observed by Sala-Trepat et al. (1978) in denaturing sucrose gradients. The isolation of high molecular weight poly(A)$^+$ RNA indicated very little degradation had occured during polysome isolation, RNA extraction, and affinity chromatography. A second cycle of poly(U)-Sepharose chromatography was omitted to avoid possible degradation which might be caused by further manipulation.

In the preincubated wheat germ extract, free polysomal poly(A)$^+$ RNA enhanced the incorporation of isotope about 16 times over background, and bound polysomal poly(A)$^+$ RNA about 11.5 times (Figure 10.9). Free polysomal RNA has been reported to be more active in wheat germ extracts (Zähringer et al., 1977). The time course of the reaction was similar to that exhibited by TMV RNA, with a linear increase in incorporation for 40-60 minutes and termination at about 120 minutes (Figure 10.9). Spermine was found to have a stimulatory effect on the incorporation of isotope when extracts were programmed with total polysomal poly(A)$^+$ RNA as has been reported (Atkins et al., 1975; Tse and Taylor, 1977; Hunter et al., 1977; Sala-Trepat et al., 1978; Rether et al., 1978). Spermine was optimal at about 80 µM (Figure 10.9). Sala-Trepat et al., (1978) reported a similar optimum for spermine in wheat germ extracts programmed with total polysomal poly(A)$^+$ RNA, and showed that polypeptides synthesized in the presence of spermine showed a size distribution markedly shifted towards higher molecular weight. The optimum concentration for potassium and magnesium was shown to be 110 mM and 2.5 mM respectively (Figure 10.10) for total polysomal poly(A)$^+$ RNA. The optimum potassium ion concentration was higher than previously reported for the translation of total rat liver polysomal RNA (Astell and Ganoza, 1974; Roewekamp et al., 1976; Sala-Trepat et al., 1978; Tse and Taylor, 1977), but may be the result of using potassium acetate instead of the usual chloride salt. The dependence of protein synthetic activity on different polysomal RNA fractions is shown in Figure 10.11. Both free and bound polysomal poly(A)$^+$ RNA saturated at 3 µg per 100 mm^3 assay, and the relatively greater incorporation supported by the free fraction was maintained over the entire RNA concentration range used. Poly(A)$^-$ RNA exhibited little synthetic activity (about one-sixth that of poly(A)$^+$ RNA at optimal RNA concentrations), saturating at 10 µg per 100 mm^3 assay. RNA concentrations greater than optimum caused a decrease in incorporation as noted for TMV RNA. This seems to be a general feature in the translation of both crude and purified mRNAs in wheat germ extracts, and has been ascribed to an effect by RNA on peptide initiation (Rhoads et al., 1973). A maximum of 33% of the isotope was incorporated into protein using free polysomal poly(A)$^+$

RNA, of which 54% was released from the ribosomes. For bound polysomal RNA 45% was released; for free polysomal poly(A)$^-$ RNA 40%; and for bound polysomal poly(A)$^-$ RNA 38%. The released polypeptides synthesized in vitro in response to poly(A)$^+$ RNA from free and bound polysomes are shown separated by SDS gel electrophoresis in Figure 10.12. As expected from a heterogeneous RNA template, considerable heterogeneity in the peptides produced was observed. The difference in synthetic capacity between free and bound polysomal poly(A)$^+$ RNA was not evident in the high molecular weight region, but was shown in the lower level of small fragments released in extract programmed with bound polysomal RNA. An examination of the peptide material still bound to ribosomes after incorporation of isotope had ceased revealed a greater proportion of higher molecular weight peptides associated with incubations using free polysomal RNA, and an almost equal amount of shorter fragments associated with both types of polysomal RNA (Figure 10.13). These short peptides, about 10 000 to 20 000 daltons, dominate the profile of synthesized products and could result from either endonucleolytically cleaved mRNA or premature termination and release of peptidyl tRNAs into the incubation medium. The latter is probably the case, since the gel electrophoretic analysis of the poly(A)$^+$ RNAs showed a proportion of RNA which should code for large polypeptides (c.f. albumin (MW 65 000) RNA is 18s)(Taylor and Schimke, 1973). The phenomenon of premature termination has also been noted by other workers in some wheat germ translation systems (Sonnenshein and Brawerman, 1977; Hofer and Sekeris, 1977; Iynedjian and Hanson, 1978; Rether et al., 1978; Sala-Trepat et al., 1978), and was shown to be due to premature termination, since translation of the same messenger fractions in a cell-free system derived from rabbit reticulocytes did not demonstrate the phenomenon (Hutchison and Holten, 1978).

The specific immunoprecipitation of released polypeptides by anti-FBPase and anti-albumin is summarized in Table 10.1. On a relative synthetic basis, poly(A)$^+$ RNA derived from bound polysomes was 130 times more active in albumin synthesis than that from free polysomes. For FBPase, poly(A)$^+$ RNA from bound polysomes was 1.8 times more active in synthesis that that from free polysomes. SDS gel electrophoretic analysis is shown for FBPase (Figure 10.14) and albumin (Figure 10.15) immunoprecipitates. For FBPase immunoprecipitates a major peak of radioactivity comigrating with the authentic protein was found in incubations which contained either free or bound polysomal poly(A)$^+$ RNA. However considerable amounts of radioactivity were associated with shorter molecular weight fragments that appeared to have been specifically immunoprecipitated, since they were not present in immunoprecipitates derived from those incubations that did not contain any added RNA. For albumin immunoprecipitates radioactivity was only associated with poly(A)$^+$ RNA derived from bound polysomes, and was found in a peak comigrating with authentic albumin protein as well as with smaller molecular weight fragments. The identity of these smaller fragments as prematurely terminated authentic protein was suggested by the absence of any such material in albumin immunoprecipitates from incubations containing poly(A)$^+$ RNA from free polysomes. The specific immunoprecipitation of authentic protein fragments has also been reported for wheat germ systems programmed with various mRNAs (Roberts and Paterson, 1973; Horikawa et al., 1977; Sonnenshein and Brawerman, 1977; Iynedjian and Hanson, 1978; Kioussis et al., 1978; Hutchison and Holten, 1978). The absence of such incomplete fragments in immunoprecipitates however, does not necessarily imply that they were absent in translation mixtures. The nature of the antigenic determinants and their dependence on the conformation of the peptides would affect any such immunoprecipitation. If only the radioactivity migrating at the position of authentic protein is used in calculating the distribution of FBPase and albumin synthesis between free and bound polysomal poly(A)$^+$ RNA, the results presented in Table 10.1 are obtained. Albumin synthesis was wholly associated with incubations containing poly(A)$^+$ RNA from bound polysomes while FBPase synthesis was associated with incubations containing poly(A)$^+$ RNA from both free and bound polysomes. On a relative synthetic basis, FBPase synthesis was 1.78-fold greater using poly(A)$^+$ RNA from bound polysomes compared to that from free polysomes. The amount of immunoprecipitable albumin and FBPase (compared to total protein synthesized) was less than the comparable in vivo rates of synthesis (chapter 6), although it was higher than a literature report comparing in vitro and in vivo rates (Iynedjian and Hanson, 1977)

The production of prematurely terminated protein, and the relative amounts of specific protein produced in wheat germ extracts may be related to the translation conditions. Tse and Taylor (1977), while examining the translation of albumin mRNA in wheat germ extracts found premature termination if translations were performed under optimal conditions for total poly(A)$^+$ RNA translation. Optimal conditions for specific albumin translation were at higher potassium and magnesium ion concentrations. The size distribution of the translated products as well as the relative efficiencies of translation were markedly altered by variations in the concentrations of potassium and magnesium ions. The synthesis of large polypeptides coded by collagen mRNA also required a higher potassium ion concentration than the optimum for translation of total poly(A)$^+$ RNA (Harwood et al., 1975; Beneviste et al., 1976). Several virus mRNAs have also required different potassium and magnesium ion concentrations for optimal translation in wheat germ extracts. The recovery of full length transcripts of rat hepatoma tyrosine aminotransferase at the expense of prematurely terminated fragments was reported when a hepatoma cell diaysate was added to wheat germ incubations containing hepatoma polysomal RNA (Rether et al., 1978). In short, it appears that different optimal conditions exist for the translation of individual mRNAs in wheat germ extracts.

The relatively low percentage of products of large molecular weight did not adequately reflect the size distribution of poly(A)$^+$ RNA observed by gel electrophoresis. This may have been due to a less efficient translation of large mRNAs in wheat germ extracts (Klein et al., 1972; Davies and Samuel, 1975; Sala-Trepat et al., 1978). It has also been reported that small mRNAs prevent the translation of larger mRNAs in unfractionated mixtures. This could account for the low percentage of high molecular weight products in the synthesis programmed with poly(A)$^+$ RNA preparations. Incorporation of amino acids into incomplete proteins, which are sometimes formed in the wheat germ system by defective termination resulting from a 'ribosome jam' near the 3' end of the mRNA (Davies and Samuel, 1975), could also contribute to this phenomenon.

Figure 10.1

RADIOCHEMICAL PURITY ANALYSIS OF L-[^{35}S]METHIONINE BY TLC ON CELLULOSE

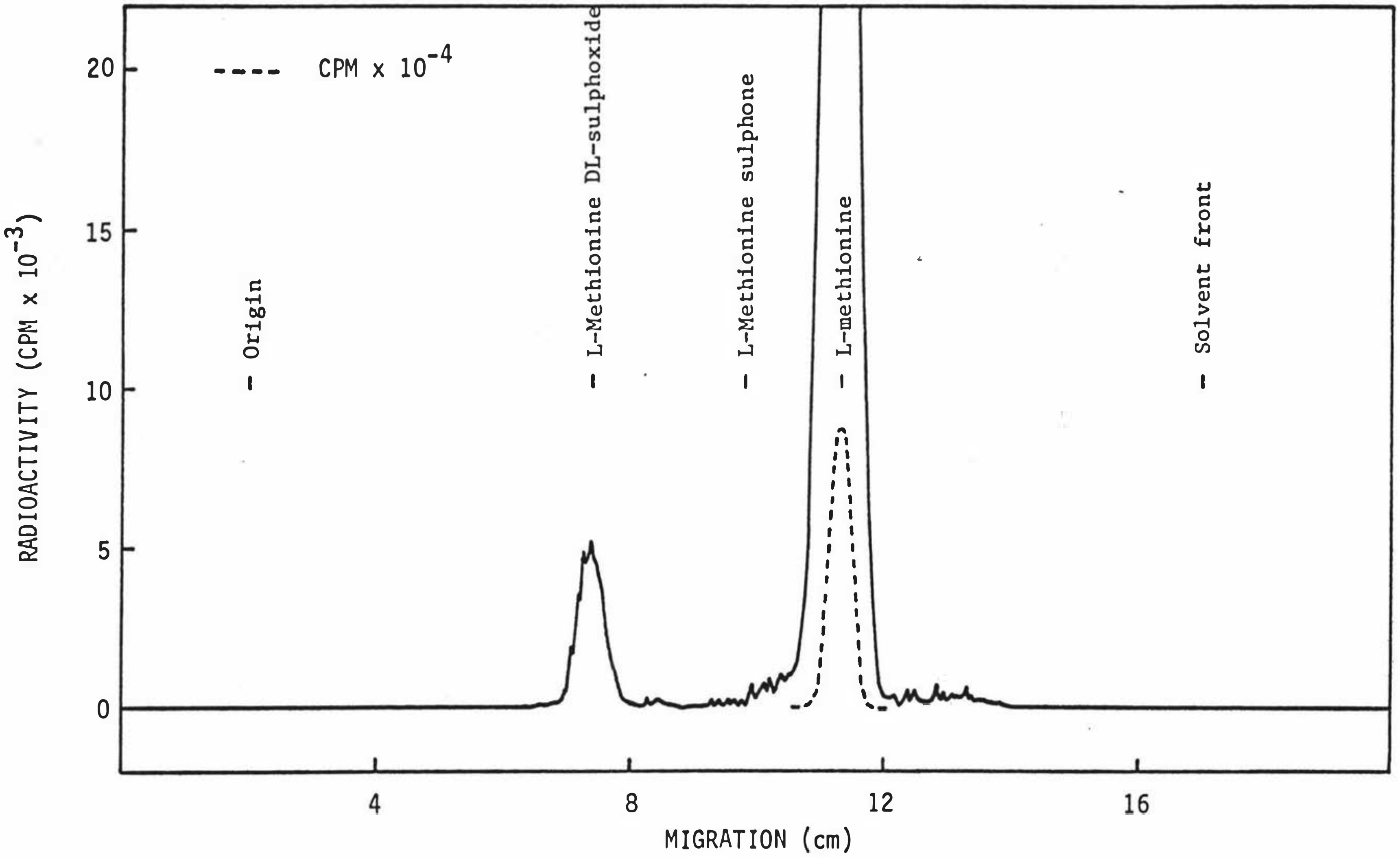


Figure 10.2

FORMAMIDE GEL (3.4%) ANALYSIS OF TMV RNA

10 μg of RNA was applied to the gel

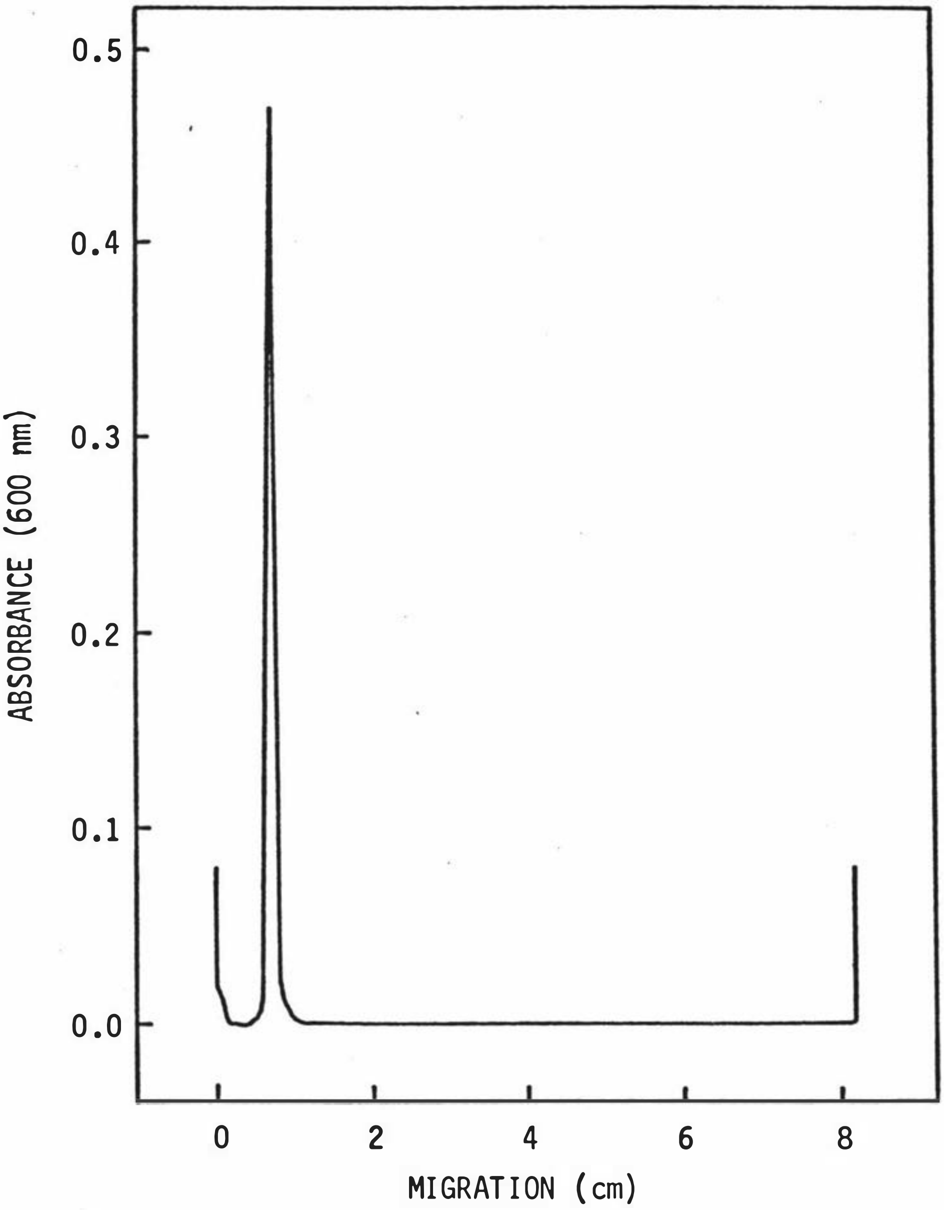


Figure 10.3

TIME COURSE OF PROTEIN SYNTHESIS AND EFFECT OF RNA CONCENTRATION ON THE TRANSLATION OF TMV RNA IN WHEAT GERM EXTRACTS

RNA was translated at optimum Mg^{2+}, K^+ and 40 μM spermine in the standard assay

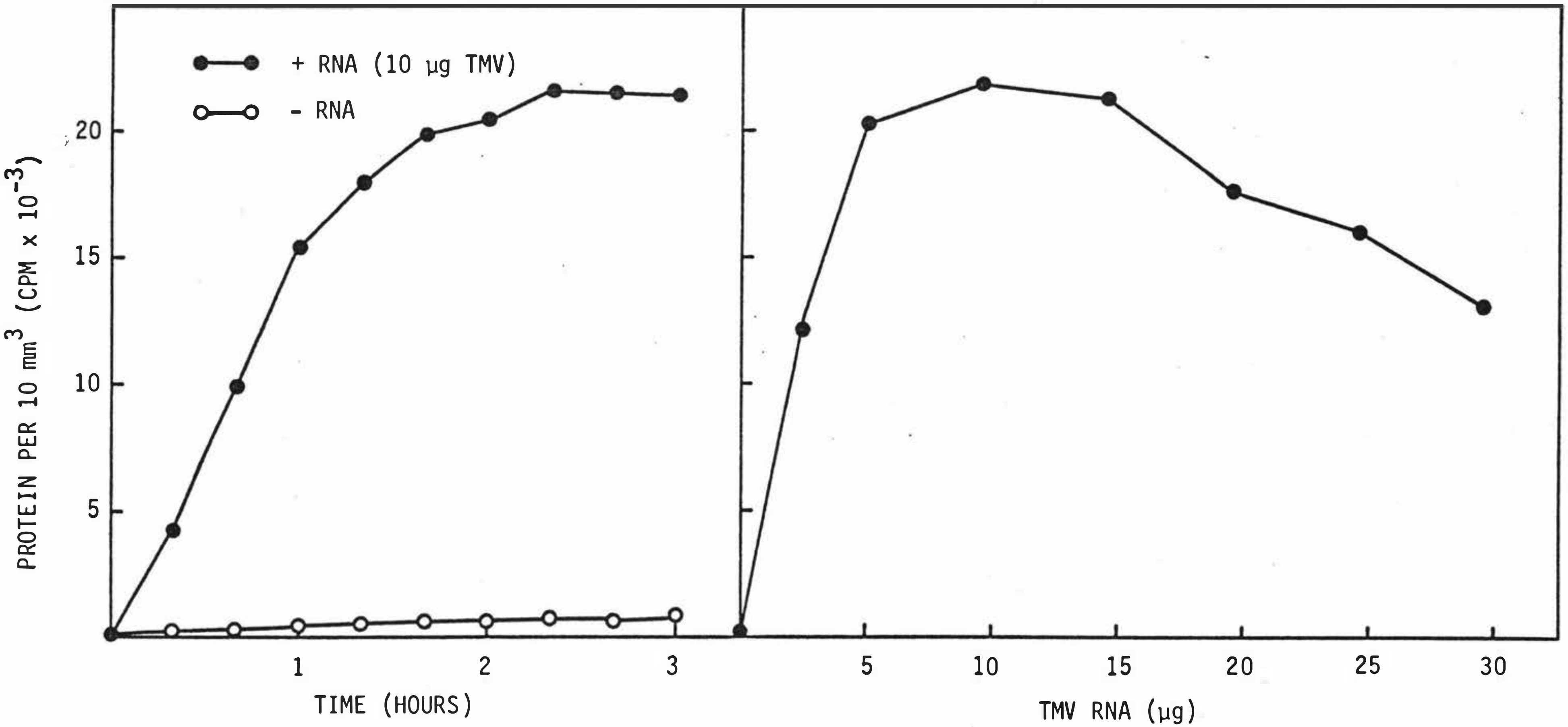


Figure 10.4

EFFECT OF K^+ AND Mg^{2+} CONCENTRATION ON THE TRANSLATION OF TMV RNA IN WHEAT GERM EXTRACTS

10 µg of TMV RNA were translated in the standard assay

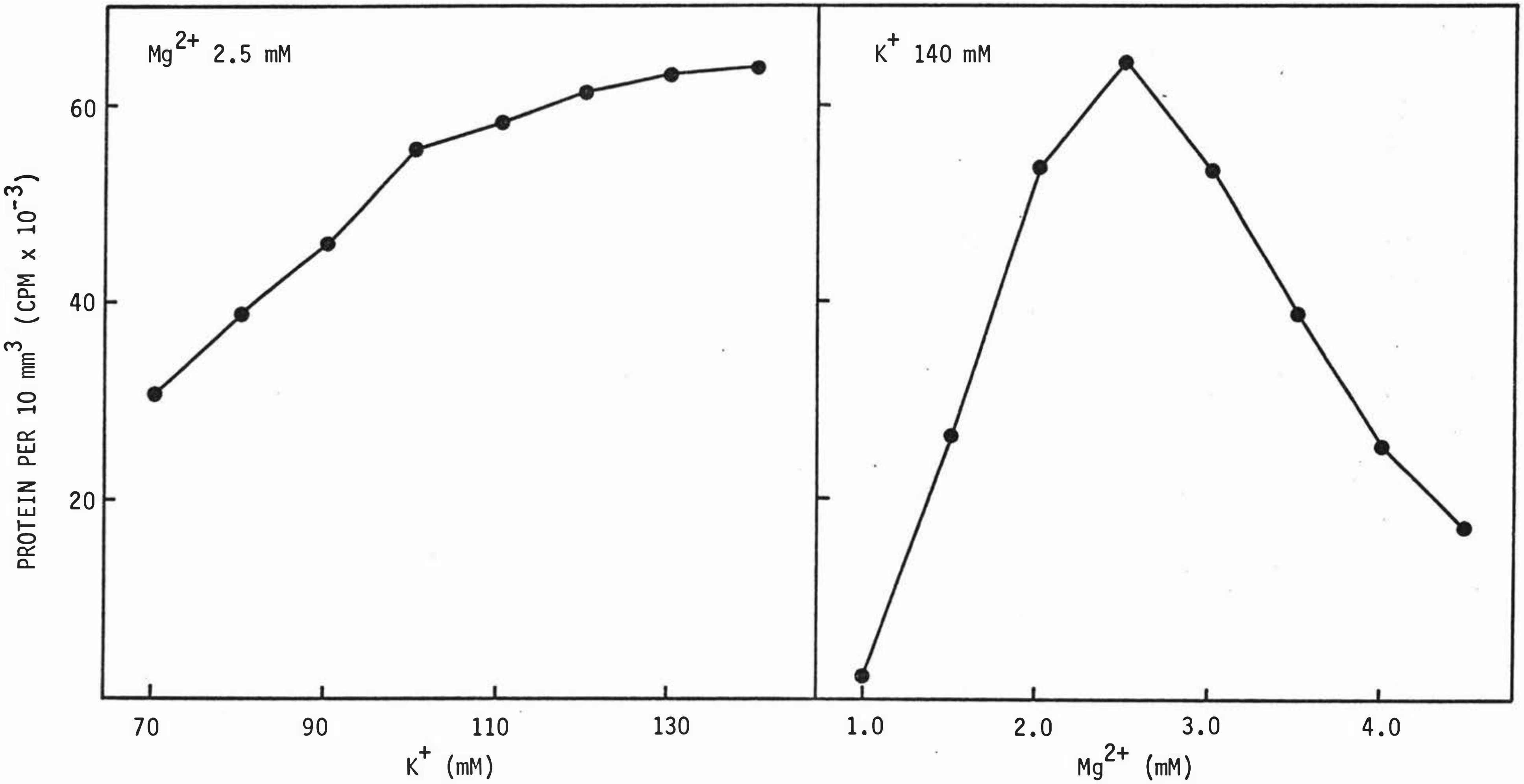


Figure 10.5

SDS GEL (7%) ANALYSIS OF TRANSLATION PRODUCTS PRODUCED IN WHEAT GERM EXTRACTS PROGRAMMED WITH TMV RNA

10 µg of TMV RNA were translated at optimum Mg^{2+}, K^+ and 40 µM spermine in the standard assay

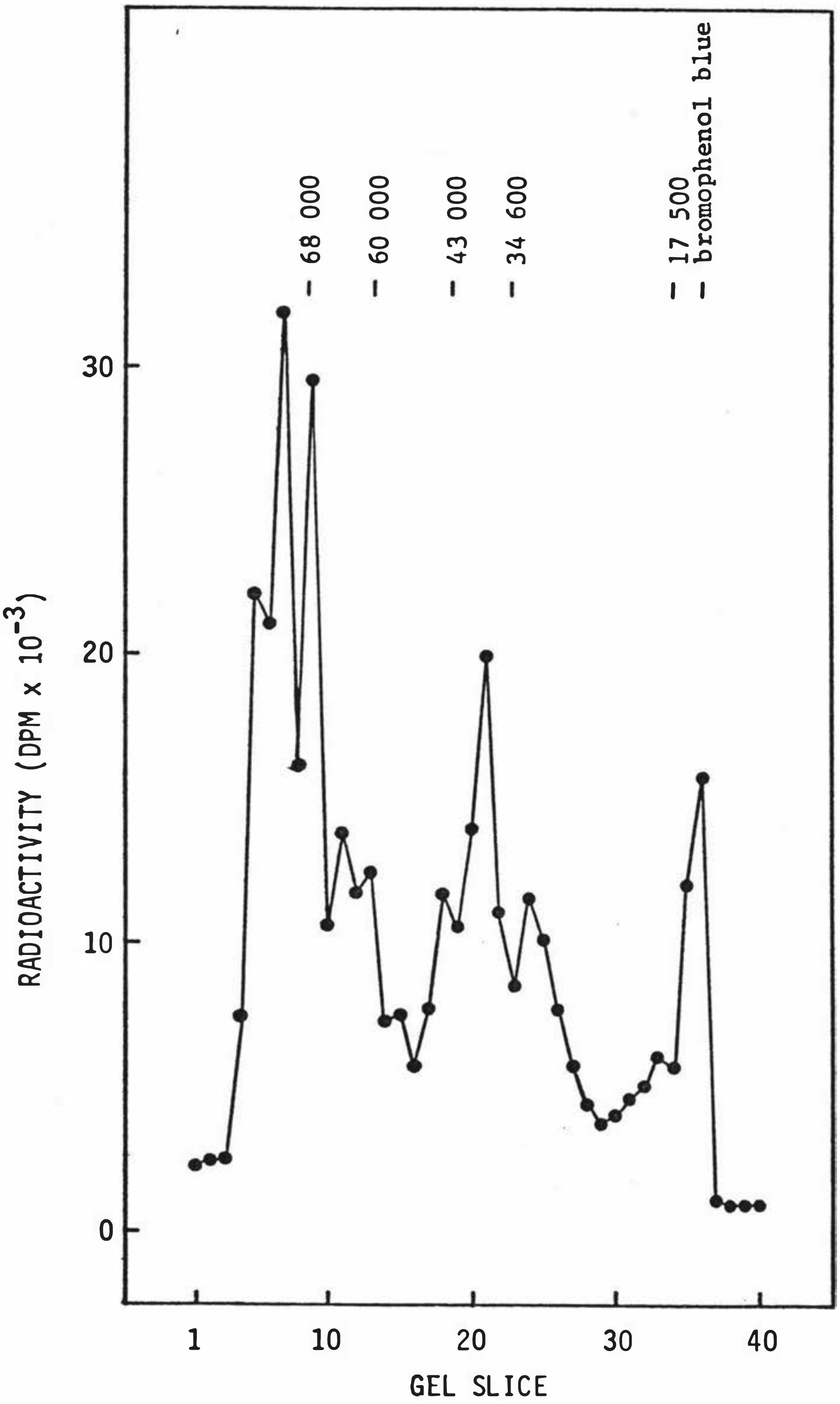


Figure 10.6

ISOLATION OF POLY(A)$^+$ RNA BY CHROMATOGRAPHY OF RAT LIVER POLYSOMAL RNA ON POLY(U)-SEPHAROSE

1200 $A_{260\ nm}$ units of RNA from free polysomes were chromatographyed on a 10 cm^3 column of poly(U)-Sepharose

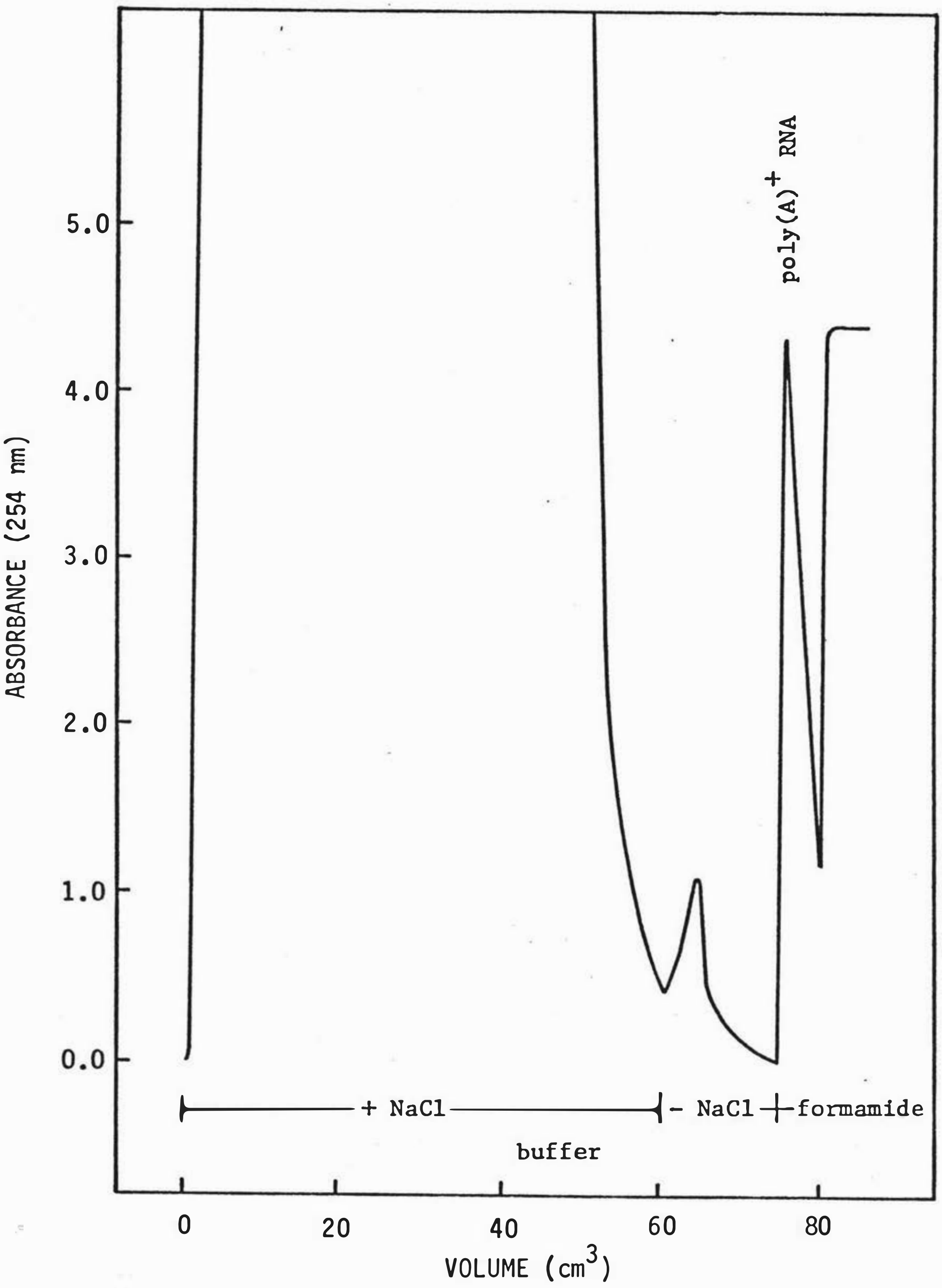


Figure 10.7

NON-DENATURING GEL (2.4%) ANALYSIS OF POLY(A)$^+$ AND POLY(A)$^-$ RNA FROM FREE POLYSOMES

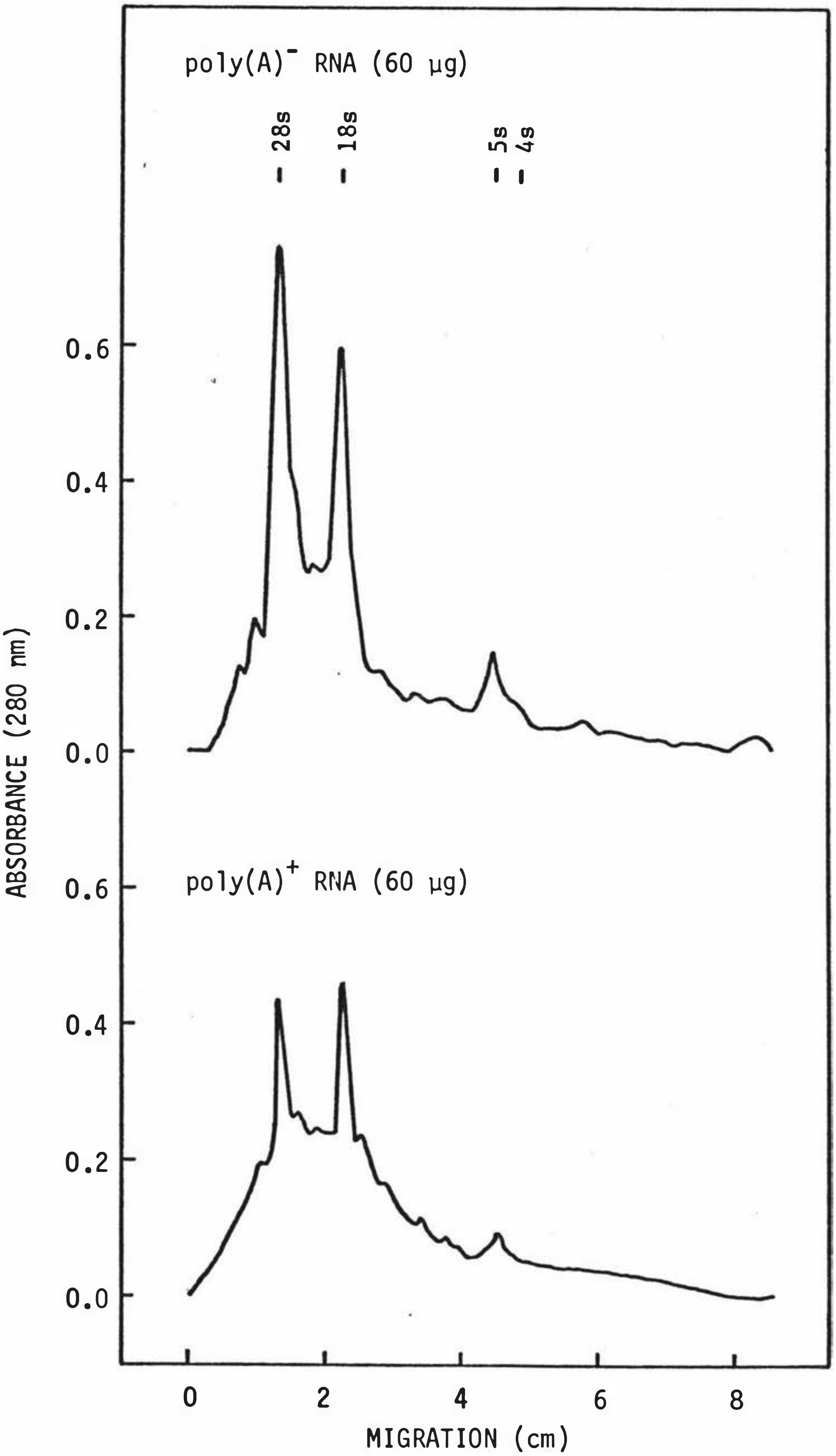


Figure 10.8

NON-DENATURING GEL (2.4%) ANALYSIS OF POLY(A)$^+$ AND POLY(A)$^-$ RNA FROM BOUND POLYSOMES

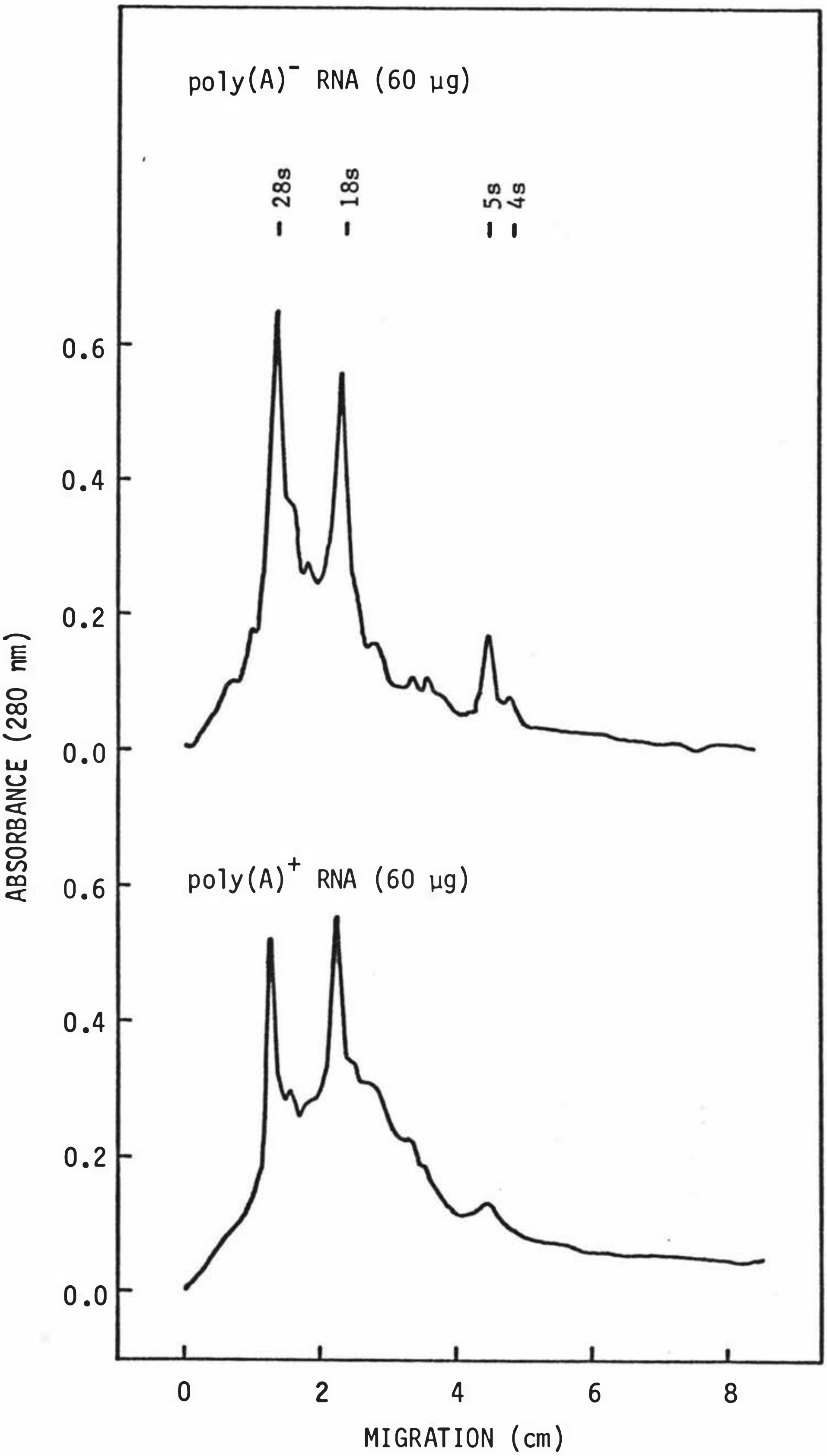


Figure 10.9

TIME COURSE OF PROTEIN SYNTHESIS AND EFFECT OF SPERMINE CONCENTRATION ON THE TRANSLATION OF POLY(A)$^+$ RNA IN WHEAT GERM EXTRACTS

3 μg of RNA was translated at optimum Mg^{2+}, K^+ and spermine concentrations in the standard assay

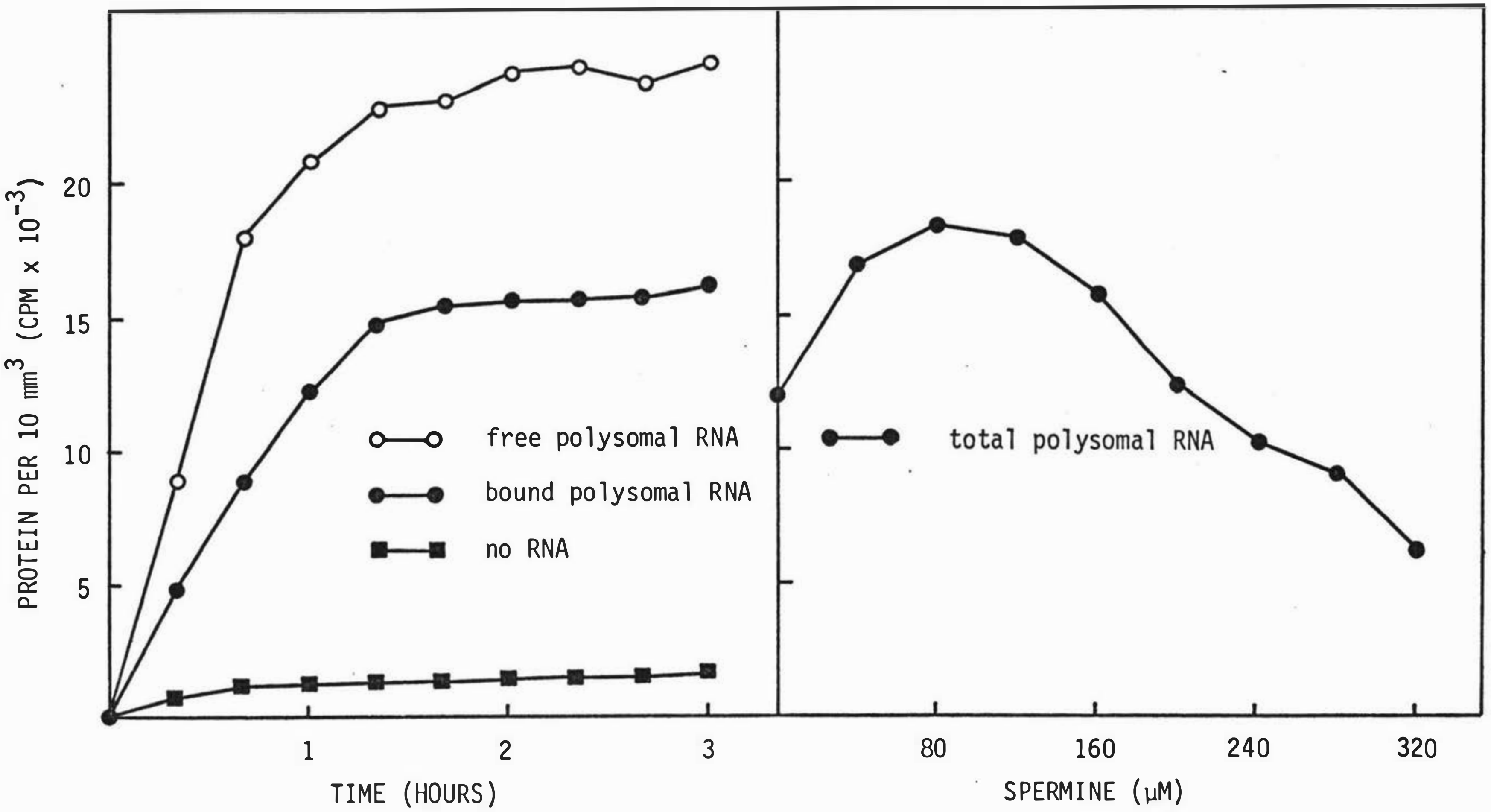


Figure 10.10

THE EFFECT OF K^+ AND Mg^{2+} CONCENTRATIONS ON THE TRANSLATION OF POLY(A)$^+$ RNA FROM TOTAL POLYSOMES IN WHEAT GERM EXTRACTS

3 µg of RNA were translated at optimum spermine concentration in the standard assay

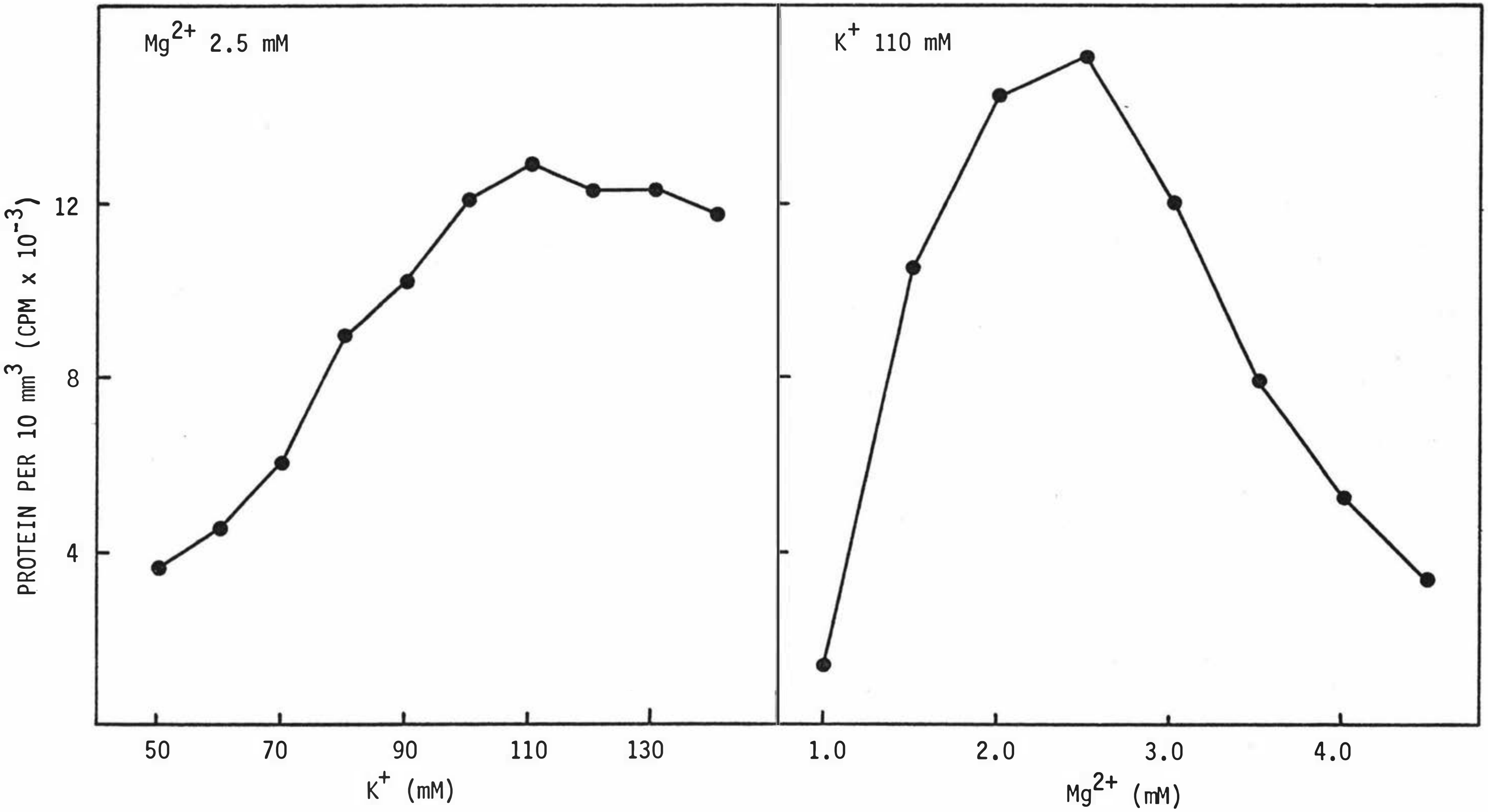


Figure 10.11

EFFECT OF RNA CONCENTRATION ON THE TRANSLATION OF POLY(A)$^+$ AND POLY(A)$^-$ RNA FROM FREE AND BOUND POLYSOMES IN WHEAT GERM EXTRACTS

RNA was translated at optimum Mg^{2+}, K^+, and spermine concentrations in the standard assay

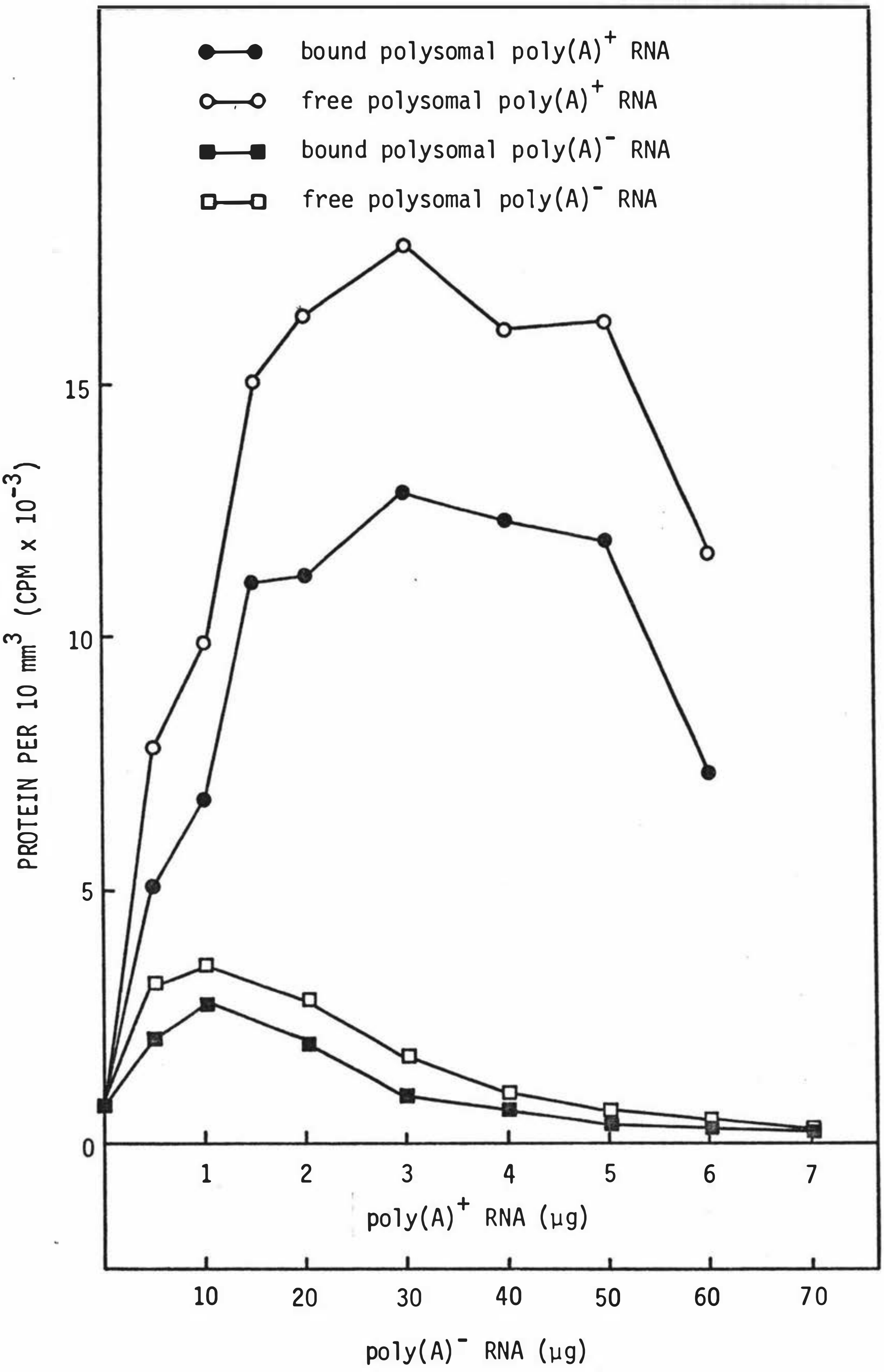


Figure 10.12

SDS GEL (7%) ANALYSIS OF RELEASED TRANSLATION PRODUCTS PRODUCED IN WHEAT GERM EXTRACTS PROGRAMMED WITH POLY(A)$^+$ RNA FROM FREE AND BOUND POLYSOMES

RNA was translated at optimum Mg^{2+}, K$^+$, spermine and RNA concentrations in the standard assay

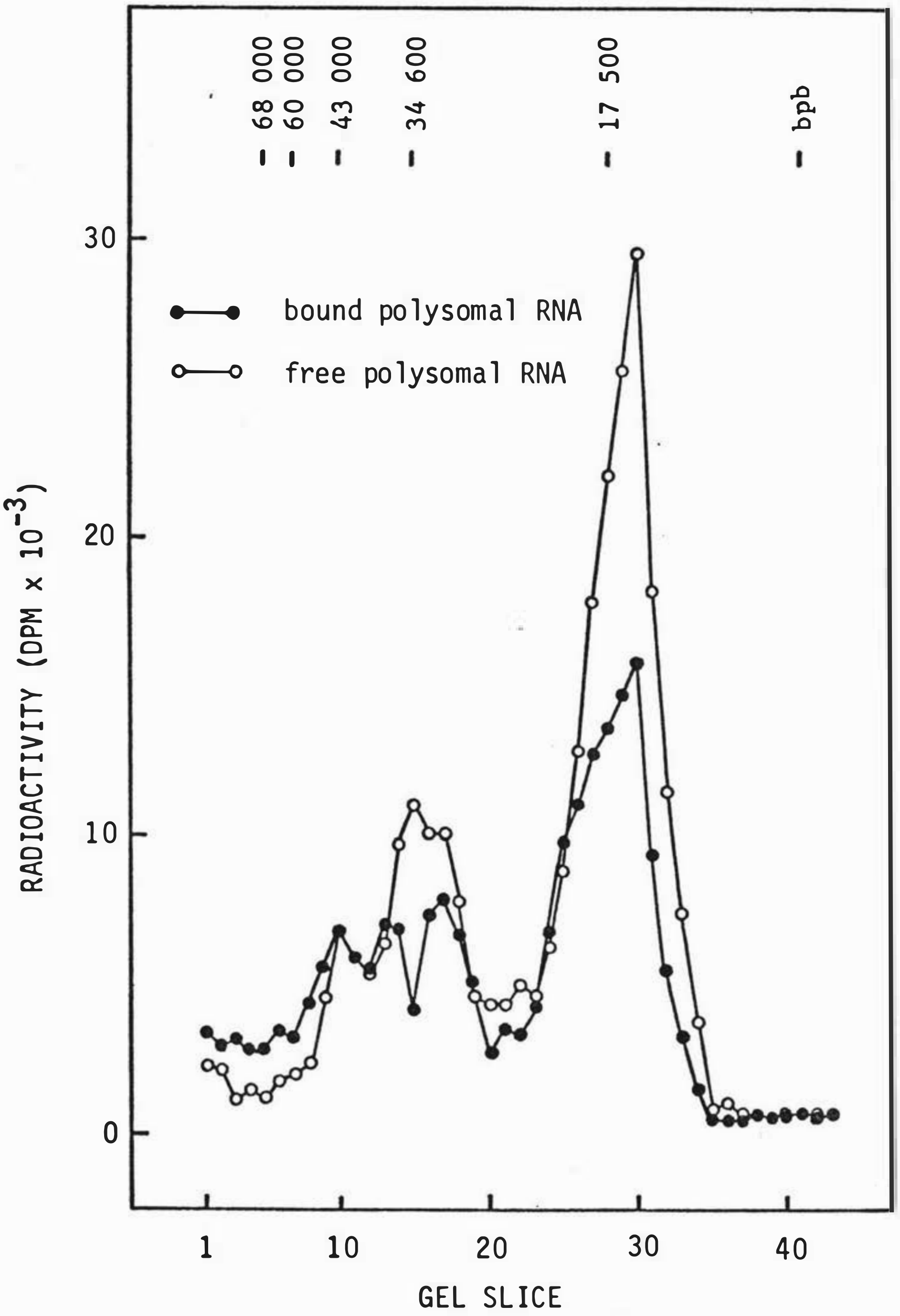


Figure 10.13

SDS GEL (7%) ANALYSIS OF RIBOSOME-BOUND TRANSLATION PRODUCTS PRODUCED IN WHEAT GERM EXTRACTS PROGRAMMED WITH POLY(A)$^+$ RNA FROM FREE AND BOUND POLYSOMES

RNA was translated at optimum Mg^{2+}, K^+, spermine and RNA concentrations in the standard assay

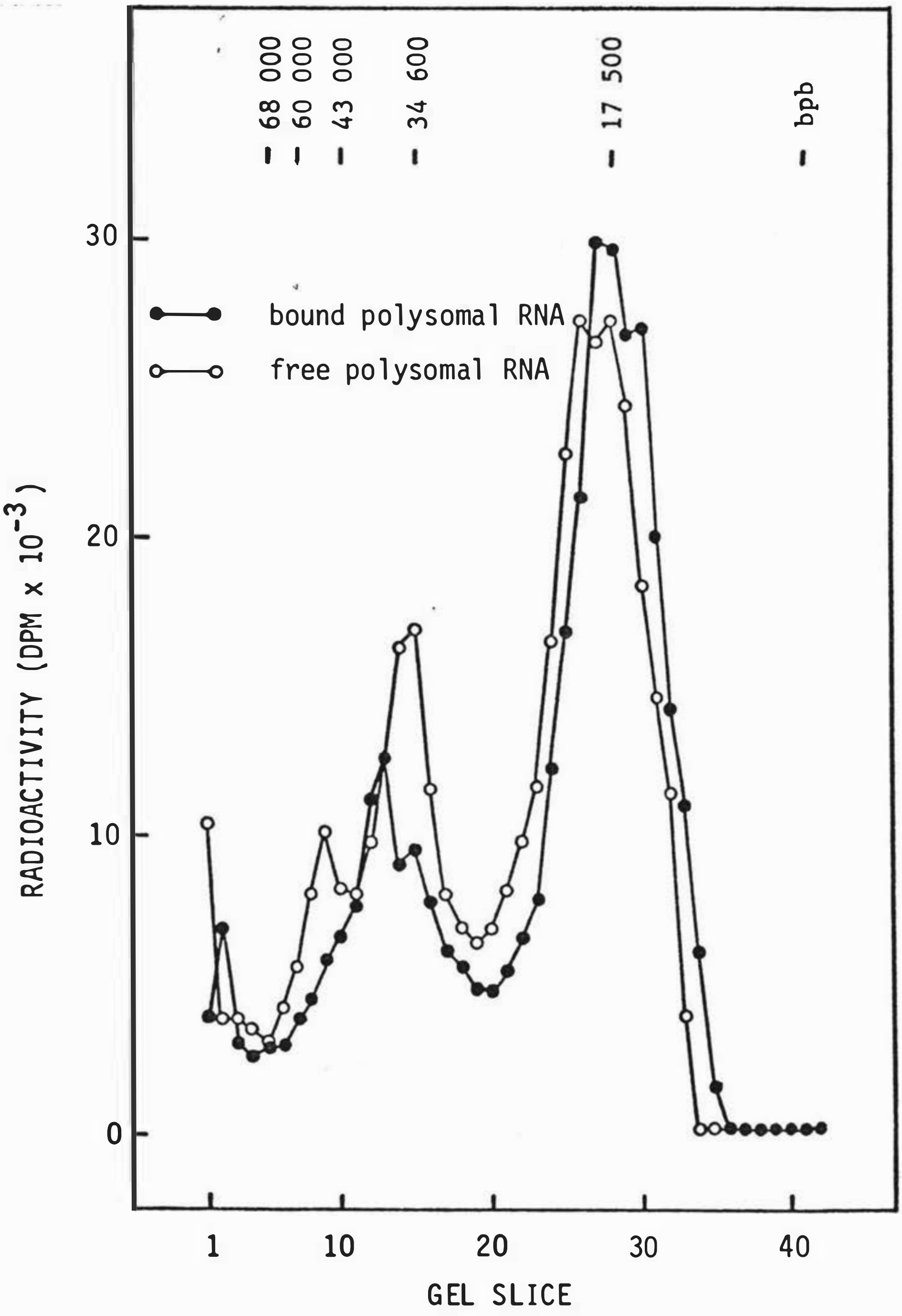


Table 10.1

DISTRIBUTION OF IMMUNOPRECIPITABLE ALBUMIN AND FRUCTOSE-1,6-BISPHOSPHATASE PEPTIDES PRODUCED IN WHEAT GERM EXTRACTS PROGRAMMED WITH POLY(A)$^+$ RNA FROM FREE AND BOUND POLYSOMES

		Radioactivity in	
RNA fraction	Total protein	Albumin	FBPase
		(DPM/100 mm^3)	
No RNA	12 496	30 (0.25%)	14 (0.12%)
Free polysomal	248 200	118 (0.04%)	555 (0.22%)
Bound polysomal	118 680	6 913 (5.22%)	478 (0.40%)

Distribution of immunoprecipitable albumin and FBPase migrating with authentic protein on the above SDS gels

		0 (0.00%)	0 (0.00%)
		0 (0.00%)	181 (0.07%)
		911 (0.77%)	148 (0.12%)

Figure 10.14

SDS GEL (10%) ANALYSIS OF TRANSLATION PRODUCTS PRODUCED IN WHEAT GERM EXTRACTS PROGRAMMED WITH POLY(A)$^+$ RNA FROM FREE AND BOUND POLYSOMES WHICH WERE IMMUNOPRECIPITATED WITH ANTI-FRUCTOSE-1,6-BISPHOSPHATASE

RNA was translated at optimum Mg^{2+}, K^+, spermine and RNA concentrations in the standard assay. 100 mm^3 of supernatant were immunoprecipitated.

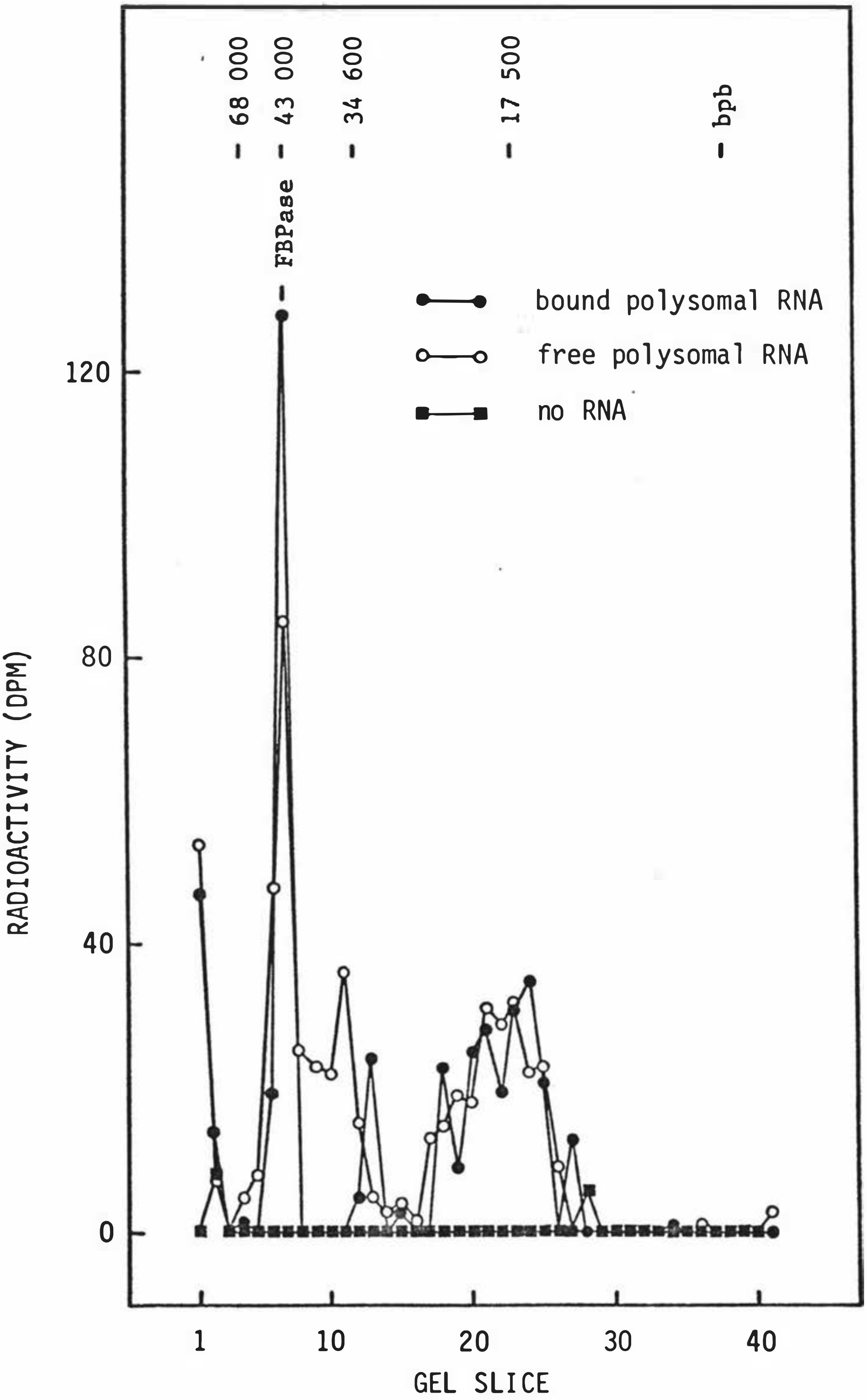
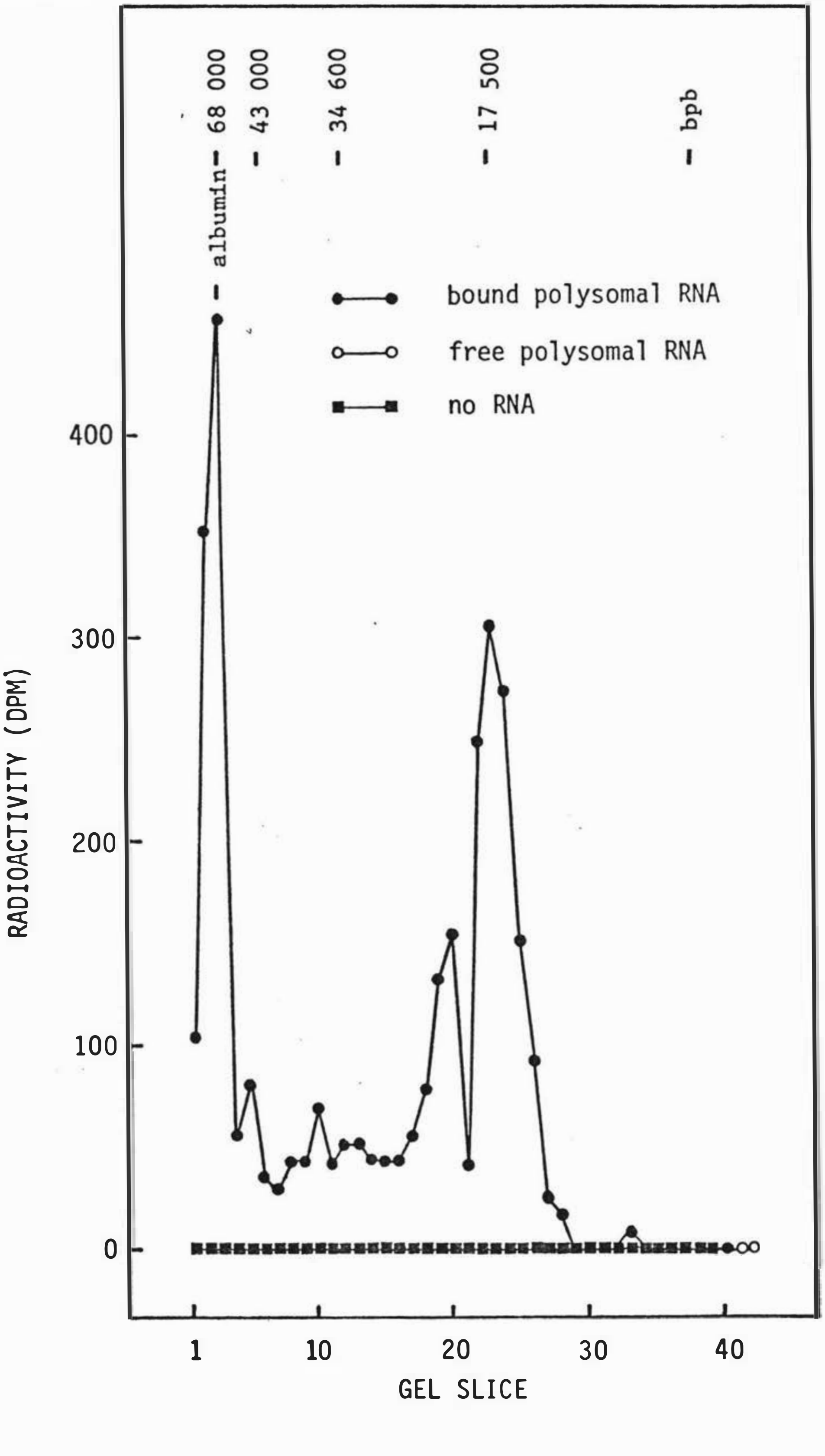


Figure 10.15

SDS GEL (10%) ANALYSIS OF TRANSLATION PRODUCTS PRODUCED IN WHEAT GERM EXTRACTS PROGRAMMED WITH POLY(A)$^+$ RNA FROM FREE AND BOUND POLYSOMES WHICH WERE IMMUNOPRECIPITATED WITH ANTI-ALBUMIN

RNA was translated at optimum Mg^{2+}, K^+, spermine and RNA concentrations in the standard assay. 50 mm^3 of supernatant were immunoprecipitated.



CHAPTER ELEVEN

DISCUSSION

This investigation has been directed towards several aspects of the biochemistry and molecular biology of rat liver FBPase.

Induction and purification of FBPase

Previous reports have indicated that the level of FBPase in rat liver could be increased by alloxan diabetes and triamcinolone treatment (Kvam and Parks, 1960; Weber et al., 1961, 1962, 1964, 1965). These results were interpreted as a specific adaptation of FBPase levels to conditions favouring gluconeogenesis. However in the present study neither the induction of enzyme by alloxan diabetes or triamcinolone treatment could be demonstrated. The non-inducibility of the enzyme under such conditions was supported by the work of Zalitis and Pitot (1977). In detailed studies of the rate of synthesis and degradation of the enzyme they concluded that the enzyme was produced constitutively. Several possibilities exist for discrepancies regarding the inducibility of FBPase in rat liver. Classically, FBPase was assayed by following the rate of phosphate release from FBP at pH 9.5. The choice of pH, 2 units above the enzyme optimum, and the possibility of interference from non-specific phosphatases makes such data difficult to interpret. The practice of reporting enzyme levels as units of enzyme activity per 100 g body weight or per liver is complicated by the quite marked changes in body and liver weight in alloxan diabetes and during triamcinolone treatment, which would tend to inflate apparent FBPase levels under such conditions.

The rate of FBPase synthesis, determined by pulse labelling liver protein synthesis, was 0.89% relative to the synthesis of total soluble protein. This figure is in good aggrement with the 0.8% reported by Zalitis and Pitot (1977), who also reported that the half-life of the enzyme was 46 hours under steady state conditions. This is considerably shorter than the half-life of total soluble liver proteins, estimated at 120 hours (Schimke et al., 1968; Stark and Frenkel, 1978), and 89 hours (Glass and Doyle, 1972).

The production of antibodies for subsequent investigations into the subcellular location of FBPase synthesis required the isolation of this enzyme at a high degree of purity.

The rat liver cytoplasmic enzyme FBPase was purified to homogeneity from rats fed ad libitum. An approximately 100-fold purification gave rise to a preparation which appeared homogeneous when examined by both denaturing and non-denaturing gel electrophoresis. The enzyme showed no evidence of proteolysis and had a single activity optimum at pH 7.5 in the presence of EDTA. Previous workers have reported that proteolytic cleavage of the enzyme occured during purification. These problems were overcome by incorporating a heat step at neutral pH into the purification. The problem of endogenous FBP interfering with the enzyme assay was also overcome by the heat step which destroyed this substrate. The specificity of elution from phosphocellulose enabled the preparation of the enzyme to be accomplished in 24 hours, which further reduced the chances of proteolysis occuring during preparation. This degradation was evident in earlier preparations of this enzyme using procedures which took several days to complete. The use of chow-fed rats may also decrease the chance of proteolysis. The enzyme prepared from rabbit liver after induction with alloxan, fasting or triamcinolone treatment showed considerably more proteolysis in the purified material (Pontremoli et al., 1973, 1974, 1975). This may have been caused by increased lysosomal fragility under these conditions and a consequent release of lysosomal enzymes during homogenization of the liver.

Intracellular protein synthesis and degradation

Proteins are synthesized by ribosomes in the cell cytoplasm. Although many proteins remain there, others have to be segregated to achieve their characteristic location in various intracellular and extracellular compartments. In many cases, proteins have to be transfered across one or two membranes. Examples are secretory, lysosomal, peroxisomal, glyoxysomal and nuclear proteins; certain mitochondrial and chloroplast proteins which are synthesized in the cytoplasm, as well as membrane proteins whose partial or complete penetration of the hydrophobic core of the lipid bilayer is a prerequisite for their asymmetric distribution in the membrane. Two distinct mechanisms have so far been uncovered for the transfer of proteins across intracellular membranes. In co-translational transfer, passage across the membrane is tightly coupled to translation and proceeds only during synthesis of the protein on membrane-bound ribosomes (Blobel and Dobberstein, 1975). In post-translational transfer, passage through membranes occurs after protein synthesis and is not mediated by a ribosome- membrane junction (Dobberstein et al., 1977).

The signal hypothesis was first proposed by Milstein et al., (1972) to account for the selective translation of mRNAs for secretory proteins on membrane-bound polysomes. They postulated that mRNAs for secretory proteins contained a sequence of codons localized at the 3' end of the AUG initiation codon. Translation of these codons would give rise to a signal sequence which would trigger binding of the nascent chain to the ER membrane. Ribosome receptor proteins in the membrane were also postulated, and it was proposed that they would aggergate to form a proteinaceous tunnel in the membrane upon interaction with the signal peptide. These events would effectively segregate the signal peptide from the lipid bilayer in preparation for its own exit as well as the co-translational passage of the remainder of the nascent chain. During or soon after transfer of the signal peptide into the luminal space of the ER, a 'signal peptidase' would cleave the signal peptide from the still uncompleted chain.

Considerable experimental evidence has lent support to this hypothesis. It has been shown now for a great number of secretory proteins that when their mRNAs are translated in a cell-free system, larger molecules are synthesized which contain an amino terminal extension of up to 30 amino acid residues. These signal sequences were characterised by a predominance and clustering of hydrophobic residues, two features consistent with the proposed role of the transient amino terminal extension in binding to presumed hydrophobic membrane receptors. Further evidence for the signal hypothesis came from experiments in which microsomes were added to cell-free translation experiments. The polysomes translating secretory proteins became bound to the microsomal membrane and these proteins were segregated in the luminal space of the microsomal vesicles. Such a segregation was not observed when microsomal vesicles were added after protein synthesis was complete. The protein recovered from inside the microsomes indicated that the signal peptides had been cleaved to give the normal mature form of the protein (Blobel and Dobberstein, 1975; Schmekpeper, 1975; Campbell and Blobel, 1976;

Habener et al., 1976; Lingappa et al., 1977; Birken et al., 1977; Jackson and Blobel, 1977; Schields and Blobel, 1977, 1978).

A large number of chloroplast proteins are synthesized in the cytoplasm and have to cross one or two membranes in order to reach the chloroplast stroma. Since ribosomes do not appear to be associated with the chloroplast envelope, the signal hypothesis cannot apply to the segregation of these proteins. When the mRNA for the small subunit of ribulose-1,5-bisphosphate carboxylase was translated in vitro, an N-terminal extension of 40-50 amino acid residues was detected. In contrast to secretory proteins, this protein was synthesized entirely on free polysomes and cleavage of the N-terminal extension could be achieved post-translationally (Dobberstein et al., 1977). It has been further suggested that this extension functions in a receptor mediated transfer of the protein through the chloroplast envelope. This example of post-translational transfer does however share some conceptually common features with co-translational transfer. The information for membrane transfer is encoded in part in the newly synthesized protein as a signal sequence that is removed during or after passage across the membrane. There should be several structurally distinct signal sequences, different for co- and post-translational transfers, and specific for each of the intracellular membranes across which transfer proceeds. It would also seem likely that additional factors are operating to account for the fact that only some signal sequences (i.e. for secretory proteins) cause binding of polysomes to membranes.

Further examples of post-translational transfer are encountered in the synthesis of the peroxisomal proteins uricase and catalase (Goldman and Blobel, 1978). These proteins are synthesized entirely on free polysomes and are not segregated either co- or post-translationally into microsomal vesicles. These results are surprising because ultrastructural studies had shown that peroxisomes bud from the ER (Novikoff and Shin, 1964), and the synthesis of peroxisomal proteins had been assumed to take place in a manner analogous to secreted proteins, except that peroxisomal proteins were segregated into intracellular vesicles after being released into the intracisternal space. N-terminal extensions acting as putative signals were also implicated in the post-translational transfer of these proteins. An analogous situation exists with malate dehydrogenase from glyoxysomes (the plant equivalent of peroxisomes) which is initially synthesized with a N-terminal extension (Walk and Hock, 1978).

The asymmetric distribution of membrane proteins has been accounted for by proposing three different types of synthesis and transfer. Proteins exposed to the cell cytoplasm would be synthesized by free polysomes, and after folding would be inserted from the cytoplasm into appropriate sites. In contrast, the schemes formulated in the signal hypothesis could account for those proteins exposed to the lumen side of membranes. Such a scheme could also account for proteins which span the membrane. Synthesis would proceed in a manner analogous to that of a secretory protein, except that the transfer of nascent chains across the ER could be incomplete. Cessation of transfer could be caused by a portion of the nascent chain (possibly hydrophobic in nature) causing disruption of the tunnel and the ribosome-membrane junction. Evidence for this mechanism has come from studies on the vesicular stomatitis virus membrane glycoprotein G. Translation of the mRNA for this protein in vitro gave rise to an unglycosylated protein with an N-terminal extension. Co-translational addition of microsomes from HeLa cells resulted in the binding of ribosomes synthesizing this protein to the membrane. Segregation of the protein occured as noted in vivo; a portion of the polypeptide chain (about 3000 daltons) remained exposed on the cytoplasmic side of the microsomes and the amino terminus penetrated into the microsomal lumen and became glycosylated (Katz et al., 1977; Toneguzzo and Ghosh, 1978).

It remains to be seen whether the compartmentation of those mitochondrial proteins synthesized in the cytoplasm follow a similar pattern to that proposed for the topologically analogous chloroplasts. As yet there is no experimental evidence pertaining to the segregation of nuclear proteins. Nuclear pores could possibly play some role in sorting out those proteins destined for compartmentation inside the nucleus.

It is a possibility that all proteins are synthsized with a N-terminus carrying the information concerning the required fate of the protein — whether it is to be cytoplasmic, secreted, or incorporated into a specific subcellular organelle. This may be achieved by a N-terminal extension which is subsequentially cleaved, or alternatively the normal N-termini of some proteins may act as the signal as seems to be the case for ovalbumin. Presumably different signal peptides could carry such information; the fate of the protein being dependent on specific cellular receptors for particular signal peptides. There should be several structurally distinct signal sequences, different for co- and post-translational transfer, and specific for each of the intracellular membranes across which transfer proceeds. The failure to find any difference between the size of mature cytoplasmic proteins and those synthesized _in vitro_, may be due to endogenous proteases present in _in vitro_ translational systems. Evidence for the cleavage of N-terminal signal peptides of glyoxisomal malate dehydrogenase (Walk and Hock, 1978), and human placental lactogen (Peeters et al., 1979), and cleavage of N-terminal methionine and acetylation (Palmiter et al., 1977, 1978) have been reported from wheat germ translation systems. All these events occured with the same degree of specificity as in the tissue from which the RNA was extracted. Direct information about initial protein N-terminal sequences will come from the sequence analysis of specific mRNAs. The 5' end of human globin α and β chains have been sequenced and shown not to have any sequences coding for a possible leader sequence (Chang et al., 1977).

Previous studies on the site of synthesis of cytoplasmic proteins have yielded conflicting results. Electron micrograph studies showed that cells active in protein secretion contained a high proportion of ribosomes bound to the ER. In contrast, cells which did not secrete their synthesized protein showed fewer membrane-bound ribosomes, but numerous ribosomes free in the cytoplasm (Siekevitz and Palade, 1960). These observations led to the concept that proteins destined for export from the cell were made on bound polysomes, and proteins retained by the cell were made on free polysomes. However experiments designed to locate the site of synthesis of specific cytoplasmic proteins have shown that this hypothesis is not correct for all cytoplasmic proteins. Rabbit reticulocyte globin (Woodward et al., 1973) and rat liver ferritin (Hicks et al., 1969; Puro and Richter, 1971; Konijin et al., 1973; Zähringer et al., 1977) have been shown to be synthesized on both free and bound polysomes. Rat liver serine dehydratase was synthesized to a greater extent on bound polysomes, and the induction of this enzyme by glucagon and high protein diets was associated initially with an increase in synthesis on bound polysomes (Pitot and Jost, 1968; McLaughlin and Pitot, 1976b). However rat liver arginase synthesis appears only to be associated with free polysomes (Tanaka and Ogata, 1971).

Experimental objectives and conclusions

A major objective of this investigation was the study of the intracellular location of FBPase synthesis, in particular to determine on which class of polysome this protein is synthesized.

The binding of affinity-purified ^{125}I-labelled antibodies against FBPase and albumin to isolated free and bound polysomes showed that nascent albumin chains appeared to be almost entirely confined to bound polysomes, as has been shown by other workers using this method (McLaughlin and Pitot, 1976a). FBPase antibody bound with almost equal affinity to both classes of polysome. An advantage of this method for quantitating protein synthesis is that the functional ability of polysomes for protein synthesis *in vitro* is not required. To compare amounts of binding using this method it is necessary to assume that messengers on both free and bound polysomes are translated with equal efficiency.

Incubation of isolated free and bound polysomes in a cell-free system derived from rat liver cytoplasm confirmed that albumin synthesis was confined to bound polysomes (Zähringer et al., 1977; Goldman and Blobel, 1978), and FBPase synthesis was found to be associated with both types of polysome. Although some full-length protein transcripts co-migrating with authentic protein were found in immunoprecipitates, a large amount of shorter polypeptide material was specifically immunoprecipitated. These shorter polypeptides were presumed to arise from premature termination and release of nascent albumin and FBPase chains. To be immunoprecipitated they must obviously contain antigenic determinants recognisable by the specific antisera. The lower levels of relative synthesis of FBPase and albumin on isolated polysomes *in vitro* compared to *in vivo*, may have resulted from several factors including: (a), the incubation conditions *in vitro* favouring the synthesis of particular proteins, (b), the fall-off in elongation and release of proteins synthesized on large polysomes (Ramsey and Steele, 1976), and (c), the production of albumin and FBPase peptides without any recognizable antigenic determinants. The synthesis of some full-length transcripts of both albumin and FBPase was an indication of the functional integrity of the isolated polysomes.

Both antibody binding to polysomes, and *in vitro* protein synthesis by polysomes showed albumin synthesis to be associated only with bound polysomes, and FBPase synthesis to be associated with both free and bound polysomes. However the translation of isolated mRNA from each type of polysome in a cell-free protein synthesizing system should give an indication as to whether the synthesis was a result of the partitioning of specific messengers between free and bound polysomes, or a translational phenomenon. The possibility exists that specific messengers may be present on free or bound polysomes, but the expression of the messenger is being affected by the rate of initiation and/or elongation of that messenger. This situation appears to be the case with rat liver ferritin mRNA, which is equally abundant in both free and bound polysomes. However isolated free polysomes synthesize three times more ferritin than bound polysomes when incubated *in vitro* (Zähringer et al., 1977).

Poly(A)$^+$ RNA was isolated from free and bound polysomal RNA by poly(U)-Sepharose chromatography, and was found to contain most of the mRNA activity when translated in a cell-free protein-synthesizing system derived from wheat germ. Albumin mRNA activity was confined to bound polysomal poly(A)$^+$ RNA, and FBPase mRNA activity was present in poly(A)$^+$ RNA from both classes of polysome. As with polysomes incubated *in vitro*, wheat germ extracts programmed with mRNA produced both full-length transcripts and prematurely-terminated albumin and FBPase peptides, which were immunoprecipitated. The ability of such extracts to produce full-length transcripts has been reported to be dependent on the incubation conditions employed, and the messenger being translated (Taylor and Tse, 1977). Premature termination and release may be associated with the choice of translation conditions, specifically the potassium and magnesium ion concentrations. Endonucleolytically-cleaved mRNA could also have given rise to small molecular weight peptides, although this was considered unlikely on the basis of the molecular weight distribution of poly(A)$^+$ RNA on polyacrylamide gels. TMV RNA when translated in the same wheat germ system, did not give rise to prematurely-terminated peptides.

All three types of experiment have demonstrated that albumin is synthesized only on bound polysomes, whereas FBPase synthesis takes place on both free and bound polysomes. This segregation of synthetic activity was shown to be a reflection of mRNA distribution and was not due to differential translation of mRNA. However pulse-labelling of liver protein synthesis in vivo revealed that newly synthesized FBPase was segregated into the cell cytoplasm, whereas albumin was associated within the microsomal vesicles, and amounted to 16.7% of total microsomal protein synthesis. Clearly the synthesis of proteins on bound polysomes does not necessarily result in their segregation into the intermembrane space of the ER.

The conclusions reached in this investigation are critically dependent on the separation of free and bound polysomes. It is of considerable importance to realize that these polysomes subclasses are purely operational definitions. As yet no evidence is available to precisely link the ultrastructural observations of electron microscopy — which shows polysomes apparently free in the cell cytoplasm, and bound to membrane structures — to the polysome subclasses isolated by various centrifugation techniques. The ultrastructural observations also do not show whether the free and bound polysomes visualized are homogeneous subclasses.

Polysomes were prepared by the differential centrifugation method of Steele et al. (Vankatesan and Steele, 1972; Ramsey and Steele, 1976, 1977), rather than by a isopycnic method (Blobel and Potter, 1967). The latter method, in which polysomes are prepared from a post-mitochondrial supernatant, leads to the loss of up to 70% of the bound polysomes and about 10% of the free polysomes into the mitochondrial pellet. This approach is subject to the criticism that the post-mitochondrial supernatant contains only a small, possibly non-representative portion of the bound polysomes (Palade and Siekevitz, 1956; Blobel and Potter, 1967; Vankatesan and Steele, 1972), and that free ribosomes can account for up to 30% of the bound fraction (Blobel and Potter, 1967; Lowe et al., 1970; Bont et al., 1972). In the present investigation, attempts made to prepare free and bound polysomes using isopycnic procedures met with little success. Polysomes prepared in this manner appeared to be significantly degraded when examined on isokinetic sucrose gradients in comparison to those isolated by differential centrifugation. The degradation most probably stemmed from the long centrifugations necessary in this method, and appears to be difficult to avoid using this procedure judging from published polysome profiles (Noll, 1969). The separation of free and bound polysomes by differential centrifugation however, produced undegraded polysomes in yields identical to those published for this method (Ramsey and Steele, 1976, 1977). Both classes of polsome were very active in protein synthesis. An estimate of the contamination of bound polysomes by free was obtained by suspending the microsomal pellet and recentrifuging. Less than 12% of the polysomes remained in the supernatant. A second resuspension of the pellet and recentrifugation showed that about 1% of the polysomes remained in the supernatant. This result suggests that a maximum of 12% of the bound polysome fraction normally isolated could be free polysomes. Since the ratio of bound to free polysomes in this tissue is approximately 2:1, this means that approximately 24% of the free polysomes could be in the bound fraction. Since all three methods used to study the distribution of FBPase synthesis showed that both types of polysome were about equally active, it is unlikely that this was due to contamination of bound polysomes by free. The clear-cut segregation of albumin synthesis on bound polysomes during translation experiments was indicative of very little contamination of free polysomes by bound.

The possibility that there are two different types of FBPase subunits (implying two different mRNAs), one syntheiszed on bound polysomes, and the other on free, seems unlikely. The rat liver enzyme appears to be composed of four identical subunits with blocked N-termini and C-terminal leucine (Tejwani et al., 1976). The amino acid composition indicates a close homology with the rabbit liver enzyme, the subunits of which have been shown to be identical by X-ray diffraction studies (Soloway and McPherson, 1978).

There is no reason to presume that because bound polysomes are the exclusive site of synthesis of secreted proteins, that these polysomes are only involved in this function. The presence of a hydrophobic leader sequence for secreted proteins has led to the conclusion that this is the mode of attachment of polysomes, i.e., that whether a ribosome is free or bound is determined by a hydrophobic signal sequence at the N-terminus of the nascent peptide. This view must be abandoned in the light of recent experiments. The most convincing evidence comes from the observation that when isolated microsomes were incubated *in vitro*, only about half the newly synthesized protein was transported inside the vesicles during translation (Redman and Sabatini, 1966; Andrews and Tata, 1971b; Bevan, 1971). In these experiments, those proteins destined for export from the cell were wholly segregated into the microsomal vesicles. Although HeLa cells export less than 2% of their newly synthesized protein (Rosbash, 1970), about 15% of the ribosomes are found to be associated with membranes (Attardi et al., 1969). The translation of ovalbumin on bound polysomes (Pennequin et al., 1978) also remains to be explained, as this protein is translated without a N-terminal hydrophobic sequence (Palmiter et al., 1978).

The existence of a previously unrecognized class of polysomes that is bound to the ER has been reported (Palmer et al., 1978). These centrifugally-labile polysomes were susceptible to carcinogen-induced degranulation, and included those susceptible to degranulation by lipid peroxidation. These polysomes were not detached from membranes by exposure to high KCl concentration ('loosely bound'), or high KCl concentrations in the presence of puromycin ('tightly bound'). The polysomes responsible for histone synthesis appear to be in this class (Zauderer et al., 1973).

How the distribution of FBPase mRNA between free and bound polysomes is achieved remains unresolved. The possibility exists that the blocked N-terminus of the enzyme subunit is acetylated (Since the enzyme from rabbit liver has been shown to be acetylated), and it could be envisioned that acetylation of the enzyme may proceed via a membrane-bound acetylase. This would suggest a new type of membrane-protein interaction, analogous to the signal hypothesis, in which the sequence of the newly-syntheisized N-terminus triggers a transient association with the ER, and acetylation of the nascent peptide chain. Such a theory must however account for the fact that about half the polysomes synthesizing FBPase appear to be free in the cytoplasm. Perhaps these polysomes contain nascent chains which have yet to be acetylated. A parallel may exist in the membrane association and transport of ovalbumin into the intravesicular space in oviduct tissue. The absence of any hydrophobic extension and of any naturally hydrophobic region at the N-terminus (Palmiter et al., 1978), suggests that a feature of the normal N-terminal sequence may function as a signal. Perhaps the N-terminal acetylation of this protein, which has been shown to occur (at least *in vitro*) when the nascent chain is about 44 amino acid residues long, may function as a putative signal, but which in this case also results in the transport of the ovalbumin across the membrane, rather than remaining in the cytoplasm as is the case for FBPase.

The sequence analysis of a number of acetylated proteins has shown that the N-terminal acetylated amino acid is either alanine, glycine, serine or methionine (Jörnvall, 1975). Additional factors in determining whether a protein is to be acetylated must be involved, as not all proteins with the above N-termini are acetylated. The N-terminal distribution of branched-chain residues is different in acetylated proteins than that found in general. Isoleucine is over-represented, long hydrophobic segments are absent, and at some positions charged residues are common. These properties are observed regardless of the origin or function of the protein (Jörnvall, 1975). It is also of interest to note that vertebrate cytochrome c, bovine fibrinopeptide B, mammalian α-MSH, sheep, pig and bovine luteinizing hormone β-chain and a bovine mitochondrial structural protein are acetylated (Jörnvall, 1975). These proteins are either secreted from various tissues or are segregated into organelles. Some histones are also acetylated, and this may be related to their synthesis on a special type of bound polysome (Zauderer et al., 1973). This may be analogous to the finding that FBPase syntheis takes place, in part, on bound polysomes.

A criticism of this hypothesis is that ovalbumin synthesized in vitro, in a rabbit reticulocyte cell-free protein-synthesizing system, is acetylated by a soluble enzyme (Palmiter et al., 1978). However it cannot be ruled out that the enzyme was solubilized in the preparation of the reticulocyte lysate, as has been suggested for the putative signal peptidase responsible for cleaving catalase and uricase N-terminal extensions (Goldman and Blobel, 1978).

Future work

Logical extensions to the present work would include a more systematic investigation into the translation of FBPase and albumin mRNAs in an in vitro translation system. The translation of poly(A)$^+$ RNA at only a single Mg^{2+} and K$^+$ concentration (optimal for total polysomal poly(A)$^+$ RNA) imposes limitations on the interpretation of albumin and FBPase synthesis, although the absolute partitioning of synthetic activity should not have been affected. A more thorough investigation of the specific ion requirement for the translation of these two proteins in this particular wheat germ system is needed, as this was most probably the reason for premature termination. Due to severe limitations on scintillation counting facilities available, this investigation was not attempted. Premature termination problems associated with wheat germ systems may be overcome by employing the cell-free protein-synthesizing system derived from rabbit reticulocytes.

The recent demonstration that the peroxisomal proteins uricase and catalase are synthesized exclusively on free polysomes (Goldman and Blobel, 1978), will provide a further internal control to verify the separation of free and bound polysomes. The exclusive synthesis of albumin on bound polysomes confirmed that free polysomes were not contaminated by bound polysomes. These two peroxisomal proteins should permit an answer to the unresolved question of contamination of bound polysomes by free polysomes.

Preliminary attempts were made to purify FBPase mRNA by immunological methods. Immunoprecipitation of affinity-purified anti-FBPase bound to polysomes by sheep anti-rabbit gamma globulin, according to the method of Taylor and Tse (1976) was unsuccessful because of the low avidity of the sheep antibody and a consequential long incubation time required for precipitation, which resulted in polysome degradation. The covalent attachment of affinity-purified sheep anti-rabbit gamma globulin (Schütz et al., 1977) to _m_-aminobenzyloxymethylcellulose (Mougdal and Porter, 1963) also failed to give sufficient binding to polysomes in the short incubation times necessary to preserve polysome integrity. This problem may be circumvented by the use of Protein A, the membrane protein from _Staphylococcus aureus_, which acts in a manner analogous to anti-gamma globulin antibody.

Ultimately the mRNA for FBPase might be purified and sequenced, which would provide information on structure-function relationships. Sequencing of the mRNA would enable any transient signal peptides to be identified. Possible mechanisms of mRNA-membrane interactions may also become evident when more eukaryotic mRNAs are sequenced.

Further work on the fractionation and identification of different polysome classes in relation to their function and intracellular location is badly needed. In particular the relationship between the association of polysomes with membranes, and the protein being translated from particular mRNAs should shed some light on the nature of polysome-membrane interactions, especially as more information on eukaryotic mRNA structure becomes available.

Binding of electron-dense labelled specific antibodies to electron microscope sections _in situ_ should enable some correlation of ultrastructural observations to the cellular site of synthesis of specific proteins. Further work on protein synthesis by isolated microsomes is necessary to elucidate the features which determine the intracellular location of newly synthesized protein. Specifically what determines the segregation of synthesis of cytoplasmic proteins onto polysomes associated with membranes needs to be investigated. This specificity could reside in either the sequence of the protein being translated, or the mRNA coding for that protein. The different types of nascent peptide chains synthesized on membrane-bound polysomes, viz., whether they are being vectorially discharged across a membrane, or released into the cytoplasm, should enable some differentiation and separation of membrane-bound polysomes according to the type of protein being synthesized.

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