SYNTHESIS IN VITRO OF A PUTATIVE PRECURSOR OF MITOCHONDRIAL MALATE DEHYDROGENASE

By

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ABSTRACT

Although mitochondria have their own genetic system, their DNA codes for less than 10% of their protein mass. The bulk of mitochondrial protein is coded for by the nucleus, synthesized in the cytoplasm and imported. Much attention has recently been focussed on the question of how these proteins are specifically directed to the mitochondrion.

It has been found that many mitochondrial proteins are synthesized as higher molecular weight precursors, which might suggest a role in directing their uptake by mitochondria - thus it was decided to determine if rat liver mitochondrial malate dehydrogenase (mMDH) is also first synthesized as a higher molecular weight precursor.

Rat liver mMDH was purified by a rapid procedure developed here and antibodies to the denatured rat liver and beef heart (commercial) enzyme were raised in white rabbits. These antisera were shown to cross-react with denatured rat and beef mMDH and were highly specific for these antigens. Total RNA and free polysomes isolated from rat liver were translated in a micrococcal nuclease-treated reticulocyte lysate cell free protein synthesizing system in the presence of [^{35}S]methionine. The translation products were denatured and incubated with antiserum after which antigen-antibody

complexes were removed using Protein A Sepharose. The complexes were subsequently stripped from the Protein A then examined by SDS polyacrylamide gel electrophoresis and fluorography.

It was found that a protein 1,500-2,000 daltons larger than mature rat liver mMDH (34,500 daltons) was immunoprecipitated using either free polysomes or total RNA to prime synthesis. The recovery of this protein could be prevented by competition with excess unlabelled mMDH. This suggests that this 36,000 dalton protein is a higher molecular weight precursor of rat liver mMDH. It was also found that ten times more of this protein could be immunoprecipitated using free polysomes rather than total RNA suggesting that the free polysome fraction is involved in the synthesis of this enzyme.

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ABBREVIATIONS

mMDH	mitochondrial malate dehydrogenase
sMDH	soluble malate dehydrogenase
AMP	adenosine monophosphate
ATP	adenosine triphosphate
GTP	guanosine triphosphate
NADH	nicotinamide adenine dinucleotide
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
MSH	2-mercaptoethanol
SDS	sodium dodecyl sulphate
PAGE	polyacrylamide gel electrophoresis
MEM	minimum essential medium
RNA	ribonucleic acid
mRNA	messenger ribonucleic acid
tRNA	transfer ribonucleic acid

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I. INTRODUCTION

As knowledge of the intricate structure of eukaryotic organelles has developed and the sites of synthesis of their components were determined, a most intriguing aspect of cellular biogenesis became apparent: the sequestration of proteins synthesized in the cytosol into many distinct sites in the cell. In the large and varied volume of intracellular protein "traffic" many proteins must be localized in a particular organelle. The challenge is to elucidate how information contained in the protein and the organelle conveys this specificity.

The biogenesis of highly compartmentalized organelles such as mitochondria and chloroplasts (Fig. 1) may be quite complex. Some mitochondrial proteins must traverse up to two membranes and some chloroplast proteins up to three membranes before reaching their final destination (Chua and Schmidt, 1979). There may be an assymetrical integration into one of the membranes or transport may be completely across, if the final residence is to be an intermembrane or matrix space. This variety of destinations within a single organelle serves to illustrate that many separate questions can be raised if one is to adequately describe the mechanism by which organelles import proteins: 1) in what form do the newly synthesized proteins travel to the

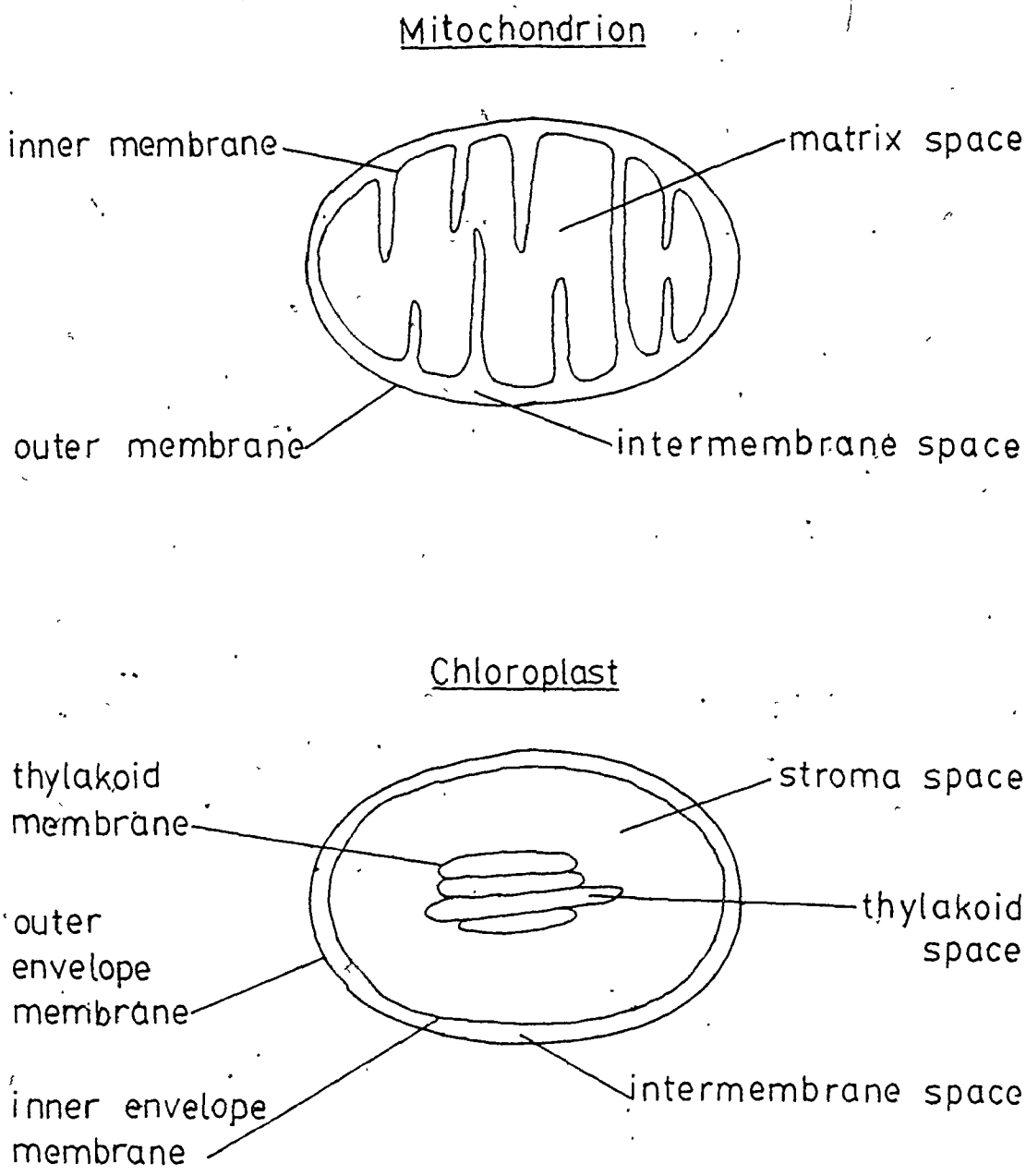


Fig. 1: Structure of mitochondria and chloroplasts.

organelle? 2) How is the specific organelle recognized? 3) How is the actual transport achieved (transport here refers simply to movement) given that certain thermodynamic problems arise? 4) What conveys irreversibility to the process? Much attention has recently been focussed on these aspects of the import process and it has been the subject of several recent reviews (Blobel et al., 1979; Wickner, 1979, 1980; Chua and Schmidt, 1979; Waksman et al., 1980; Neupert and Schatz, 1981a; Kreil, 1981).

Secretion of Protein from Cells

Before attention was directed at the problem of the intracellular localization of protein, the problem of how proteins are secreted from cells was examined. Here similar problems are involved. In particular, how are proteins destined for export selected and how is export achieved? Advances made in understanding how these proteins are directed to and transferred across the endoplasmic reticulum as the first step in secretion from cells was a great stimulus for research in organelle biogenesis. The development of the "signal hypthesis" for secretion by Blobel and his colleagues (Blobel and Dobberstein, 1971, 1975a,b; Blobel et al., 1979) has been a useful unifying hypothesis against which to examine assembly of organelles. The hypthesis will be outlined briefly.

It has been observed that many proteins destined for export were synthesized on ribosomes bound to endoplasmic

reticulum (reviewed by Palade, 1975). Subsequently it was demonstrated that proteins synthesized on these membrane bound polysomes were vectorially transferred during synthesis into the lumen of the endoplasmic reticulum (Redman et al., 1966; Redman and Sabatini, 1966; Sabatini and Blobel, 1970; Adelman et al., 1973). Milstein et al. (1972) discovered that the light chain of Ig6 which is secreted from mouse myeloma tumor cells, is synthesized in a higher molecular weight precursor form. This finding was subsequently confirmed by several laboratories (e.g. Swan et al., 1972, Mach et al., 1973; Schechter et al., 1974). The "signal hypothesis" proposed by Blobel and Dobberstein in 1975 postulated that all proteins destined for export would be synthesized with an amino-terminal extension. This sequence would serve as a signal. The mRNA coding for these proteins would first associate with ribosomes free in the cytosol. When the signal was synthesized it could specifically initiate binding to the endoplasmic reticulum where the ribosome signal complex would recruit membrane proteins to form a translocation complex. A pore would be formed in the membrane through which the growing polypeptide chain would be extruded into the lumen of the endoplasmic reticulum. Transport would be passive in that it would be driven by polypeptide chain elongation and the signal sequence would be cleaved by a specific peptidase located on the cisternal side of the

membrane. Irreversibility would be provided by this cleavage, conformational changes and synthesis on one side of the membrane. The evidence for this mechanism is compelling and has been reviewed many times (Blobel et al., 1979; Chua and Schmidt, 1979; Wickner, 1979, 1980). Recently, a membrane-associated protein complex has been purified from dog pancreas microsomes which is required for protein translocation across the endoplasmic reticulum (Walter and Blobel, 1980).

The primary structure of the signal sequences examined so far have revealed a highly hydrophobic central region flanked by polar and often basic residues, but no other obvious sequence homologies exist (Blobel et al., 1979; Kreil, 1981). The physical basis for the signal function is not clear except that there may be a requirement for a certain minimum hydrophobicity. If rat preprolactin or human placental prelactogen (which contain leucine-rich signal sequences) are synthesized in vitro in the presence of a polar leucine analogue, translocation into microsomal vesicles is prevented (Hortin and Boime, 1980). Studies on the hydrophobicities of signal sequences have led von Heijne (1981) to suggest that the sequences partition directly into the membrane interior.

Palmiter (1978, ref. add.) discovered that the secreted protein chicken ovalbumin is not synthesized as a higher

molecular weight precursor, indicating that the signal need not be an amino-terminal extension which is subsequently cleaved. The location of the signal peptide of ovalbumin has not been established (Kreil, 1981).

After segregation of the secretory proteins into the lumen of the endoplasmic reticulum, several more steps of the export process follow. The proteins are transferred to the Golgi apparatus where further covalent modifications such as glycosylation can occur and then to condensing vacuoles where they are concentrated and stored to await export. Export is achieved by exocytosis (Palade, 1975).

Intracellular Localization of Protein to the Plasma Membrane

The description of plasma membrane assembly provided the first example of how some intracellular proteins may be localized. Research in this area was complementary to and a natural extension to the work on secretory proteins as both types of protein reach their final destination via the endoplasmic reticulum (Blobel and Dobberstein, 1975a,b; Blobel et al., 1979). It was found that the initial events in the synthesis of certain plasma membrane proteins and secretory proteins were probably identical. Much of the evidence has been obtained using assembly of proteins into the viral membrane in virus-infected animal cells as a model. Studies by Ghosh, Lodish and others (Rothman and Lodish, 1977; Toneguzzo and Ghosh, 1978; ref. add.) showed that the glycoproteins of

vesicular stomatitis virus was synthesized with an amino-terminal extension on ribosomes bound to the endoplasmic reticulum. Instead of vectorial transfer completely into the lumen of the endoplasmic reticulum, the protein remains embedded in the membrane near the carboxy-terminus. The protein is partially glycosylated in the endoplasmic reticulum, then transferred to the Golgi apparatus where the remaining sugars are added. Fusion of granules containing glycoprotein with the plasma membrane allows the asymmetry achieved during synthesis to be maintained.

Sequencing of the signal peptides from plasma membrane proteins has revealed the same pattern of amino acid residues that is found in pre-secretory proteins (Blobel et al., 1979; von Heijne, 1981; Kreil, 1981). This similarity most likely reflect the similar (possibly identical) roles of recognizing the endoplasmic reticulum and initiation of translocation.

It is thus clear that the localization of certain plasma membrane proteins closely follows the route established for secretory proteins, however it has become apparent that different mechanisms of insertion must exist for other proteins. Several membrane proteins such as cytochrome P-450 and epoxide hydratase, which have been shown to be synthesized on membrane-bound ribosomes, have their amino-terminal embedded in the membrane. Other proteins, such as erythrocyte band III may span the bilayer several times.

Furthermore, cytochrome b_5 and NADH cytochrome b_5 reductase are synthesized on free polysomes (reviewed by Wickner, 1980; Kreil, 1981). The inability to reconcile these variations with the "signal hypothesis" has led to the proposal of alternative models of assembly (Wickner, 1979; 1980; Blobel, 1980).

Localization of Protein Into Bacterial Membranes

The study of bacteria, particularly Esherichia coli, has provided insight into mechanisms of specific protein translocation and possible roles of the signal sequence. This bacterium is surrounded by an envelope consisting of an inner and outer membrane. Between these lie the periplasmic space containing the peptidoglycan cell wall (Osborne et al., 1972). Proteins destined for the periplasmic space or outer membrane must first traverse the inner membrane. The translocation of several major envelope proteins across the inner membrane has been shown to occur by a mechanism similar to secretion (Inouye and Halegoua, 1980; Kreil, 1981). These proteins are synthesized as higher molecular weight precursors on ribosomes bound to the inner membrane and vectorially transferred across the membrane. The composition of the amino terminal extensions, regardless of the final location (periplasmic space, inner or outer membrane) are remarkably similar and resemble the signal peptides of eukaryotic secretory and plasma membrane proteins (Inouye and Halegoua, 1980; von Heijne,

1981). It has been suggested that the similarities among envelope proteins reflect the common function of specific recognition and initiation of transfer across the inner membrane (Inouye and Halegoua, 1980). This is supported by evidence of Halegoua and Inouye (1979) that the E. coli outer membrane protein tol G has its signal sequence cleaved before insertion into the outer membrane.

The use of bacterial mutants containing hybrid proteins has yielded interesting information regarding the role of the signal sequence. When part of the gene coding for the amino-terminus of the E. coli outer membrane protein lamB was fused to the amino-terminus of the gene for the cytoplasmic enzyme β-galactosidase, the enzyme was relocated to the outer membrane. However, the presence of the signal sequence alone fused to β-galactosidase was not sufficient to cause relocation (Emr et al., 1980).

It is clear that all bacterial envelope proteins are not assembled in a manner similar to eukaryotic protein secretion. Deletion mutants of the periplasmic protein β-lactamse in Salmonella have been made. These proteins lack varying amounts of the carboxy-terminus of the protein and cannot be exported to the periplasm (Koshland and Botstein, 1980). There is evidence for post-translational transport for several bacterial envelope proteins (Kreil, 1981).

Localization of Protein Into Mitochondria

Mitochondria are semi-autonomous organelles, the biogenesis of which requires co-operation between the nucleo-cytoplasmic genetic system and that of the mitochondria. Mitochondria actually code for less than 10% of their own protein mass, the rest being coded by the nucleus, synthesized in the cytoplasm and somehow imported into various locations in the organelle (Mason and Schatz, 1974; Milner, 1976; Tzagoloff and Macino, 1979). Proteins destined for mitochondria will reside in one of four sites: outer membrane (e.g. NADH-cytochrome c reductase), intermembrane space (e.g. cytochrome c peroxidase), inner membrane (e.g. ADP/ATP translocator) or matrix (e.g. malate dehydrogenase).

Specificity of proteins for excretion or localization into the plasma membrane depends in large part on the information in their amino-terminal extension and its interaction with the endoplasmic reticulum. It is not surprising that mitochondrial proteins have been examined for similar extensions in their newly synthesized form. The latter are isolated by immunological techniques from pulse-labelled cells or from cell-free protein synthesizing systems. Most work has been done with Neurospora crassa and the yeast Saccharomyces cerevisiae, but recently attention has been turned to mammalian cells.

All mitochondrial proteins examined so far except

for cytochrome c from yeast, Neurospora and rat liver and the ADP/ATP translocator of yeast and Neurospora have been shown to be synthesized as precursors 2,000 to 6,000 daltons larger than the mature protein (Table 1). This is a larger variation in size than is seen in the precursors of secretory or plasma membrane proteins. Little is known about the nature of the amino-terminal extensions of mitochondrial proteins. In cells, three unidentified mitochondrial precursor proteins have higher pI values than the mature products indicating that the extra peptides are highly basic (Anderson, 1981). This is a significantly different composition from the signal peptides discussed earlier (Blobel et. al., 1979; Anderson, 1981). It is interesting to note that the precursor to the pea chloroplast protein ribulose-1,5-biphosphate carboxylase is synthesized with an amino-terminal extension which is acidic in nature (Highfield and Ellis, 1978). These differences might suggest the role of organelle recognition for the extra peptides.

Following from the "signal hypothesis" it would be expected that the extension would interact with the mitochondrial membrane during protein synthesis. That is, transfer to mitochondria would be a co-translational event. However, most evidence indicates that import is a post-translational event in Neurospora and mammalian cells. In yeast the situation might be more complex. If transport was co-

TABLE 1: Mitochondrial proteins and their precursors. Abbreviations: aspartate aminotransferase (AAT), carbamyl phosphate synthetase (CPS), δ-aminolevulinate synthetase (δ-ALAS), ornithine transcarbamylase (OTCase).

Location	Protein	Source	Apparent Molecular Weight		Reference
			Mature	Precursor	
matrix	AAT	chicken liver	45,000	+3,000	(93)
	CPS	rat liver	160,000	+5,000 -5,500	(84,70)
	δ-ALAS	rat liver	45,000	+6,000	(108)
	OCTase	rat liver	39,000	+4,000	(71,16)
	citrate synthetase	Neurospora	45,000	+2,000	(33)
	ATPase	yeast			(56)
	α-subunit		58,000	+6,000	
	β-subunit		54,000	+2,000	
	γ-subunit		34,000	+6,000	
inner membrane	ADP/ATP translocator	yeast Neurospora	32,000	-	(73) (109)
	cytochrome c	yeast Neurospora	13,000	-	(112) (32)
	ATPase proteolipid	Neurospora	8,000	+4,000	(67)
	bc_1 complex subunit V	yeast	25,000	+2,000	(18)
intermembrane space	cytochrome c peroxidase	yeast	33,500	+6,000	(57)

translational, it should be dependent on concurrent protein synthesis and the cytosol should not contain any pools of mitochondrial proteins or their precursors. However, this is not the case. Hallermeyer et al., (1977) and Harmey et al., (1977) found that in pulse-labelled Neurospora there was a lag in the labelling of mitochondrial protein compared to other subcellular fractions, and protein continued to accumulate in the mitochondria even after inhibition of cytosolic protein synthesis with cycloheximide.

Further evidence that import was post-translational followed the identification of high molecular weight precursors to a number of mitochondrial proteins. Using pulse-labelled rat liver cells it has been possible to demonstrate cytoplasmic pools of the precursor to carbamyl phosphate synthetase (Raymond and Shore, 1979), pre-δ-aminolevulinate synthetase (Yamauchi et al., 1980) and pre-ornithine transcarbamylase (Morita et al., 1981). By pulse-labelling Neurospora Harmey et al., (1980) were able to immunoprecipitate cytochrome c, the ADP/ATP translocator and citrate sythetase from a post-ribosomal supernatant. Citrate synthetase is the only protein of these three that is synthesized as a higher molecular weight precursor.

In contrast, no higher molecular weight precursors to mitochondrial proteins have been identified in the cytoplasm of yeast. Schatz and co-workers (Schatz, 1979) could

immunoprecipitate the three large subunits of the F_1-ATPase in their mature form from a post mitochondrial fraction of pulse-labelled yeast. The subunits from this fraction were much more heavily labelled than the corresponding subunits from the mitochondria at the early time points. The high molecular weight precursors to these subunits could be detected *in vivo* only by mixing the pulse-labelled spheroplasts with several protease inhibitors and dropping the suspension directly into a 20% SDS solution at 100°C (Schatz, 1979). Its subcellular location is not known.

If synthesis and uptake of mitochondrial proteins are separate events in cells, it follows that one should be able to show the uptake of the precursor form by isolated mitochondria. Using heterologous protein synthesizing systems and isolated mitochondria the uptake and processing of precursors to their mature form has been demonstrated, after protein synthesis was inhibited by cycloheximide. This has been shown for the three large subunits of yeast F_1-ATPase (Maccheccini *et al.*, 1979a), yeast cytochrome *c* peroxidase (Maccheccini *et al.*, 1979b), rat liver carbamyl phosphate synthetase (Morita *et al.*, 1979) and rat liver ornithine transcarbamylase (Conboy and Rosenberg, 1981). The mature form is protease resistant indicating its location within a membrane. However, this uptake process *in vitro* is inefficient compared to that in whole cells.

A protease involved in the processing of pre-ornithine transcarbamylase has been identified in the matrix of rat liver mitochondria (Morita et al., 1980) and a protease from yeast and rat liver mitochondrial matrix has been shown to process several matrix proteins to their mature form (Neupert and Schatz, 1981a). These proteases are insensitive to most serine protease inhibitors, but are strongly inhibited by EDTA. Energy is required for the transport of several mitochondrial proteins into yeast and Neurospora (Nelson and Schatz, 1979; Neupert and Schatz, 1981a).

An interesting correlate of post translational uptake is the demonstration that several mitochondrial matrix proteins (carbamyl phosphate synthetase (Shore et al., 1979); δ -aminolevulinate synthetase (Yamauchi et al., 1980) and ornithine transcarbamylase (Morita et al., 1981)) and cytochrome c (Cordova et al., 1980), all from rat liver, are synthesized on free ribosomes. This was shown by incubating free and bound polysomes in a reticulocyte cell-free protein synthesizing system. In contrast, glutamate dehydrogenase synthesis may deviate from this pattern. This enzyme could be immunoprecipitated from rat liver microsomes sedimenting at 105,000 g_{av} that were isolated from pulse labelled rat liver cells (Godinot and Lardy, 1973). Kawajiri et al., (1977) showed that nascent polypeptide chains released from

rough microsomes by EDTA and non-ionic detergents could be immunoprecipitated by antibodies against glutamate dehydrogenase. Free polysomes on the other hand, contained no glutamate dehydrogenase antigenic determinants. However, this protein by virtue of its accessibility to antibody, susceptibility to proteases and release from the microsomes by high ionic strength buffers (Kawajiri et al., 1977) is not segregated into the lumen of the endoplasmic reticulum. The nature of its association is not known.

In the Fungi, the situation is different. Neurospora has little endoplasmic reticulum so it is likely that mitochondrial proteins are synthesized on free polysomes. This has been demonstrated for the ADP/ATP translocator (Zimmerman et al., 1979a) and cytochrome c (Zimmerman et al., 1979b).

There is evidence that synthesis of yeast mitochondrial proteins occurs on ribosomes bound to the mitochondrial membrane. Cytoplasmic-type 80 S ribosomes have been shown to be associated with yeast mitochondria (Kellems and Butow, 1972; Kellems et al., 1974; Kellems and Butow, 1974; Kellems et al., 1975). These mitochondria-associated polysomes are enriched in mRNA which codes for mitochondrial proteins (Ades and Butow, 1980a; Neupert and Schatz, 1981). In studies by Ades and Butow (1980b) using pulse and pulse-chase experiments in the presence of cycloheximide and aurintricarboxylic acid, the labelling kinetics of mitochondrial protein

indicated that both co- and post-translational transport may be present. It has yet to be demonstrated if this co-translational mechanism is analagous to that involved in secretion. Evidence for post-translational transport comes from the work of Maccheccini et al., (1979a) who showed that the precursors of yeast F_1-ATPase subunits could be taken up into isolated mitochondria and processed in the absence of protein synthesis.

It is clear that the mechanism of specific import of mitochondrial proteins will be different from that of the localization of plasma membrane proteins. For example, the amino-terminal extension need not fulfill an identical role. Moreover, there is evidence that the mature enzymes (or their subunits) aspartate aminotransferase (Marra et al., 1977, 1978, 1979, 1980), δ-aminolevulinate synthetase (Ohashi and Sinohara, 1978) and malate dehydrogenase (Passerella et al., 1981, ref. add) from rat liver can be taken up into isolated mitochondria. However, the relevance of these results to the in vivo process has been questioned due to the high concentrations of mature enzymes required to observe uptake (Neupert and Schatz, 1981b). Furthermore, chicken liver aspartate aminotransferase and rat liver δ-aminolevulinate synthetase are synthesized as higher molecular weight precursors (Table 1).

Cleavage of the signal sequence may contribute to the

irreversibility of transport into mitochondria, but so might several other processes.

The interaction of the imported protein with mitochondrially synthesized subunits of the same enzyme complex, such as occurs with cytochrome oxidase is a situation unique to semi-autonomous organelles (Thalenfeld and Tzagoloff, 1980) and may play a role. Many mitochondrial proteins are oligomers so that polymerization may be required for irreversibility. This could be important for proteins such as the ADP/ATP translocator which is not synthesized as a higher molecular weight precursor in Neurospora and yeast (Harmey et al., 1980; Neupert and Schatz, 1981a). There is evidence that the covalent attachment of heme to apo-cytochrome c in Neurospora and yeast plays a role in the sequestration (Korb and Neupert, 1978; Zimmerman et al., 1979b).

Post-translational transport present special thermodynamic problems. Unlike co-translational transport, entire integral membrane proteins must first be transported through the aquaeous compartments. Neupert et al., (1980) have presented evidence that in Neurospora the ADP/ATP translocator of the mitochondrial inner membrane occurs in a large aggregate in the cytosol. The nature of this aggregate is not known. On the other hand completed hydrophilic proteins must somehow pass through the hydrophobic membrane barrier.

In light of the several differences between aspects

of specific localization of protein in mitochondria and the selection of protein for plasma membrane assembly or secretion, several alternative models to the "signal hypothesis" have been proposed for the import of mitochondrial proteins. These models emphasize the importance of conformational change in the migrating protein (Wickner, 1979, 1980; Webster et al., 1979; Waksman et al., 1980; Blobel, 1980). There is no comprehensive evidence to support any one of these models. Thus, the question of how proteins are localized in the mitochondria remains unanswered though some steps of the import process have been determined.

Rationale

As previously discussed, the role of the amino-terminal extension of mitochondrial precursor proteins in the import process is unknown. It has yet to be established whether many mitochondrial proteins are synthesized as high molecular weight precursors and whether the large variation in the size of the extra peptides is important to the specific localization. Mitochondrial matrix enzymes might be a particular enigma as the mature rat liver enzymes (or their subunits) aspartate aminotransferase, δ-aminolevulinate synthetase and malate dehydrogenase can be taken up into isolated mitochondria. Furthermore, rat liver mitochondrial aspartate aminotransferase (reviewed by Braunstein, 1973) and mitochondrial malate dehydrogenase (reviewed by Banaszak and Bradshaw,

1975) have cytosolic counterparts, and these functionally equivalent, but structurally different isozymes are excluded from the mitochondria under the same conditions (Marra et al., 1977; Passeralla et al., 1980). It has been suggested by Waksman and his colleagues (Waksman and Rendon, 1974; Waksman et al., 1977; Hubert et al., 1979) that these two mitochondrial enzymes can shift their intramitochondrial location in response to changing levels of substrates. These characteristics of the mature enzymes may provide insight into the possible role of an amino-terminal extension. Aspartate aminotransferase, as well as other matrix enzymes (see Table 1) have been shown to be synthesized as higher molecular weight precursors. It was decided to establish if rat liver mitochondrial malate dehydrogenase is also synthesized in a higher molecular weight form.

The detection of precursors has been greatly facilitated by the use of heterologous systems for cell-free protein synthesis. The unfractionated reticulocyte lysate has been particularly helpful. The method of preparation (Villa-Komaroff et al., 1974) allows rapid removal of mitochondria, minimizing the risk of contamination of the lysate with mitochondrial "signal" peptidase. The lysate, treated with micrococcal nuclease by the method of Pelham and Jackson (1976) efficiently translates exogenous mRNA. Precursors can be immunoprecipitated from the translation products using

antibody raised against highly purified, mature enzyme.

Mitochondrial malate dehydrogenase is a dimer (subunit molecular weight 33,000 to 35,000 in mammals) and accounts for less than 0.3% of mitochondrial protein mass (Strasberg et al., 1979). This has made the isolation of large quantities of high purity, active enzyme difficult. The use of affinity chromatography on 5'-AMP Sepharose has greatly facilitated the isolation of other dehydrogenase enzymes (Kaplan et al., 1974; Walk and Hock, 1976) and might be applied to the isolation of mitochondrial malate dehydrogenase.

II. MATERIALS

Protein A Sepharose and 5'AMP Sepharose were purchased from Pharmacia Fine Chemicals. Ultra pure sucrose was from Schwartz Mann. [^{14}C]formaldehyde and [^{35}S]methionine, each at the highest specific activity available were purchased from New England Nuclear, Canada. Bovine heart mitochondrial malate dehydrogenase was from Sigma Chemical Co. and porcine heart soluble malate dehydrogenase was from Research Plus Laboratories. Micrococcal nuclease and creatine kinase and creatine phosphate were from Boehringer Mannheim. Amino acids were all of the L-configuration and were purchased from Sigma Chemical Co. ATP and GTP were from Sigma Chemical Co. Most other chemicals were of analytical grade and were purchased from either Sigma Chemical Co. or Fisher Scientific.

III. METHODS

1. Isolation of Enzymes for Antibody Production and Cross-Reactivity Studies.

A. Rat Liver Mitochondrial Malate Dehydrogenase (mMDH)

A procedure was developed for the rapid isolation of rat liver mMDH using affinity chromatography on N^6-amino-hexyl 5'-AMP sepharose CL 4B. The procedure involves an initial ammonium sulphate fractionation step followed by affinity chromatography and Sephadex G-100 chromatography. All steps were carried out at 0-4°C.

Ammonium Sulphate Fractionation of Rat Liver Mitochondrial Protein

Livers from 150-250 g male Sprague-Dawley rats were homogenized using a loose fitting Potter-Elvehjem homogenizer in 0.3 M sucrose, 2.0 mM Tris-HCl, pH 7.4, 2 mM EDTA, (medium B) with 3 vol/g rat liver. The homogenate was centrifuged at 500 g_{av} for 5 min, the pellet discarded and the supernatant centrifuged at 12,000 g_{av} for 10 min. After decanting the supernatant, the surface of the mitochondrial pellet was washed 3 times with a small volume of medium B. The mitochondria were not further washed in order to keep the loss of mMDH due to leakage from the mitochondria to a minimum. The pellet was resuspended in hypotonic medium

containing 20 mM sodium phosphate, 2 mM EDTA, 4 mM 2-mercaptoethanol (MSH), pH 7.0 (PEM 7.0) at 25 ml per 30-40 g of rat liver. The mitochondria were sonicated using either a bath or probe sonicator for three 2 min intervals taking care that the temperature did not rise more than 4 to 5°C. Sonication in the bath sonicator was done using 3 ml volumes, whereas the entire sample (approx. 25 ml) could be done at once using a 2 cm probe sonicator. Sonicated mitochondria were centrifuged at 12,000 g_{av} for 30 min and the supernatant saved. The pellets were re-extracted and the supernatants pooled. The protein concentration was then adjusted to 10±2 mg/ml with PEM 7.0. Solid $(NH_4)_2SO_4$ was added slowly with stirring over a period of 1 hour to 30% saturation.

After stirring for a further hour, the precipitate was recovered by centrifugation at 12,000 g_{av} for 30 min. The same procedure was followed to collect the 31-50%, 51-70%, and 71-95% fractions. The pH of the ammonium sulphate suspensions was monitored periodically and adjusted to pH 7.0 when necessary using 1 N NaOH. The precipitated protein from each fraction was resuspended in PEM 7.0 and dialysed against this buffer. Dialysis was considered complete when the pH and conductivity of the dialysis buffer did not change.

Chromatography on 5'-AMP Sepharose

The $(NH_4)_2SO_4$ fraction which contained the most

mMDH activity and which also always had the highest specific activity was applied to a 0.9 x 30 cm column of 5'-AMP Sepharose equilibrated with PEM 7.0. (The capacity of the column to bind mMDH from the $(NH_4)_2SO_4$ fraction was determined in separate experiments by applying sample until MDH activity could be detected in the eluent. These indicated that it was necessary to use a high protein concentration for maximal binding.) The column was rinsed with 3 to 4 column volumes of PEM 7.0 and then bound mMDH was eluted using a 500 ml linear gradient of 0 to 100 μM NADH in PEM 7.0. After each experiment the column was stripped of any remaining protein with a solution of 0.5 M NaCl and 300 μM NADH in PEM 7.0 and equilibrated in PEM 7.0.

Sephadex G-100 Chromatography

Fractions containing high specific activity mMDH from the affinity chromatography step were pooled and dialysed against 100% $(NH_4)_2SO_4$ in 100 mM sodium phosphate, 1 mM EDTA, 2 mM MSH, pH 7.5 (PEM 7.5). The precipitated protein was centrifuged at 12,000 g_{av} for 45 min and the pellet was re-suspended in PEM 7.5. The sample was applied to a 1.5 x 90 cm column Sephadex G-100 equilibrated in PEM 7.5 containing 100 μM NADH. NADH is known to stabilize dilute solutions of the mMDH dimer of certain species (Glatthaar et al., 1974). Fractions containing mMDH activity were pooled and concentrated by dialysis against 100% $(NH_4)_2SO_4$ in PEM 7.5, and

stored in this form at 4°C. The purity of the protein was determined by SDS PAGE and starch gel electrophoresis.

B. Beef Heart mMDH

Beef heart mMDH from Sigma Chemical Co. was freed of contaminating protein by chromatography on 5'-AMP Sepharose. Up to 10 mg of protein was applied at a time to the column. The purity of the protein was determined by SDS-PAGE.

C. Cytoplasmic (Soluble) Malate Dehydrogenase (sMDH)

The enzyme was partially purified from rat liver by the procedure of Glatthaar et al., (1974) except that the last G-100 chromatography step was not done. Purity was determined by SDS-PAGE. The pig heart enzyme was purchased from Research Plus Laboratories.

2. Rabbit Anti-mMDH Antisera

A. Immunization

Antibodies were induced in New Zealand white rabbits against denatured beef heart mMDH and denatured rat liver mMDH. The enzymes were denatured essentially by the method of Goldman and Blobel (1978). Purified protein was dissolved to a final concentration of 0.5 to 1.0 mg per ml of denaturing solution which contained 0.5% SDS, 140 mM NaCl and 10 mM MSH and boiled for 2 min. After cooling to room temperature 50 µmol of iodoacetamide was added and the mixture was incubated at 37°C for 1 hour. For the first injection,

the antigen was mixed 1:1 (v/v) with Freund's complete adjuvant, and the subsequent injections were made at 2 week to 1 month intervals with antigen alone. Injections were made into the thigh muscles and subcutaneously at the nape of the neck using 200 to 300 μg of rat liver enzyme and 400 to 500 μg of beef heart enzyme per rabbit. Blood samples were taken by bleeding from the ear vein 8 to 9 days after the third and subsequent injections. The blood was allowed to clot for 2 to 3 hours at room temperature and then it was centrifuged at 12,000 g_{av} for 20 min at 0°C to collect the serum. Serum from a non-immune rabbit was collected to use as a control for immunoprecipitation experiments. Sera was stored at -20°C or below.

B. Efficacy and Cross-Reactivity of Antisera

The anti-rat liver and anti-beef heart mMDH antisera were tested for their ability to immunoprecipitate, a) purified rat liver mMDH in the native and denatured forms, b) purified beef heart mMDH in the native and denatured forms, c) partially purified sMDH from porcine heart in the denatured form and d) partially purified sMDH from rat liver in the denatured form. The enzymes were labelled with [^{14}C]formaldehyde by the reductive methylation procedure of Rice and Means (1971), and denatured as described for immunization when necessary. Antigen-antibody complexes were recovered using protein A Sepharose (Schaffhausen et al., 1978) which was swollen in

140 mM NaCl, 50 mM Tris HCl, 5 mM EDTA, pH 7.6. Native enzymes were dissolved in 50 mM Tris-HCl, 140 mM NaCl, 1mM EDTA, 2 mM MSH, pH 7.6 for immunoprecipitation.

A number of preliminary experiments were necessary to find optimal conditions for immunoprecipitation of denatured enzymes. In particular it was necessary to denature the enzyme with SDS (having sufficient SDS to protein) and then remove SDS in triton micelles and exchange SDS bound to protein for triton (using an appropriate ratio of triton X-100 to SDS) (Tanford and Reynolds, 1976). Assuming approximately 1.4 g SDS can be bound to 1.0 g of protein, (Tanford and Reynolds. 1976) the amount of SDS present in these experiments was always in excess of the amount required to effect denaturation. The molar ratio of Triton X-100 to SDS was varied from 0 to 24.

Early experiments were attemped using high salt concentrations ($\geq$ 500 mM) and high pH ($\geq$ 8.5) to reduce non-specific association of protein (Schaffhausen et al., 1978) but the recovery of mMDH was greatly reduced. Subsequent experiments were performed using physiological pH and salt concentrations. The final immunoprecipitation curves were done using antigen denatured as described for immunization in the absence of iodoacetaminde, to which was added one half volume of a buffer containing 50 mM Tris-HCl, 5 mM EDTA, 140 mM NaCl, 10 mM MSH, 3% Triton X-100, pH 7.5

(3% TENT).

The efficacy and cross- reactivity of the antisera for various antigens was determined with 5.5 µg of native or denatured beef or rat mMDH with 25 to 175 µl of anti-beef mMDH antiserum or 3.5 µg of the antigens with 25 to 175 µl and the anti-rat mMDH antiserum. A lower quantity of antigen was used with the anti-rat antiserum as preliminary experiments suggested that the titre was lower. The antisera were also tested against denatured soluble (cytoplasmic) MDH from rat and pig using approximately 10 µg of enzyme and 50 µl of antiserum. All experiments were performed using non-immune serum as control. The mixtures were allowed to incubate for 1 hr at 37°C then for 12 to 16 hr at 4°C. A volume of settled Protein A Sepharose equal to the volume of antiserum present was then added and the mixture was rotated at room temperature for 3 to 4 hours. The bound immune complexes were washed 4 times with 500 µl of a buffer containing 125 mM NaCl, 50 mM Tris-HCl, 5 mM EDTA, 10 mM MSH, pH 7.6 (1X wash buffer). One further wash was done with this buffer at 250 mM NaCl (2X wash buffer). In preliminary experiments, changing the pH from 7.6 to either 6.0 or 9.0, or increasing the salt concentration to 500 mM NaCl in the wash buffer resulted in a decreased recovery of antigen. The washed immune complexes were then stripped from the Protein A Sepharose by heating to 95°C for 5 min in two consecutive washes with a volume

of stripping buffer equal to the volume of antiserum originally added. The stripping buffer contained 6% (w/v) SDS, 10% (w/v) MSH, 125 mM Tris-HCl, pH 8.8. The two washes were pooled and radioactivity determined directly in 10 ml of ACS scintillant and counted in a Beckman LS200 B scintillation counter.

C. Specificity of Antisera

The above experiments demonstrated that the antisera reacted with denatured mMDH (results section) but it was necessary to show that they were specific for the enzyme. This was demonstrated in two ways. 1) Mitochondrial MDH was immunoprecipitated from [^{35}S]methionine-labelled mitochondrial protein from bovine MDBK cells labelled in suspension and 2) [^{14}C]mMDH was added to and then immunoprecipitated from protein synthesized in vitro by a reticulocyte lysate in the presence of [^{35}S]methionine (to be discussed below).

Immunoprecipitation of mMDH from MDBK Mitochondrial Protein

MDBK cells were grown in monolayer culture to confluence in α-MEM supplemented with 10% new born calf serum. The medium was decanted and the surface of the cells was washed once with PBS (96 mM Na/K phosphate, 140 mM NaCl, pH 7.4). After decanting the PBS, 2 ml of 5 mg trypsin per ml PBS containing 3 mM EDTA was added and allowed to cover the surface of the cells for 5 to 10 min until they detached from the flask. Approximately 10^8 cells were diluted

into 200 ml of α-MEM then pelleted by centrifugation for 2 min at room temperature at 1000 rpm in the International centrifuge. The cells were resuspended in 50 ml of α-MEM minus methionine with 10% new born calf serum. After adding 1 mCi of ^{35}[S]methionine, the cells were incubated for 1 to 2 hours. They were washed and mitochondria isolated according to Freeman (1965). The mitochondrial pellet was dissolved in 2% SDS, 140 mM NaCl and 10 mM MSH and the acid precipitable radioactivity determined. The sample was stored frozen at -20°C for less than 1 week before use. For each immunoprecipitation $5X10^5$ cpm (approximately 1 mg) of mitochondrial protein was mixed with 4 vol of 3% TENT and 75 µl of antiserum. After incubation with Protein A, the complexes were washed 5 times with 500 µl of 1X wash buffer at 5 mM EDTA and 20 mM methionine, then 2 times with 500 µl of 2X wash buffer at 20 mM methionine and 5 mM EDTA. After stripping part of the sample was counted in ACS and the rest was saved for analysis by SDS PAGE and fluorography.

Immunoprecipitation of Added [^{14}C]mMDH from Protein Synthesized In Vitro in the Presence of [^{35}S]Methionine

For these experiments it was necessary to first determine the optimal conditions for immunoprecipitation using [^{14}C]mMDH and cold reticulocyte lysate. In a first group of experiments the effects of lysate and SDS concentration were examined by adding 10^4 cpm of previously

denatured [^{14}C]mMDH to 20 µl lysate which had been denatured at 0%, 2% and 6% SDS, and exchanged with 4 volumes of TENT at 0%, 3% and 9% Triton X-100 respectively to keep a constant Triton:SDS ratio. After the addition of 75 µl of antiserum, the samples were treated as described for the cross-reactivity studies. In a second group of experiments 10^4 cpm of the native enzyme was added to 20 µl of the native lysate, and they were denatured and exchanged together under the conditions described above. Denatured mMDH was immunoprecipitated in the absence of lysate as control. In a third experiment using the same protocol with lysate in 6% SDS the effect of Triton X-100 in the exchange buffer at 0%, 3%, 9% and 15% was determined. Finally, denaturation was done at room temperature because heating the lysate yielded a fine precipitate that was difficult to remove. As a result of these experiments, subsequent immunoprecipitations were performed with the lysate denatured in 6% SDS and exchanged with 4 vol of 9% TENT.

Preliminary experiments showed that [^{14}C]mMDH could be precipitated from the protein synthesizing system but the presence of highly [^{35}S]-labelled protein and free [^{35}S]methionine necessitated examining the procedure for washing the Protein A-bound immune complexes. About 10^4 cpm of [^{14}C]mMDH was added to 5×10^5 acid-precipitable counts of protein synthesized in vitro primed with rat liver

polysomes (to be described below). The mixture was denatured in the presence of 6% SDS, exchanged using 4 volumes of 9% TENT, and incubated with 75 µl of antiserum. The basic wash buffer (1X wash buffer) contained 125 mM NaCl, 50 mM Tris-HCl, 10 mM MSH, pH 7.6 to which was added a) nothing, b) 20 mM cold methionine, c) 5 mM EDTA, d) 20 mM cold methionine plus 5 mM EDTA and e) 0.1% SDS. The complexes were washed six times with 400 µl of the appropriate buffer, then each was washed four times with 400 µl of basic wash buffer before stripping the bound immune complexes and counting the radioactivity. From these experiments it was determined that the 1X wash buffer should include 20 mM methionine and 5 mM EDTA to help reduce background radioactivity.

With these two types of control experiments as background, it was possible to clearly demonstrate the precipitation of [^{14}C]mMDH from [^{35}S]methionine-labelled reticulocyte lysate primed with rat liver polysomes. The lysate was denatured with 6% SDS, 6 mM EDTA and 10 mM MSH, exchanged with 4 vol of 9% TENT and centrifuged for 5 min on an Eppendorf microfuge. For each immunoprecipitation, 10^6 acid precipitable cpm were mixed with approximately $5X10^5$ cpm (2 µg) of previously denatured [^{14}C]beef heart mMDH. In this experiment 150 µl of antiserum was used and the volume of wash buffer was increased to 750 µl. The immunoprecipitates were examined by SDS PAGE and fluorography.

3. Isolation and Characterization of Free Polysomes from Rat Liver

In all work involving polysomes and the reticulocyte lysate precautions were taken against contamination by RNase. Glassware was baked for several hours at 175°C and acid washed prior to baking where feasible. Apparati that could not be baked were either autoclaved or soaked in 1% SDS at 37°C then rinsed in sterile water. Sucrose solutions were prepared by adding RNase-free sucrose to sterile 2X concentrated stock solutions. After adjusting the volume and pH, the solutions were filter sterilized using 0.22 μm pore size filters. Similar precautions were taken with other solutions which could not be autoclaved. Unless otherwise indicated, work was done at 0 to 4°C.

A. Isolation of Polysomes from Rat Liver

The free polysome fraction of rat liver was isolated by the procedure of Shore *et al.* (1979). Livers (10 to 15 g) from 150 to 200 g male Sprague-Dawley rats which had been starved for 16 hours were homogenized in 8 vol of 0.35 M sucrose, 200 mM Tris-HCl, 50 mM KCl, 10 mM $Mg(CH_3COO)_2$, pH 8.5 (0.35S, $T_{200}K_{50}M_{10}$, 8.5). The homogenization was done using a Potter-Elvehjem type homogenizer with 5 strokes of a medium tightness pestle. The homogenate was centrifuged at 520 g_{av} for 2 min in the SW27 rotor of the Beckman ultracentrifuge, after which the force was increased to 52,200

g_{av} for 16 minutes. The top three-quarters of the supernatant was aspirated using a 50 ml glass syringe with a large bore needle, avoiding the layer of lipid on top. After making the supernatant 1% in Triton X-100, 9 ml was layered over 3 ml of 1.5 M sucrose in $T_{200}K_{50}M_{10}$,8.5 and the samples were centrifuged in the SW41 rotor (Beckman) at 110,000 g_{av} for 105 minutes. The solutions above the pellet were aspirated and the tubes were immediately inverted. The top three-quarters of the centrifuge tube was cut away and the surface of the polysome pellet was washed three times with resuspension buffer containing 10 mM Tris-HCl, 20 mM KCl, 5 mM Mg-$(CH_3COO)_2$, pH 7.6. The pellets were transferred to a Dounce homogenizer using a sterile spatula and resuspended in a minimum volume of the resuspension buffer using 3 strokes of the homogenizer at 3 one-half hour intervals. Polysomes were either used fresh or stored at -70°C until use.

B. Sucrose Gradient Analysis of Polysomes

Polysomes (0.5 to 1.0 OD_{260} units) were layered over 12 ml of a 15 to 30% (w/v) sucrose gradient in $T_{20}K_{75}M_5$,pH 7.6, prepared by using a Pharmacia gradient mixer (single chamber capacity 40 ml). The polysomes were centrifuged through the gradients at 110,000 g_{av} for 90 min in the SW41 rotor. Gradients were fractionated by pumping 45% sucrose through the bottom of the tube, and the absorption was monitored continuously at 254 nm using an ISCO UV analysing

system.

C. Preparation of 80 S Ribosomal Marker for Sucrose Gradients

Free polysomes were used to prepare mRNA-fragment ribosomes. A polysome suspension was made 1 mM in $CaCl_2$ and 10 μg/ml in micrococcal nuclease. After incubation at room temperature for 20 min, the sample was made 2 mM in EGTA, pH 7.0 and layered over 5 ml of 25% sucrose in $T_{20}K_{75}M_5$, pH 7.6. Centrifugation was for 2 h. at 160,000 g_{av} in the Beckman type 65 rotor. The ribosomes in the pellet were characterized by sucrose gradient analysis as described in the previous section, except that the centrifugation time was increased to 3 hours. The sedimentation co-efficient corresponding to the major peak was calculated according to McEwan (1967). The optical density of polysomes was determined using a Zeiss spectrophotometer.

4. Preparation of Total RNA from Rat Liver

Total RNA from rat liver was prepared by S.M. Chien using the method of Haffner et al. (1978).

5. Protein Synthesis In Vitro Using Reticulocyte Lysates and Immunoprecipitation of Translation Products

A. Induction of Reticulocytes and Preparation of Lysate

The procedure of Villa-Komaroff et al. (1974) was followed exactly. Four to six lb. New Zealand white rabbits

were injected with 1.2% acetylphenylhydrazine (buffered to pH 7.0 using 1.0 M HEPES, pH 7.5) on 5 consecutive days. Injection was subcutaneous using 2.0, 1.6, 1.2, 1.6 and 2.0 ml on successive days. If the hematocrit was found to be less than 25 on day 6, the rabbit was cannulated on day 7. The blood (approximately 100 ml) was collected into an equal volume of chilled saline containing 140 mM NaCl, 5 mM KCl, 1.5 mM $Mg(CH_3COO)_2$, and 0.001% heparin, then filtered through cheesecloth to remove any clots. The filtrate was centrifuged at 500 g_{av} for 5 min and the supernatant discarded. The pellets were washed 3 times with the saline solution with the final centrifugation at 600 g_{av}. The cells in the final pellet were lysed by resuspension in an equal volume of sterile distilled water, and mixing for 1 minute. Cell debris was removed by centrifugation at 12,000 g_{av} for 20 min and the supernatant (lysate) was stored in liquid nitrogen.

B. Cell-Free Protein Synthesis

The components for cell-free protein synthesis were those described by Villa-Komaroff et al. (1974) using both endogenous and exogenous polysomes. However, the lysate was optimized for hemin, Mg^{2+}, and K^+ concentrations for synthesis with rat liver polysomes. The lysate was treated with micrococcal nuclease by the method of Pelham and Jackson (1976) when exogenous polysomes were used.

For ease of optimization of the reticulocyte lysate

programmed with rat liver polysomes, the protocol was set up in the following manner. The thawed lysate was immediately suplemented with hemin and creatine kinase at the final concentrations in Table 2. The lysate was adjusted to 1 mM $CaCl_2$ and 10 μg micrococcal nuclease per ml and incubated at room temperature for 15 minutes. Nuclease activity was stopped by adding 100 mM EGTA, pH 7.0 to 4 mM. In a 100 μl final assay system the proportion of the components was: 75 μl of untreated or nuclease treated lysate, 5 μl of master mix (components listed in Table 2), 5 μl of KCl solution; 5 μl of [^{35}S]methionine solution; 10 μl of water or polysomes. The assay was started by the addition of [^{35}S]methionine. If exogenous polysomes were used, they were added just prior to this.

ATP, GTP, creatine kinase, hemin, creatine phosphate, amino acids and tRNA were from stock solutions stored frozen at -20°C. HEPES, KCl, $Mg(CH_3COO)_2$, $CaCl_2$, and EGTA were from stock solutions stored at 4°C. MSH was freshly diluted for addition to the master mix. The master mix was stored at -20°C for less than one month before use and once thawed was never reused.

Optimization of Hemin, Mg^{2+} and K^+ Concentrations

Using the assay system described above, the final concentration of hemin was varied from 0 to 180 μM in the presence of 1.0 mM Mg^{2+}, 50 mM KCl, 0.02 OD_{260} units polysomes

TABLE 2: Final concentration of components for cell-free protein synthesis. Concentrations do not take endogenous levels into consideration.

Component	Final Concentration
creatine kinase	150 μg/ml
hemin	150 μM or varied 0 to 180 μM
KCl	50 mM or varied 0 to 130 mM
[^{35}S]methionine	0.15 to 1.0 uCi/μl
master mix:	
ATP (pH 7.0)	900 μM
GTP (pH 7.0)	175 μM
creatine phosphate	11 mM
MSH	5 mM
HEPES (pH 7.6)	12 mM
21 amino acids-methionine (includes homoserine)	120 μM
calf liver tRNA	50 μg/ml
$Mg(CH_3COO)_2$	1 mM or varied 0 to 40 mM

per µl and 0.2 µ Ci [^{35}S]methionine per µl. The mixture was incubated and 2 µl samples were taken at 0, 10, 20, 30, 60 and 90 min and spotted on filter paper discs for the determination of radioactive amino acid incorporation into protein. In a similar manner, the KCl concentration was varied from 0 to 130 mM in the presence of 1 mM Mg^{2+} and 150 µM hemin. Mg^{2+} was varied from 0 to 4.0 mM in the presence of 150 µM hemin and 60 mM KCl. According to Palmiter (1973), 50 mM KCl and 1.0 mM Mg^{2+} represents the endogenous concentration of these components in the lysate.

Saturation of Lysate with Polysomes

Polysomes prepared by the method of Blobel and Potter (1967) by S.M. Chien in our laboratory were used in these experiments as these polysomes were highly concentrated. The volume of added polysomes was 10% of the total incubation volume. Polysomes from a 400 OD_{260} units/ml stock solution were diluted in $T_{10}K_{20}M_5$, pH 7.6 and added to the assay system to give final concentrations from 13 to 34 OD_{260} units/ml (0.97 to 2.54 mg/ml). In the final mixture the Mg^{2+}, K^+, hemin and ^{35}[S]methionine were 2 mM, 100 mM, 150 µM and 0.15 to 1.0 µCi respectively.

6. Immunoprecipitation of mMDH from Products of Cell-Free Protein Synthesis

A. Translation of Free Polysomes

In a final incubation volume of 500 µl, 10 to 12 OD_{260} units of rat liver free polysomes were incubated in the presence of 2.0 mM Mg^{2+}, 100 mM KCl, 150 µM hemin, and 1.0 µCi [^{35}S]methionine per µl. After 60 min the lysate was made 6% in SDS, 6mM in EDTA and 10 mM in MSH. After thorough mixing, the lysate was allowed to stand for 45 min at room temperature during which time the amount of radioactivity incorporated into protein was determined. Then 4 vol of TENT at 9% Triton X-100 was added and after thorough mixing 50 µl of antiserum was added to 10^7 cpm. The immunoprecipitation procedure has been described above. The bound immune complexes were examined by fluorography of SDS polyacrylamide gels.

B. Translation of Total RNA

Total RNA was translated as for free polysomes at a concentration of 40 OD_{260} units/ml. Fifty µl of antiserum was used to immunoprecipitate 6X10^6 cpm of protein.

C. Competition for Immunoprecipitation by Unlabelled mMDH

To demonstrate that the specific product of immunoprecipitation by anti-mMDH antiserum is a form of mMDH, excess unlabelled mMDH from beef heart (200 µg) was added to the <u>in vitro</u> translation products and the mixture denatured together. Immunoprecipitation was then carried out as described above using control serum and anti-beef mMDH antiserum. Disappearance of the band would indicate competition by cold enzyme for

specific antibody binding sites.

7. Miscellaneous Procedures

A. Assay of MDH Activity

The enzyme was assayed in 100 mM sodium phosphate, pH 7.5 containing 160 μM NADH and 120 μM oxaloacetate. Ten μl of appropriately diluted enzyme was added to 3 ml of assay mixture and the decrease in optical density at 340 nm was monitored using a Cary spectrophotometer. One unit of enzyme activity is defined as the amount of enzyme that will catalyse the oxidation of 1 μmol of NADH in 1 minute.

B. Assay of Protein

Protein was determined by a modification of the method of Lowry et al. (1951).

C. Starch Gel Electrophoresis

Starch gel electrophoresis was done according to Poulik (1957).

D. SDS-PAGE and Fluorography

SDS-PAGE was done essentially by the method of Laemmli (1970) and fluorography was done according to Bonner and Laskey (1974).

E. Labelling of Protein with [^{14}C]Formaldehyde for Immunoprecipitation Studies

The procedure of Rice and Means (1971) was followed

exactly. Protein was dissolved to a concentration of approximately 5 mg/ml in 0.2 M borate buffer, pH 8.0. Ten µl of 33 mM [^{14}C]formaldehyde (50 mCi/mmol) was added per 500 µg of protein. This was followed after 30 sec by four sequential additions of 2 µl $NaBH_4$ (5 mg/ml freshly made) and 10 µl 1 min later. The protein was then dialysed against the appropriate buffer to remove excess formaldehyde and to prepare the protein for the pertinent experiment.

F. Determination of Radioactivity Protein

The amount of [^{35}S]methionine incorporated into protein in vitro, and the degree of labelling by reductive methylation with [^{14}C]formaldehyde were determined by the procedure of Mans and Novelli (1961). Two µl samples of radioactive protein were spotted on 3 MM Whatman filter paper discs and dried. The discs were washed once in boiling 5% TCA (containing 1 mg cold methionine per ml for the protein synthesized in vitro) using at least 5 ml per disc. They were then washed once at 0°C with the same solution, once with ethanol:ethyl ether, 1:1 at 3 ml per disc and two times with ethyl ether at 3 ml per disc. The discs were then air dried, placed at the bottom of a vial and counted with toluene-omnifluor scintillant.

IV. RESULTS

1. Isolation of Rat Liver mMDH

In order to produce antibodies specifically against rat liver mMDH it was necessary to immunize the animals with highly purified enzyme. Mitochondrial MDH had been isolated from various sources in the past (Wolfe and Neilands 1953; Ochoa, 1955; Thorne, 1960; Gregory et al., 1971; Mathewson et al., 1973; Glatthaar et al., 1974), but these procedures are time consuming. Thus a rapid procedure was developed for the isolation of the rat liver enzyme.

From the experiments on $(NH_4)_2SO_4$ fractionation of the mitochondrial protein it was found that at a protein concentration of $10^{+}/-2$ mg/ml (approximately 60 enzyme units per ml), 75% of the enzyme activity recovered was found in the 51 to 95% $(NH_4)_2SO_4$ fraction. This was the fraction used in the final isolation procedure to be applied to the affinity column. Fig. 2 illustrates the elution profile from the affinity column.

For pig heart MDH, Walk and Hock (1976) found that the mitochondrial but not the cytoplasmic isozyme was bound to 5'-AMP Sepharose when the column was equilibrated at 20 mM sodium phosphate, pH 7.0. The enzyme could then be eluted with NADH at a concentration of 36 μM. The average concentra-

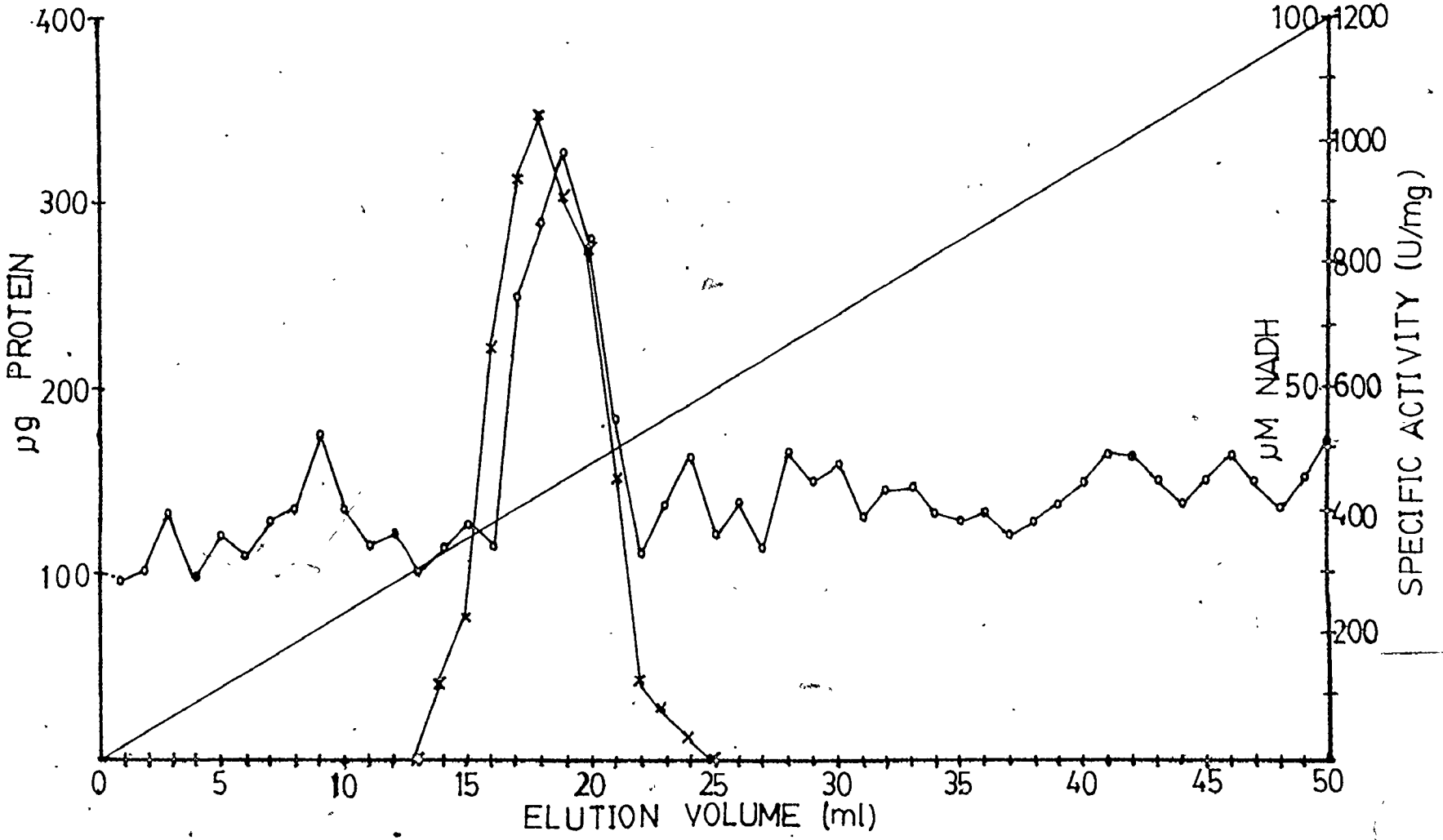


Fig. 2: Elution profile from affinity chromatography column. The ammonium sulphate fraction was applied to the column and unbound protein was removed with PEM 7.0 before applying a linear gradient of 0 to 100 µM NADH in PEM 7.0. Fractions were assayed for protein (o) and MDH activity (x).

tion at which the rat liver enzyme was eluted in these experiments was 32±5 μM under the same conditions. Raising the pH to 7.5 allowed the enzyme to be eluted at a much lower concentration of NADH (6 μM) confirming the observations of Kaplan et al. (1974) who showed that the beef heart mitochondrial enzyme was only weakly bound to the resin at this pH. Lowering the pH to 6.8 apeared to have no effect on the concentration of NADH at which the rat liver enzyme eluted. Thus the capacity of the affinity column for rat liver mMDH from the $(NH_4)_2SO_4$ step was determined using 20 mM sodium phosphate pH 7.0. If the protein concentration was approximately 6 mg/ml, 250 units of enzyme could be bound per ml of 5'-AMP Sepharose. Since the specific activity of the purified enzyme was 650 units/mg and the subunit molecular weight is 35,000 this represents 0.38 mg enzyme or $1.2X10^{-2}$ μmol bound per ml. As the concentration of ligand is 2 μmol /ml and there is only one coenzyme binding site per subunit, it can be seen that only a small fraction of ligand is actually occupied by enzyme.

The elution profile from the final Sephadex G-100 chromatography step is shown in Fig. 3. After this step, the enzyme was shown to be free of contaminating protein by starch gel electrophoresis (not shown) and by SDS PAGE (Fig. 15, lane 6). A flow chart of the final isolation procedure is illustrated on Fig. 4.

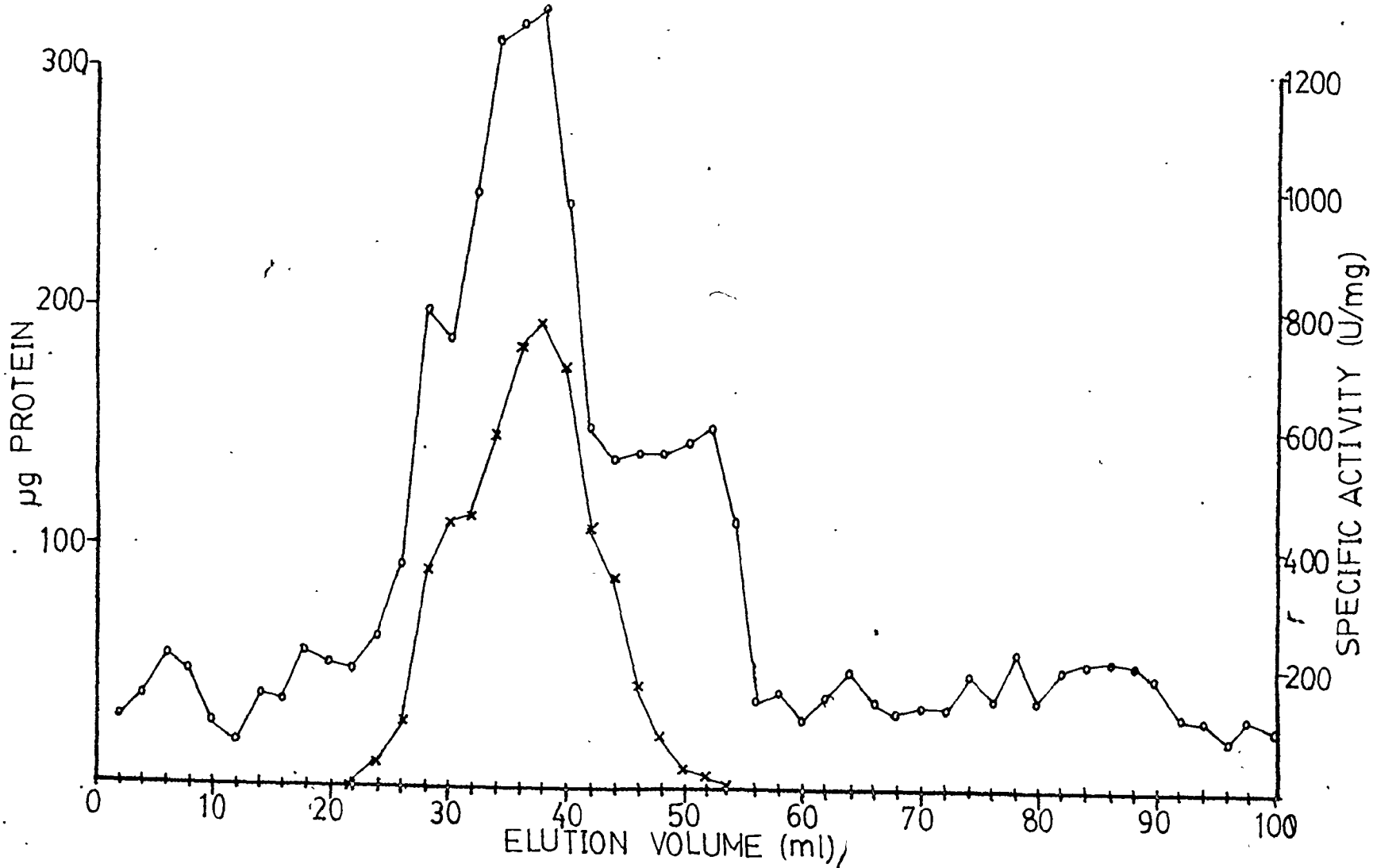
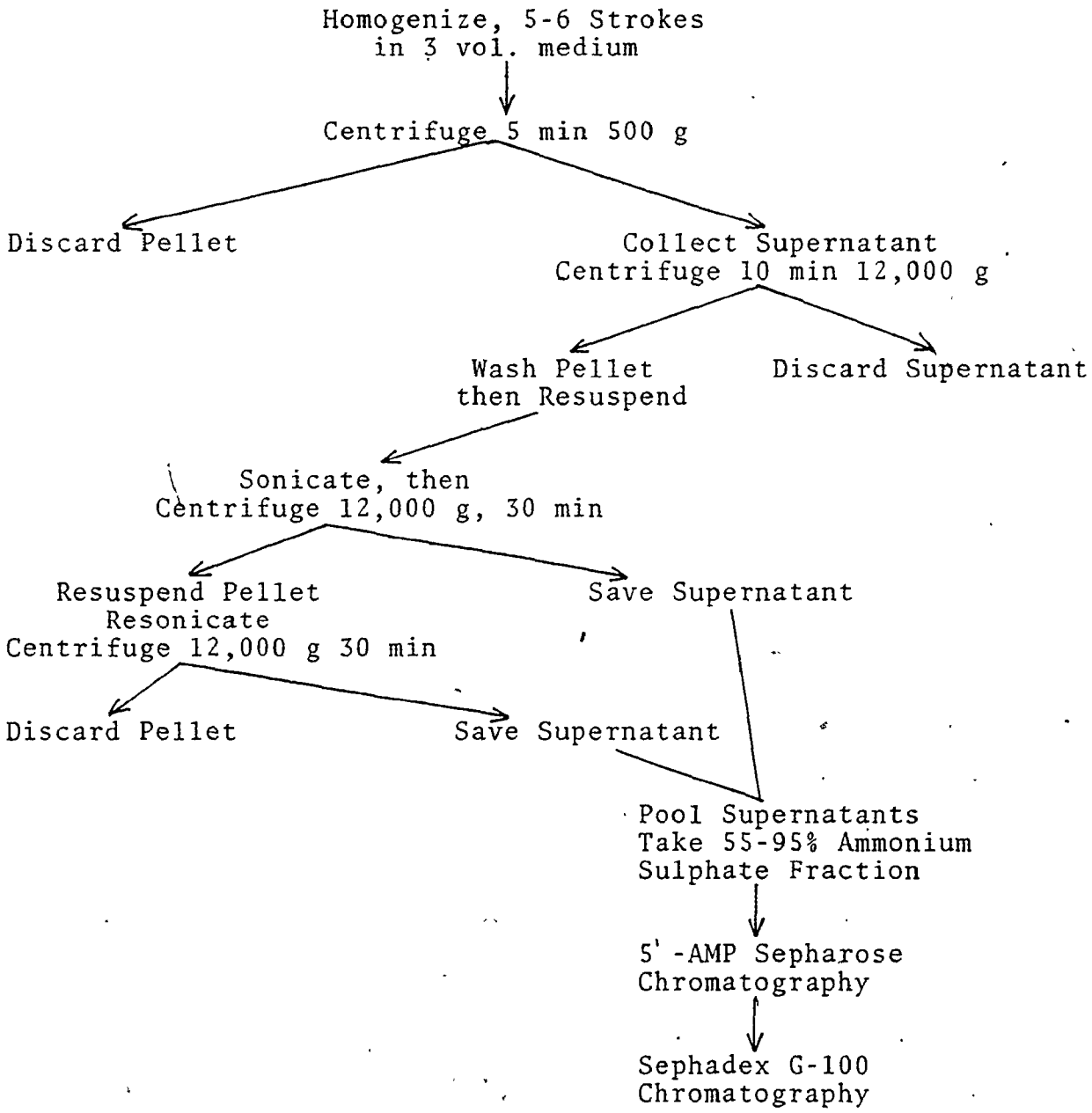


Fig. 3: Elution profile from Sephadex G-100 chromatography column. Fractions from the affinity column were concentrated and applied to a 1.5 x 90 cm column of Sephadex G-100 in PEM 7.5 containing 100 µM NADH. Fractions were assayed for protein (o) and MDH activity (x).

Fig. 4: Flow Chart for the Isolation of Rat Liver mMDH



The details of the isolation and purification are given in Table 3. A comparison with the results of others is given in Table 4. Since assay procedures vary widely, it is difficult to compare the specific activity of the purified enzyme to all others reported in the literature. The percentage yields of enzyme also vary greatly with the procedure used and the animal source.

The greatest advantage of the procedure developed here is that pure rat liver enzyme can be obtained in three days whereas procedures reported prior to this require a minimum of six days. The greatest disadvantage is that the amount of starting material is limited by the quantity of 5'-AMP Sepharose used.

Subsequent to the development of this procedure, Kuan et al. (1979) developed a rapid procedure for the isolation of the rat liver enzyme as well. Their procedure utilizes Sephadex-Blue Dextran pseudo-affinity chromatography and ion-exchange chromatography on CM cellulose. It is claimed that the procedure can be done in 2 days, and the precentage yield of enzyme is 38 percent.

B. Beef Heart mMDH

This enzyme, purchased from Sigma Chemical Co., was shown to have several contaminating proteins by SDS-PAGE. These were removed by chromatography on 5'-AMP Sepharose

TABLE 3: Summary of steps in the purification and isolation of rat liver mMDH. This isolation was started from 45 g of rat livers.

Procedure	Total Units (U)	Total Protein (mg)	Specific Activity (U/mg)	Yield of Activity (%)
whole mitochondria	4,280			
sonicated mitochondria (minus membranes)	6,800	1,596.0	4.2	100
ammonium sulphate fraction	3,205	560.0	5.7	47.1
5'-AMP Sepharose	2,723	5.4	505.0	40.1
Sephadex G-100	1,836	2.8	658.0	27.0

TABLE 4: Summary of specific activity and yield of mMDH from published procedures.

Author	Source	Specific* Activity (U/mg)	Yield (%)
Ochoa, (1955)	beef heart	750	
Wolfe and Neilands, (1956)	pig heart		10
Siegal and England, (1960)	pig heart	330	20
Gregory et al., (1971)	pig heart		40
Mathewson et al., (1973)	pig heart		50
Glatthaar et al., (1974)	pig heart		21
Kuan et al., (1979)	rat liver		38
Kuan et al., (1979)	rat heart		52
Aziz, (1979)	rat liver	658	27

*MDH assayed in the direction of oxaloacetate reduction.

(Fig. 7 , lane 2). Greater than 0.8 mg MDH per ml of sepharose could be applied because the enzyme was already highly purified and concentrated.

C. Soluble MDH

A partial purification of this enzyme from rat liver was achieved. Although there were several contaminating bands, the procedure was not completed due to low yields of enzyme. However, greater than 50% of the protein present appeared to be MDH on SDS PAGE (data not shown). The pig heart sMDH from Research Plus Laboratories was also shown to contain several contaminating proteins, but to a much lesser degree than the rat enzyme.

2. Preparation of Rabbit Anti-mMDH Antisera

A. Immunization

Although several rabbits were injected, only the antiserum from the two rabbits which gave the highest titre were used in all experiments.

B. Efficacy and Cross-Reactivity of Antisera

The ability of antisera to immunoprecipitate mMDH and cross-reactivity was determined using Protein A Sepharose to bind radiolabelled antibody-antigen complexes. The technique has the advantage that titres of antibody too low to be detected by precipitin reactions, such as Ouchterlony double diffusion, can be determined. This technique also

greatly facilitated determination of conditions of immunoprecipitation. After denaturing the enzyme in the denaturation buffer containing 0.5% SDS, one-half volume of exchange buffer (TENT) with varying amounts of Triton X-100 was added. The molar ratio of Triton X-100 to SDS used and the effect on immunoprecipitation of antigen is shown in Table 5. It can be seen that optimal immunoprecipitation occurs at a molar ratio of 2:1. In a separate experiment, it was apparent that adding iodoacetamide did not increase the amount of enzyme immunoprecipitated (data not shown). The immunoprecipitation curves for anti-beef and anti-rat MDH antisera are illustrated in Fig. 5 and Fig. 6 respectively. It was found that in order to get a linear increase in the quantitiy of antigen immunoprecipitated with increasing amounts of antiserum, the antigen had to be resuspended in 50 to 100 µl of the denaturing and exchange buffer per 100 µl of antiserum. The reason for this is not known.

A summary of the amount of antigen that could be immunoprecipitated is given in Table 6. Neither antisera would immunoprecipitate the native mitochondrial enzymes or the denatured sMDH. The rat liver and beef heart enzymes were found to be immunologically cross-reactive with each antiserum more effectively immunoprecipitating the immunogen with which it was raised. The antisera were also found to be similar in titre so that either sera could be

TABLE 5: Effect of Triton X-100 concentration on the immunoprecipitation of antigen. Approximately 5 μg of [^{14}C]beef heart mMDH denatured at 0.5% SDS, followed by the addition of one-half volume of exchange buffer with varying concentrations of Triton X-100, was incubated with 50 μl of anti-beef heart mMDH antiserum. The immunoprecipitation procedure is described in the text. A separate experiment gave similar results.

Triton X-100: SDS (molar ratio)	Counts Immunoprecipitated (%)
0:1	46
2:1	51
9:1	13
18:1	6
24:1	4

Fig. 5

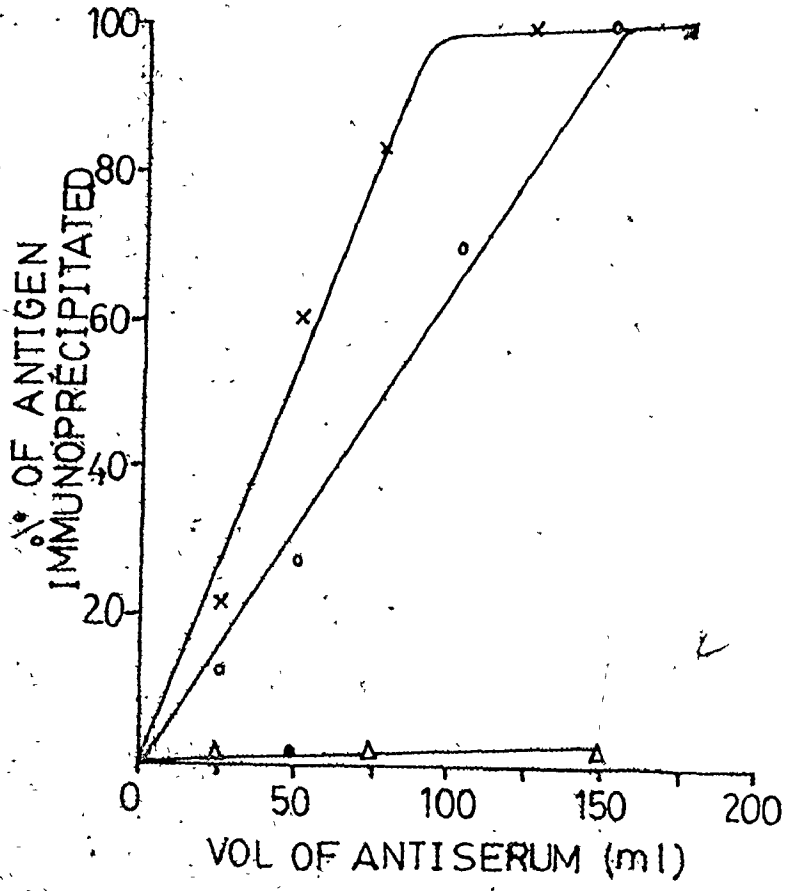


Fig. 6

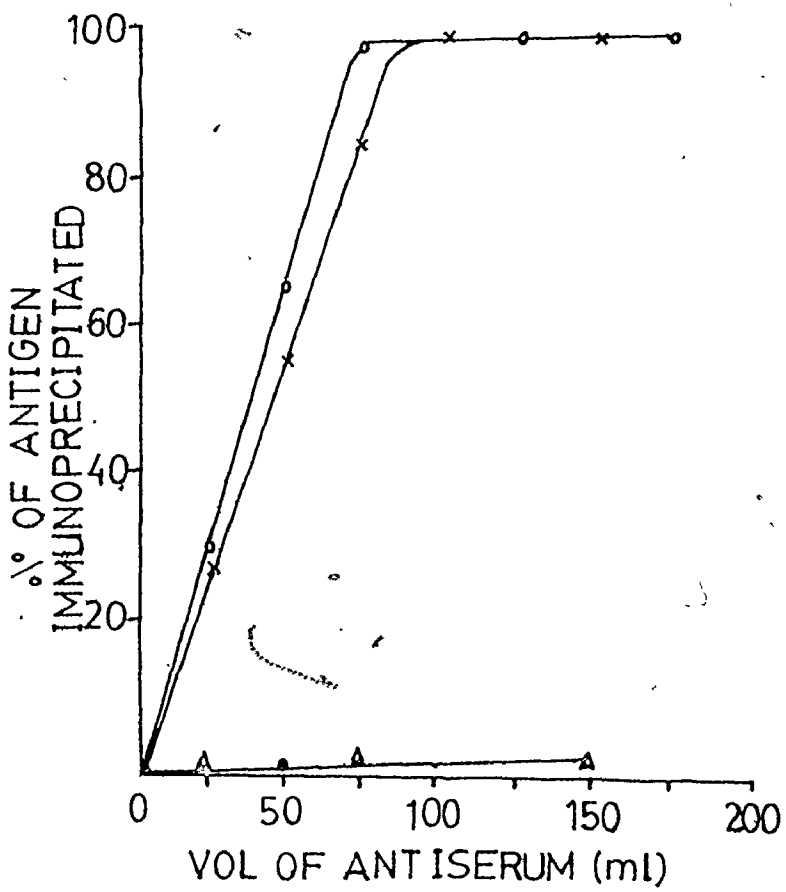


Fig. 5 and 6: Immunoprecipitation of antigen using anti-beef heart mMDH (5.5 µg of antigen, Fig. 5) and anti-rat liver mMDH (3.4 µg of antigen, Fig. 6). Enzymes were labelled with [^{14}C]formaldehyde and incubated with 25 to 175 µl of antiserum. Antigen representation: (X) denatured beef mMDH, (o) denatured rat mMDH, (●) native beef or rat mMDH, (Δ) denatured rat or pig sMDH. Results from separate experiments were similar.

TABLE 6: Amount of mMDH immunoprecipitated by anti mMDH antisera. Approximately 5.5 μg of denatured [^{14}C]beef heart or rat liver mMDH was immunoprecipitated with anti-beef heart mMDH antiserum or 3.4 μg of these antigens were immunoprecipitated with anti-rat liver mMDH antiserum. Values were taken from the graphs of Fig. 5 and 6 for 50 μl of each antiserum.

Antiserum	Antigen	μg Antigen Immunoprecipitated by 50 μl of Antiserum
Anti-beef mMDH	denatured beef mMDH	2.5
	denatured rat mMDH	1.8
Anti-rat mMDH	denatured beef mMDH	1.7
	Denatured rat mMDH	2.2

used to immunoprecipitate the rat liver mMDH synthesized *in vitro*. Anti-beef antisera were used in washing experiments and to find optimal conditions for immunoprecipitation from reticulocyte lysate.

C. Specificity of Antisera

Since the purpose of this work was to find if a single protein, mMDH, is synthesized in a higher molecular weight form, it was necessary to demonstrate that the antisera used were highly specific for mMDH. With lysates this was done after optimizing conditions of immunoprecipitation.

Immunoprecipitation of mMDH from MDBK Mitochondrial Protein

Mitochondrial protein from MDBK cells labelled with ^{35}[S]methionine, was denatured and 5×10^5 cpm (approx. 1 mg protein) were mixed with anti-mMDH antiserum to examine specific immunoprecipitation of mMDH. The products of immunoprecipitation by both antisera are illustrated in Fig. 7. Both anti-beef (lane 4) and anti-rat (lane 5) antisera but not control serum (lane 3) precipitated a protein corresponding in molecular weight to that of the marker beef enzyme. Any of the other protein bands present with the anti-MDH antisera were also present in the control serum, indicating that they may be non-specifically associated with a serum protein or the Protein A Sepharose. The faintness of the mMDH band in this experiment is due to

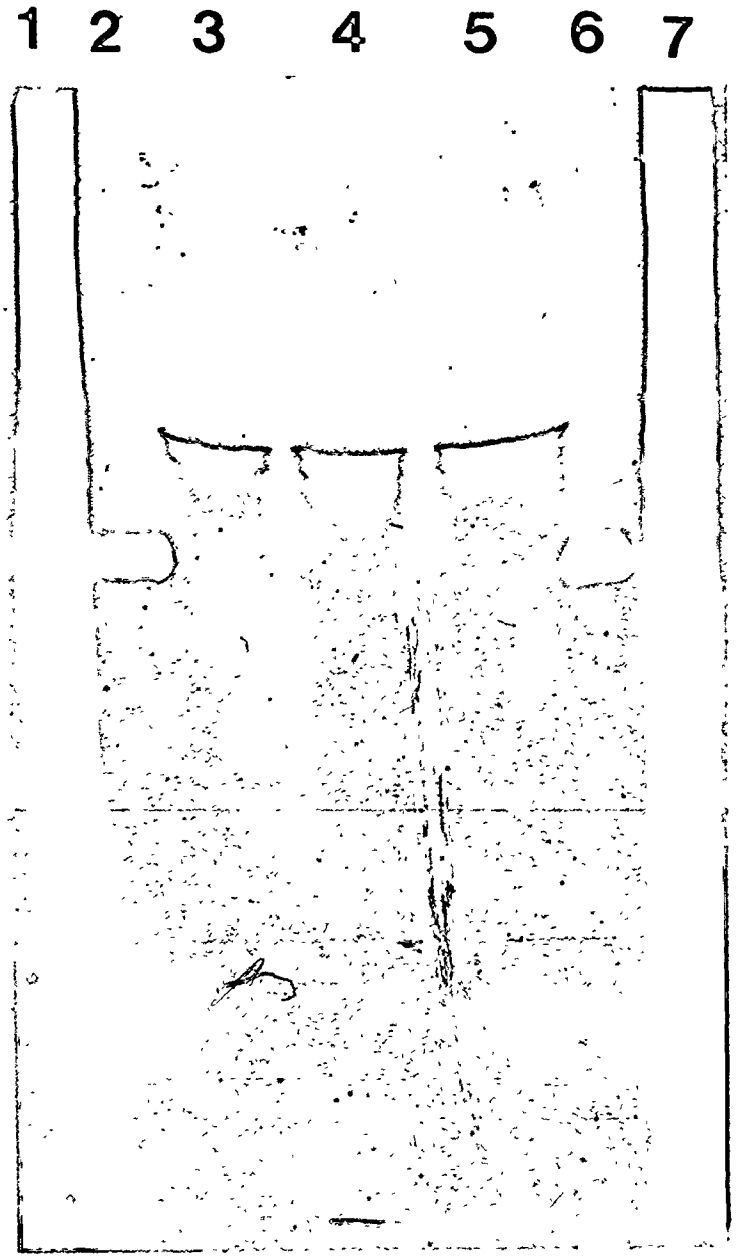


Fig. 7: Immunoprecipitation of mMDH from MDBK protein labelled with [^{35}S]methionine in suspension. Approximately 5 x 10^5 cpm of mitochondrial protein was denatured and mixed with 75 µl of antiserum and the immune complexes that were bound to Protein A Sepharose were examined by fluorography of SDS polyacrylamide gels. Lanes 1 and 7: total MDBK mitochondrial protein; lanes 2 and 6: [^{14}C]beef heart mMDH; lane 3: immunoprecipitation using non-immune serum; lane 4: immunoprecipitation using anti-beef mMDH antiserum; lane 5: immunoprecipitation using anti-rat mMDH antiserum. The details of immunoprecipitation are outlined in the text, and the results of this experiment were reproducible.

the low quantities of endogenous mMDH.

Immunoprecipitation of [^{14}C]mMDH from Protein Synthesized In Vitro in the Presence of [^{35}S]methionine

From the cross-reactivity studies it was clear that the antisera did not immunoprecipitate the native enzyme, so that it was essential that enough SDS was added to the lysate to denature the mMDH. Using denatured antigen immunoprecipitated in the absence of lysate as control, the effects of lysate and SDS was examined. The results are illustrated in Table 7. As expected, little mMDH was precipitated if the enzyme was not denatured (condition c). When denatured mMDH was added to lysate denatured at 2% (a) and 6% (b) SDS, 51% and 67% of the enzyme was immunoprecipitated, respectively, compared to control. Furthermore, when the antigen was denatured in the presence of lysate, only 37% was immunoprecipitated at 2% SDS (d) whereas 66% was immunoprecipitated at 6% SDS (e), similar to the result with previously denatured antigen. It is not known why there is incomplete recovery of mMDH. In all subsequent experiments the lysate was denatured at 6% SDS.

The need for adequate Triton X-100 to exchange with SDS in the presence of lysate is illustrated in Table 8. The difference in the amount of antigen precipitated using 3%, 9% and 15% Triton X-100 all were considerably higher

TABLE 7: Effect of SDS concentration and lysate on the immunoprecipitation of [^{14}C]beef heart mMDH. 10^4 cpm of previously denatured antigen was added to 20 µl of lysate which had been treated with 2% (a) or 6% (b) SDS and exchanged with 4 vol of TENT at 0%, 3% or 9% Triton X-100 respectively to keep a molar ratio of Triton:SDS of 2:1. Next 10^4 cpm of the native enzyme was added to 20 µl of the native lysate, and they were denatured and exchanged together under the conditions described above. Immunoprecipitation of mMDH in the absence of lysate at a molar ratio of Triton X-100:SDS of 2:1 was used as control. Separate experiments gave similar results.

Condition	% of Control Immunoprecipitated
a) mMDH and lysate denatured separately; each at 2% SDS	51
b) mMDH and lysate denatured separately; mMDH denatured at 2% and lysate denatured at 6% SDS	67
c) lysate and mMDH mixed together at 0% SDS	6
d) lysate and mMDH denatured together at 2% SDS	37
e) lysate and mMDH denatured together at 6% SDS	66

TABLE 8: Effect of Triton X-100 concentration in the exchange buffer on the immunoprecipitation of mMDH denatured in the lysate at 6% SDS. 10^4 cpm of native mMDH was added to 20 µl of native lysate and they were denatured together at 6% SDS. Four volumes of exchange buffer at 0%, 3% 9% or 15% Triton X-100 were added. Immunoprecipitation of mMDH in the absence of lysate at a molar ratio of Triton X-100:SDS of 2:1 was used as control. Separate experiments gave similar results.

Concentration of Triton X-100 in Exchange Buffer (%)	Triton X-100:SDS (molar ratio)	Amount of Control Immunoprecipitated (%)
0	0	33
3	2:3	60
9	2.1	72
15	10.3	64

than when Triton was not added. Subsequent experiments were done using 9% Triton X-100 in the exchange buffer (molar ratio of Triton: SDS of 2:1).

When immunoprecipitating from the protein synthesizing system, the presence of many different [^{35}S]methionine-labelled proteins as well as free [^{35}S]methionine necessitated increasing the number and volume of washes of the Protein A Sepharose-bound immune complexes. In addition, the effect of cold methionine and EDTA in the wash solution was examined (Table 9). In this experiment 10^4 cpm of [^{14}C]beef mMDH was added to $5X10^5$ acid precipitable cpm of protein synthesized in vitro. Using the basic wash buffer alone, more than 10^4 cpm were recovered compared to an expected $7X10^3$. This indicated non-specific association of label. The presence of methionine alone or with EDTA lowered the recovery of counts to about the expected level. EDTA, while decreasing the counts recovered, did not remove all contamination. The presence of 0.1% SDS resulted in almost all radioactivity being washed out. The composition of the washing buffer used in subsequent experiments was 125 to 250 mM NaCl, 50 mM Tris-HCl, 10mM MSH, 20 mM methionine, 5 mM EDTA, pH 7.6.

Having determined conditions for immunoprecipitation and washing the specificity of the antisera for mMDH in the lysate was determined. Beef heart enzyme was labelled with [^{14}C]formaldehyde and $5X10^3$ cpm were mixed with 10^6 cpm

TABLE 9: Effect of EDTA, methionine and SDS in the basic wash buffer on the recovery of [^{14}C]mMDH from protein labelled *in vitro* with [^{35}S]methionine. pproximately 10^4 cpm of enzyme was added to $5X10^5$ cpm of protein labelled *in vitro* and immunoprecipitated. The Protein A-bound immune complexes were washed with the basic wash buffer (125 mM NaCl, 50 mM Tris-HCl, 10 mM MSH pH 7.6) to which was added a) nothing, b) 20 mM cold methionine, c) 5 mM EDTA, d) 20 mM cold methionine plus 5 mM EDTA, e) 0.1% SDS. Values are given as the average of duplicates ± error.

Components of Wash Buffer	Control Serum (cpm)	Anti-mMDH Antiserum (cpm)	Experimental-Control (cpm)
basic wash buffer	4,458±470	17,265±1,266	12,707±1,736
+ 20 mM methionine	1,129±275	6,926±574	5,797±849
+ 5 mM EDTA	3,891±632	13,428±1,055	9,137±1,687
+ 20 mM methione and 5 mM EDTA	1,207±157	7,249±604	6,042±761
+ 0.1% SDS	740±112	839±170	99±182

of protein synthesized in vitro and immunoprecipitated. As in the experiment with MDBK protein, the anti-mMDH antisera, but not control serum immunoprecipitated a protein corresponding in molecular weight to the marker beef heart enzyme (Fig. 8, lanes 4 and 5 compared to 3). Again, all other bands that were present with anti-mMDH sera were also present with the control serum. The results of both experiments indicated that the anti-mMDH antisera are highly specific for mMDH.

3. Isolation and Characterization of Free Polysomes from Rat Liver

The mRNA which codes for carbamoyl phosphate synthetase (a mitochondrial matrix protein, like mMDH) was found to be associated with the free polysome fraction of rat liver by Shore et al. (1979). For this reason, this fraction was also used in these experiments for in vitro translation.

The free polysome fraction was isolated by the method of Ramsay and Steel, (1976) as modified by Shore et al. (1979). This procedure was chosen over the much used procedure of Blobel and Potter (1967) which utilizes a discontinuous sucrose gradient to effect the separation of free and membrane-bound polysomes. The former method uses a differential centrifugation step to separate the free and membrane-bound fractions which avoids the long centrifugation

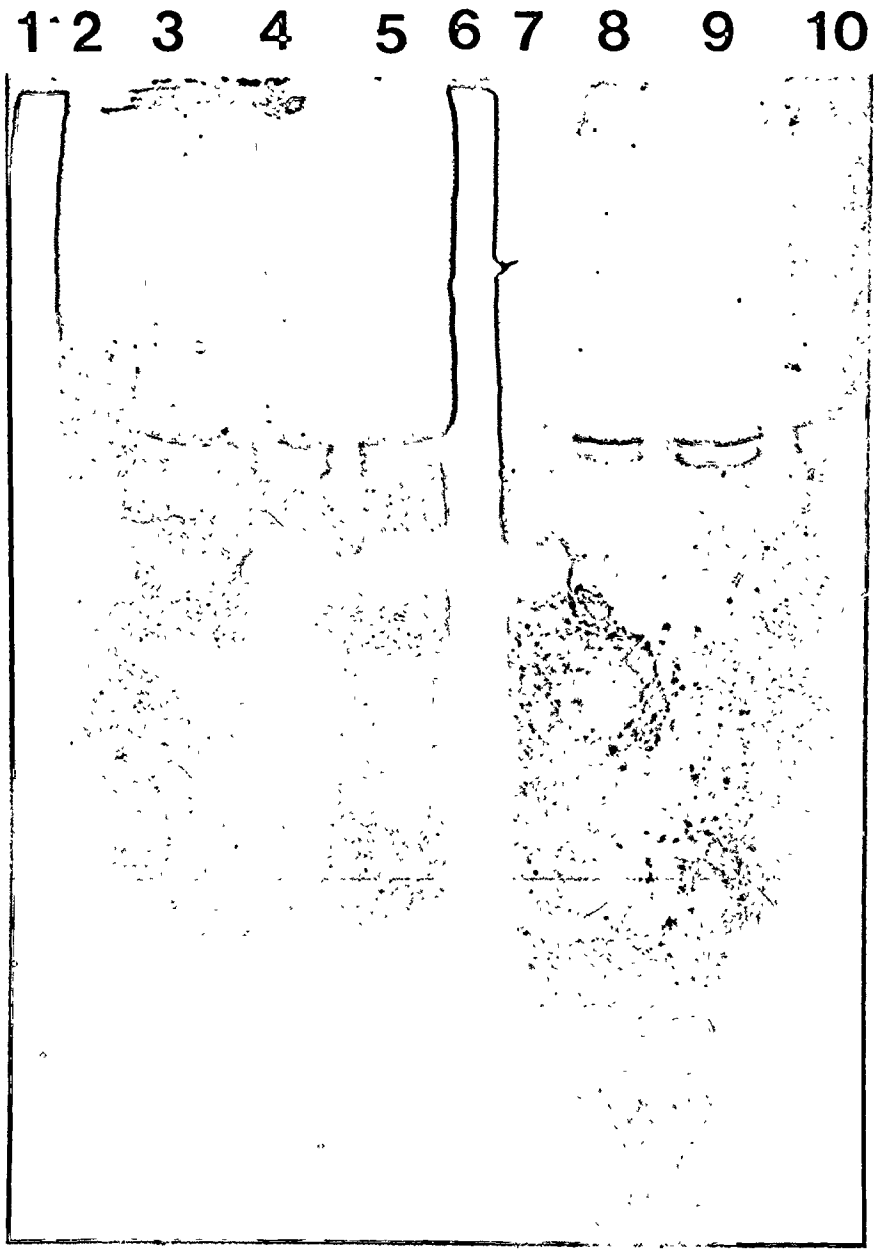


Fig. 8: Immunoprecipitation of [^{14}C]beef heart mMDH from protein labelled in vitro with [^{35}S]methionine by free polysomes in a reticulocyte lysate. Details of the incubation are outlined in the text. Immune complexes bound to Protein A Sepharose were examined by SDS PAGE and fluorography, (lanes 1 and 6: total translation products of rat liver free polysomes; lane 2: [^{14}C]beef heart mMDH; lane 3: immunoprecipitation with non-immune serum; lane 4: immunoprecipitation with anti-beef mMDH antiserum; lane 5: immunoprecipitation with anti-rat mMDH antiserum. Lanes 7 to 10: immunoprecipitation in the absence of added [^{14}C]beef heart mMDH; lane 7: [^{14}C]rat liver mMDH; lane 8: non-immune serum; lane 9: immunoprecipitation with anti-beef mMDH antiserum; lane 10: immunoprecipitation with anti-rat mMDH antiserum.

times of the sucrose gradient method. The use of high ionic strength (200 mM Tris-HCl) and high pH of 8.5 inhibits any endogenous RNase activity which precludes the addition of other RNase inhibitors such as heparin (Roth, 1953) or the high speed supernatant protein from rat liver (Roth, 1956). The method resulted in high molecular weight polysomes which were prepared in 4 to 5 hours. A sucrose gradient analysis of these polysomes is illustrated in Fig. 9. The disadvantages to this method are that the bound polysomes cannot be isolated simultaneously without the availability of two ultracentifuges, and the amount of starting material is limited because of the large volume of homogenization buffer used. A maximum of about 100 OD_{260} units of polysomes could be obtained from 10 to 15 g of rat liver. This small quantity was technically difficult to resuspend in less than 250 µl, resulting in maximum polysome concentrations of 175 to 240 OD_{260} units/ml. Polysomes prepared by the method of Blobel and Potter by S-M. Chien were resuspended to greater than 400 OD_{260} units/ml. The OD_{260}/OD_{280} ratio of polysomes prepared by the method of Shore et al. was routinely 1.6 to 1.8.

4. Preparation of Total RNA from Rat Liver

The total RNA had an OD_{260}/OD_{280} ratio of 2.0 indicating that there was very little protein contamination.

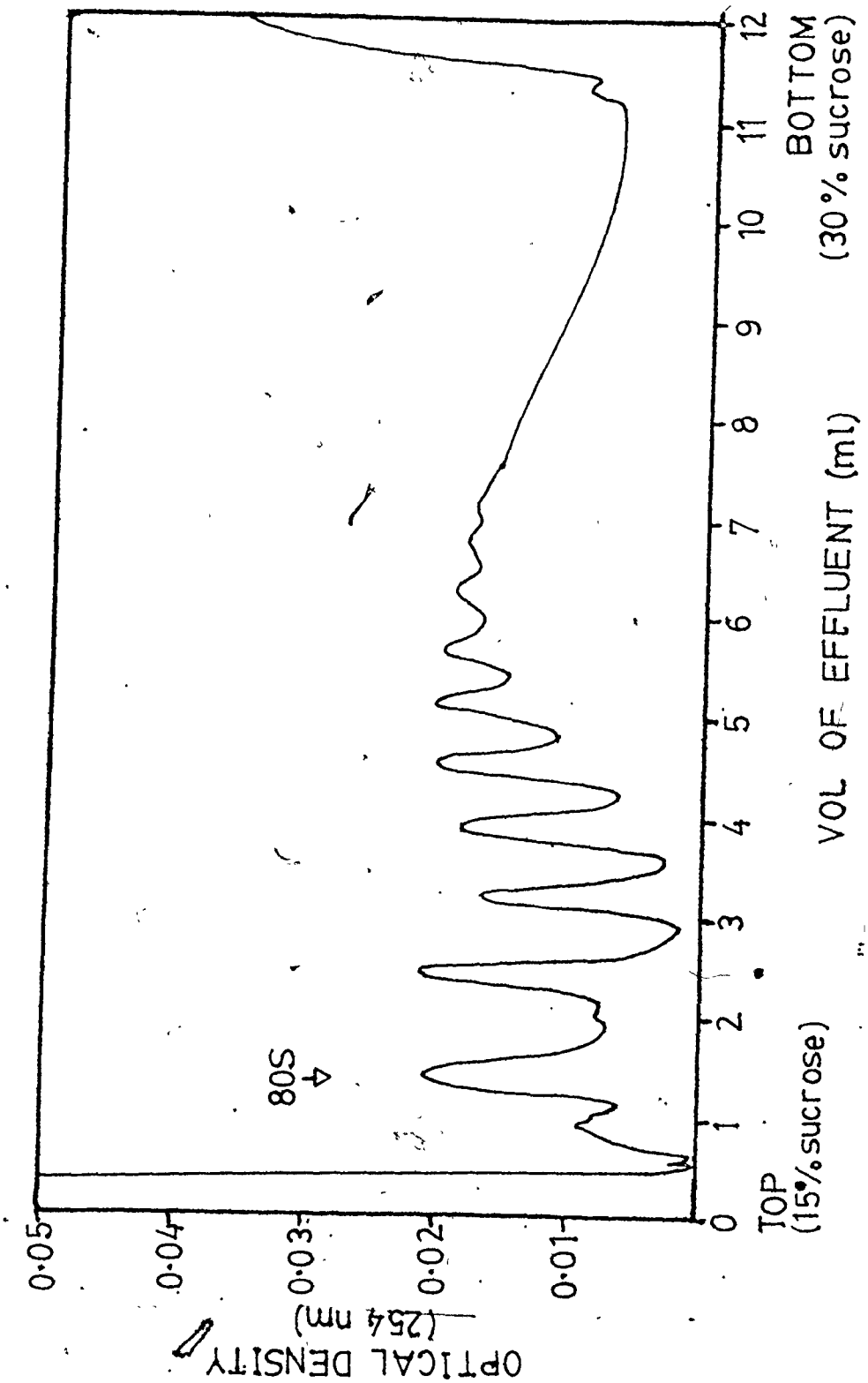


Fig. 9: Polysome profile. Free polysomes (0.5 to 1.0 OD_{260} units) were layered over a linear gradient of 15 to 30% sucrose in $T_{20}K_{75}M_5$, pH 7.6 and centrifuged at 110,000 gav for 90 min in the Beckman SW41 rotor. Absorbance at 254 nm was monitored using an ISCO UV analysing system.

5. Protein Synthesis *In Vitro* Using Reticulocyte Lysate and Immunoprecipitation of Translation Products

A. Induction of Reticulocytes and Preparation of Lysate

The endogenous protein synthetic activity of lysate prepared from the blood of each rabbit was tested, and the two lysates with highest activity were used in subsequent experiments. Incorporation of label into acid precipitable counts was linear for greater than 30 minutes. Nuclease treatment of the lysate resulted in a drastic decrease in incorporation of label and abolishment of synthesis of globin and other high molecular weigh protein as judged by SDS PAGE (data not shown).

B. Cell-Free Protein Synthesis with Exogenous mRNA

Optimization of Hemin, Mg^{2+}, and KCl Concentrations

The curves illustrated in Fig. 10 show the incorporation of label into protein with time at different hemin concentrations. From the graph inset of the incorporation at 60 min versus hemin concentration, it can be seen that the optimal hemin concentration is from 120 to 150 μM. The optimal concentration reported in the literature for protein synthesis by mammalian ribosomes varies from 30 to 100 μM (Ranu and London, 1979; Palmiter, 1973; Adamson *et al.*, 1968). The concentration of hemin (120

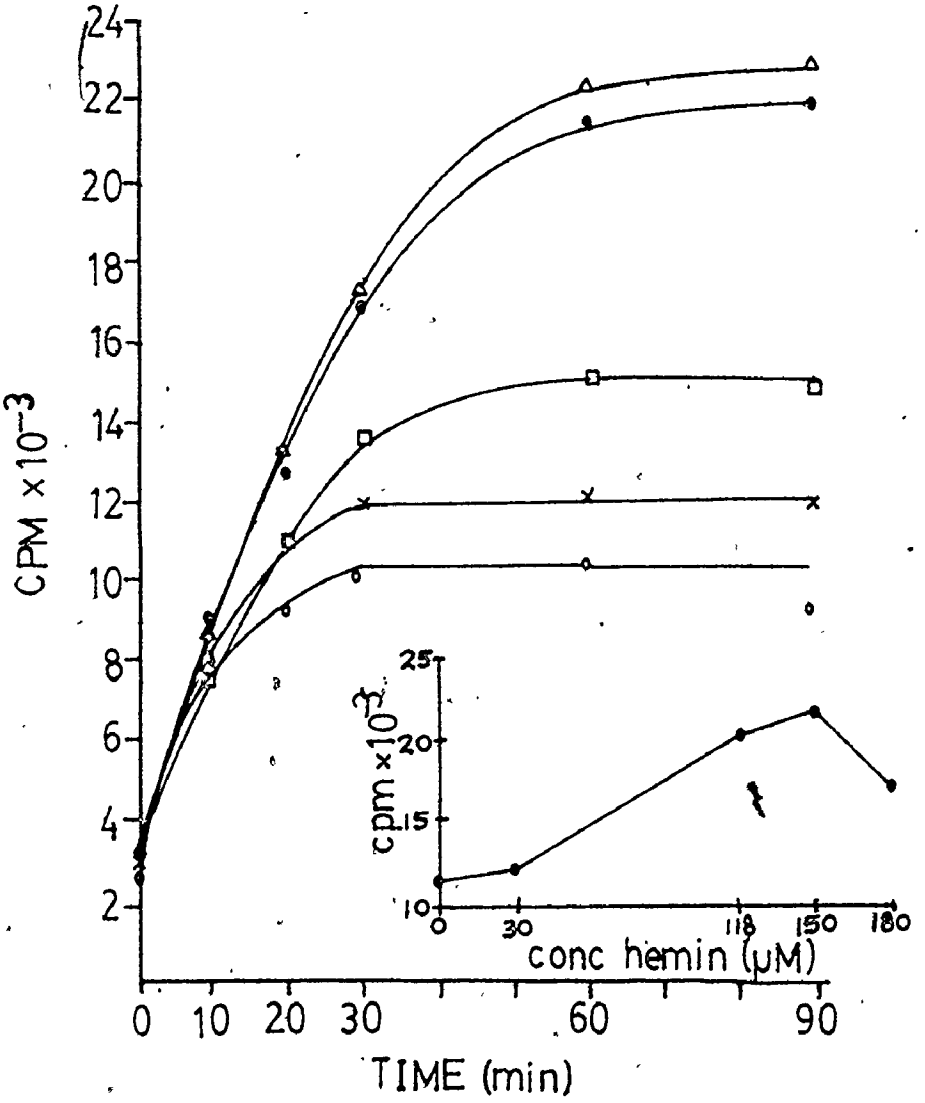


Fig. 10: Effect of hemin concentration on the incorporation of [^{35}S]methionine into acid-precipitable counts. Free polysomes were incubated in the reticulocyte lysate made 2 mM in Mg^{2+}, 50 mM KCl and varied hemin concentrations: (o) 0 μM, (x) 30 μM, (•) 120 μM, (Δ) 150 μM and (□) 180 μM. Graph inset: acid precipitable cpm at 60 min with 0 to 180 μM hemin. A separate experiment yielded similar results.

to 150 μM) was overestimated due to some precipitation of the reagent during preparation.

Figures 11 and 12 illustrate the effect of K^+ and Mg^{2+} concentrations respectively on protein synthesis. The optimal KCl concentration was between 70 and 100 mM and optimal Mg^{2+} was between 1.5 and 2.5 mM. Both values are similar to the optima (75 mM KCl and 2.0 mM Mg^{2+}) found by Shore et al., (1979) for rat liver polysomes translated in reticulocyte lysates.

Saturation of Lysate with Polysomes and the Time Course of Incorporation Using Polysomes and Total RNA

A linear increase in the amount of ^{35}[S]methionine incorporated into protein was obtained with increasing concentration of polysomes in the lysate, but incorporation appeared to start levelling off at a concentration of 2.5 mg/ml (Fig. 13). The reason for this levelling is most likely due to one of the protein synthesis factors endogenous to the lysate being limited.

The time course for incorporation is shown in Fig. 14. Translation of free polysomes under optimal conditions resulted in $3X10^5$ acid precipitable cpm in 2 μl of lysate in 60 min while under the same conditions translation of total RNA yielded $6X10^4$ cpm in 2 μl. The reason for this difference is unknown. The amount of total RNA had been determined to

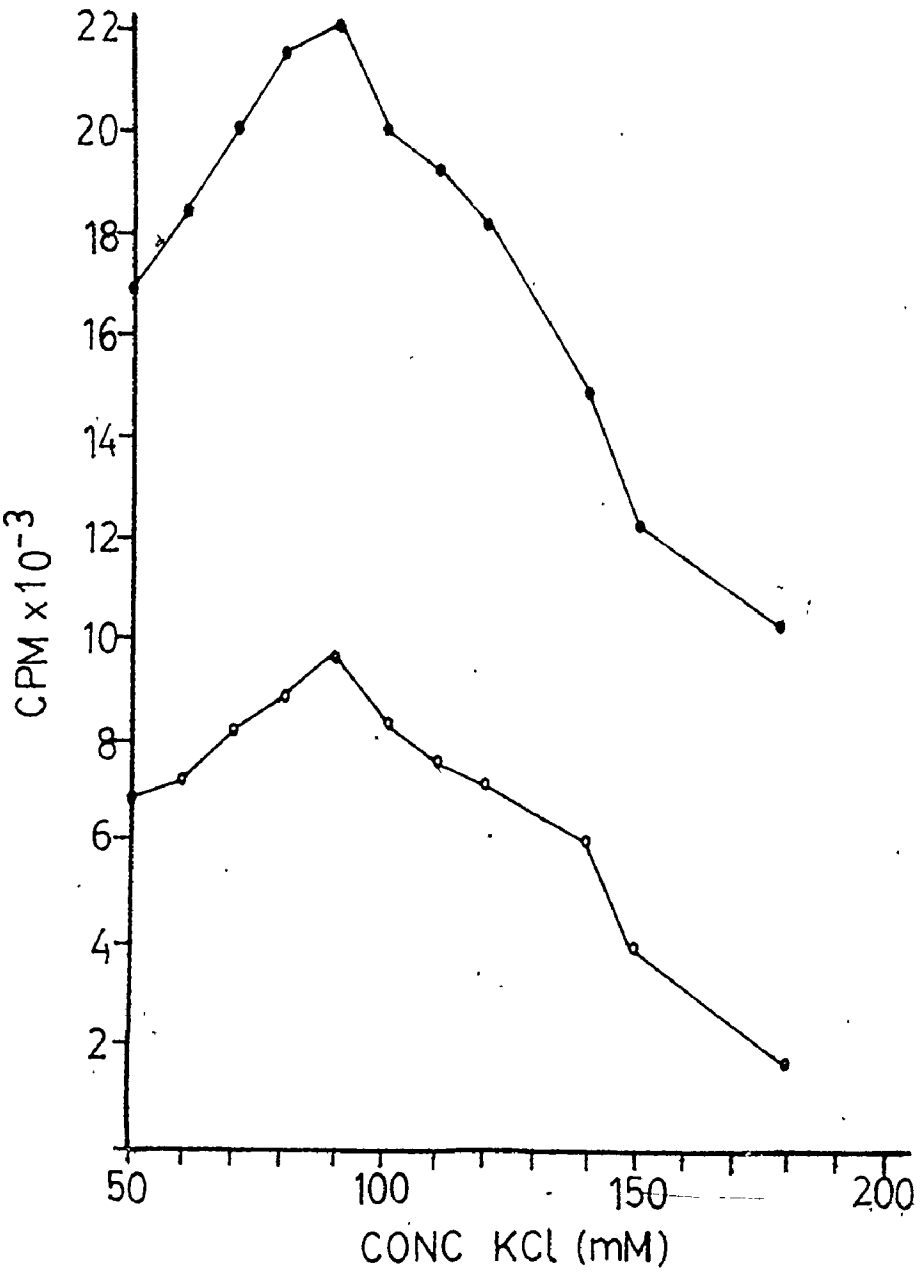


Fig. 11: Effect of K^+ concentration on the incorporation of [^{35}S]methionine into acid-precipitable cpm. Free polysomes were incubated in the reticulocyte lysate made 2 mM in Mg^{2+}, 150 µM in hemin and varied K^+ concentrations. Samples were taken at 30 (o) and 60 (•) min. Separate experiments yielded similar results.

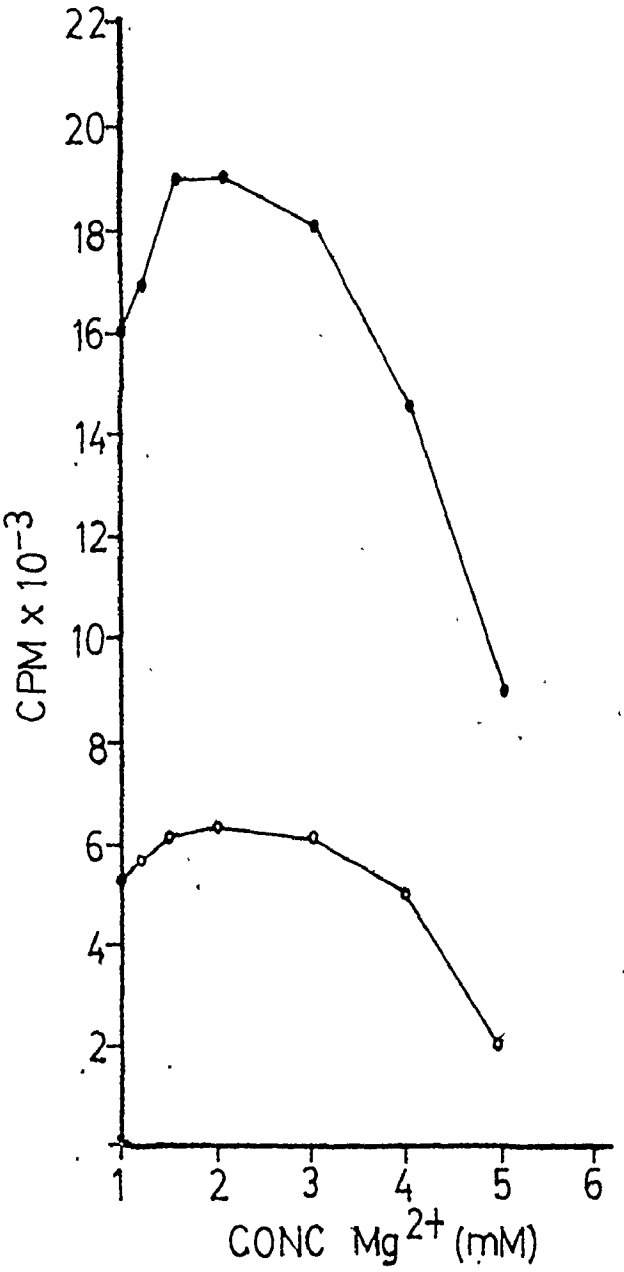


Fig. 12: Effect of Mg^{2+} concentration on the incorporation of [^{35}S]methionine into acid-precipitable counts. Free polysomes were incubated in the reticulocyte lysate made 110 mM in KCl, 150 μM hemin and varied in Mg^{2+} concentrations. Samples were taken at 30 (o) and 60 (•) min. Separate experiments yielded similar results.

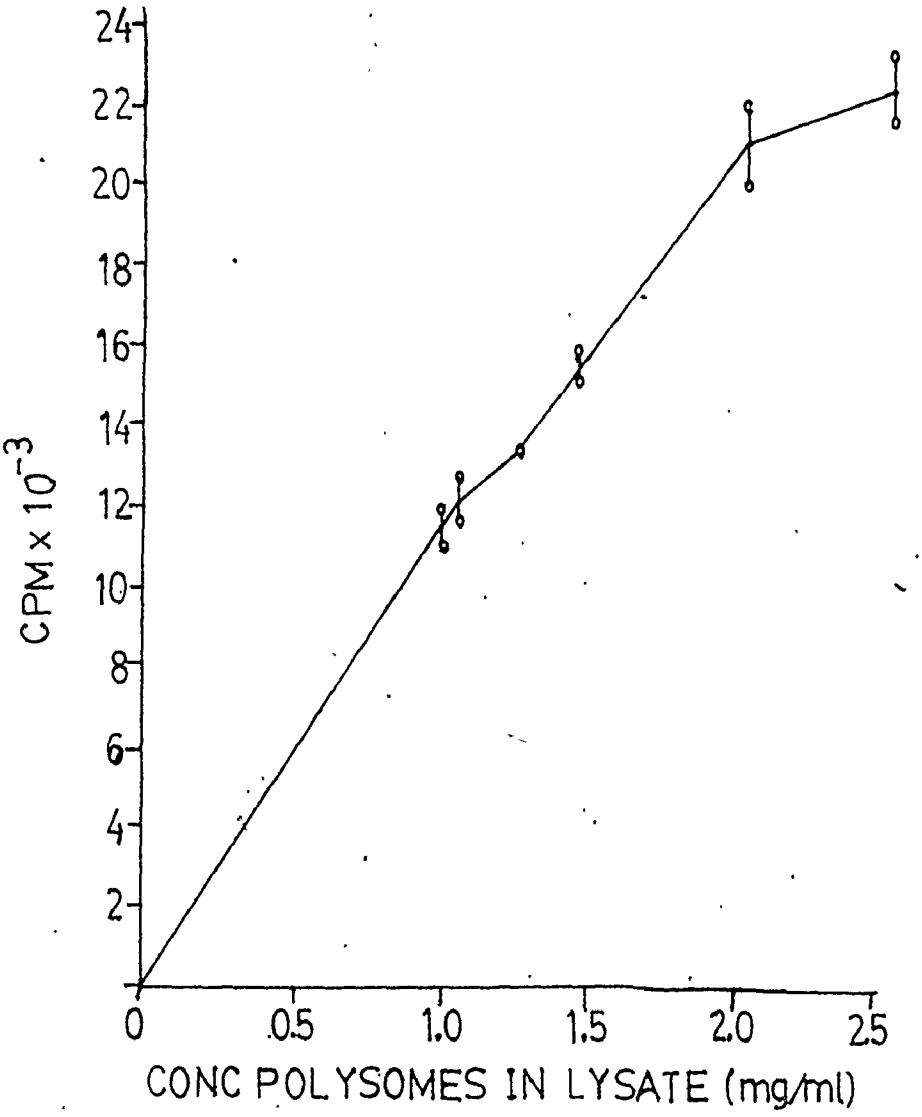


Fig. 13: Attempt to saturate lysate with polysomes. Free polysomes from a 400 OD_{260} units/ml stock solution were diluted in $T_{10}K_{20}M_5$, pH 7.6 and added to the assay system to give final concentrations from 13 to 34 OD_{260} units/ml (approx. 1 to 2.5 mg/ml. Mg^{2+}, KCl and hemin were at concentrations of 2 mM, 100 mM and 150 µM respectively.

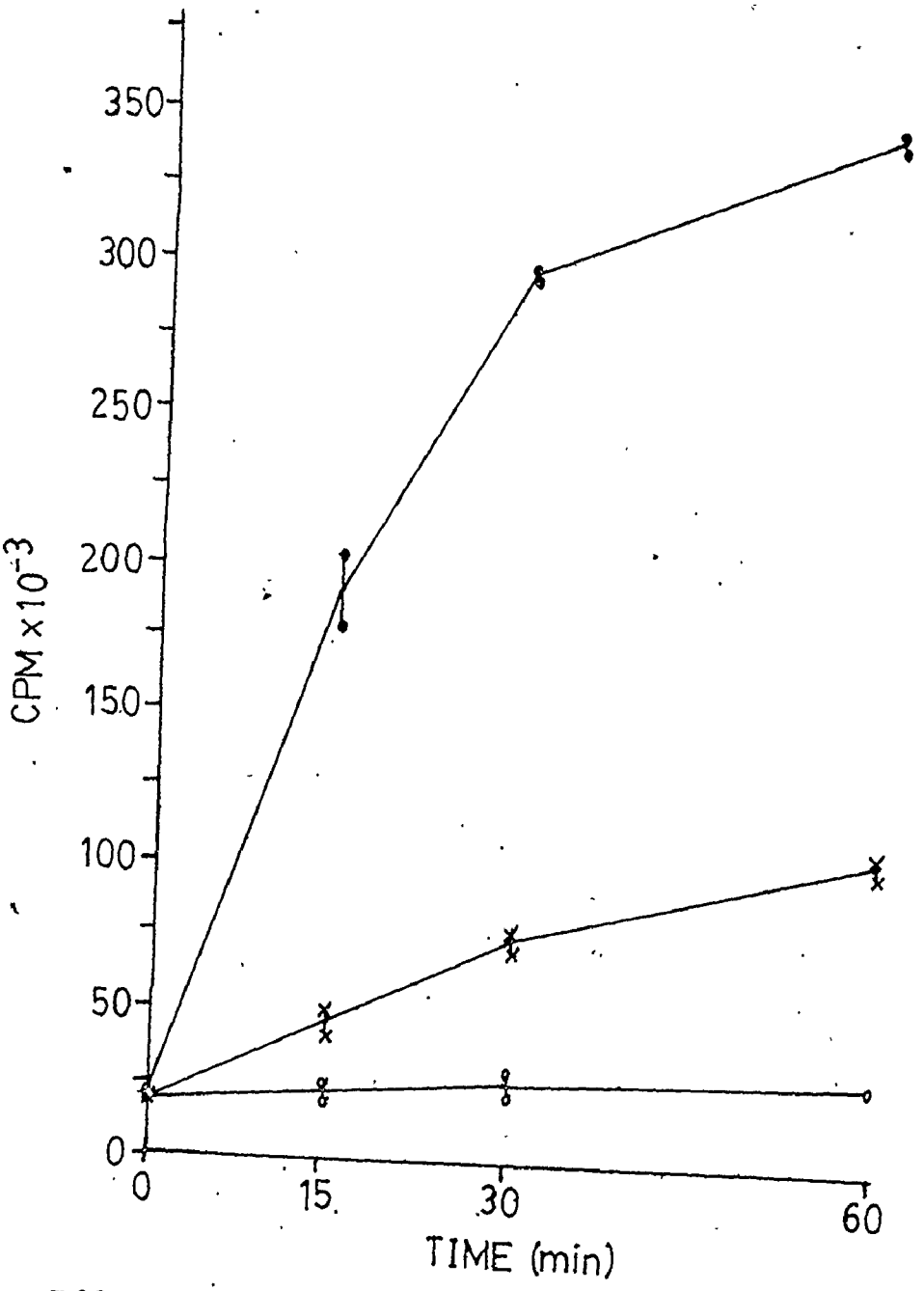


Fig. 14: Effect of free polysomes and total RNA on the incorporation of [^{35}S]methionine into acid-precipitable counts in the nuclease-treated lysate. Polysomes (●) and total RNA (x) were incubated in the presence of 1 μCi/μl [^{35}S]-methionine, 2 mM mg^{2+}, 100 mM KCl and 150 uM hemin. The absence of RNA is represented by (o).

be optimal (data not shown, experiments done by S-M. Chien), though ionic conditions had not been optimized for total RNA.

6. Immunoprecipitation of mMDH from Products of Cell-Free Protein Synthesis

The culmination of all prior experimental work was the attempt to immunoprecipitate mMDH and/or a precursor form of the enzyme from the products of cell-free protein synthesis. Several preliminary experiments using protein synthesized in vitro by free polysomes revealed that a single protein could be specifically immunoprecipitated using the anti-mMDH antisera. This protein appeared to have a higher molecular weight than mature rat liver mMDH (Fig. 8, lanes 9 and 10 compared to lane 7). Preliminary evidence also showed that the presence of excess cold mMDH during immunoprecipitation caused this band to disappear. The molecular weight was estimated to be 1,500 to 2,000 higher than mature mature rat liver mMDH.

The final results of immunoprecipitation from the translation products of both polysomes and total RNA and competition by unlabelled mMDH can be seen in Fig. 15. Anti-mMDH antiserum appears to immunoprecipitate a protein of the same molecular weight for both total RNA and polysomes (lanes 3 and 6 respectively). Addition of cold mMDH resulted in the abolition of the band in both cases

(lanes 2 and 7) so that the results are indistinguishable from the immunoprecipitation using non-immune serum (lanes 1 and 8). Estimation of the molecular weight of this protein from Fig. 16 shows it to be 36,000 or 1,500 higher than mature rat liver mMDH (Fig. 15, lane 6).

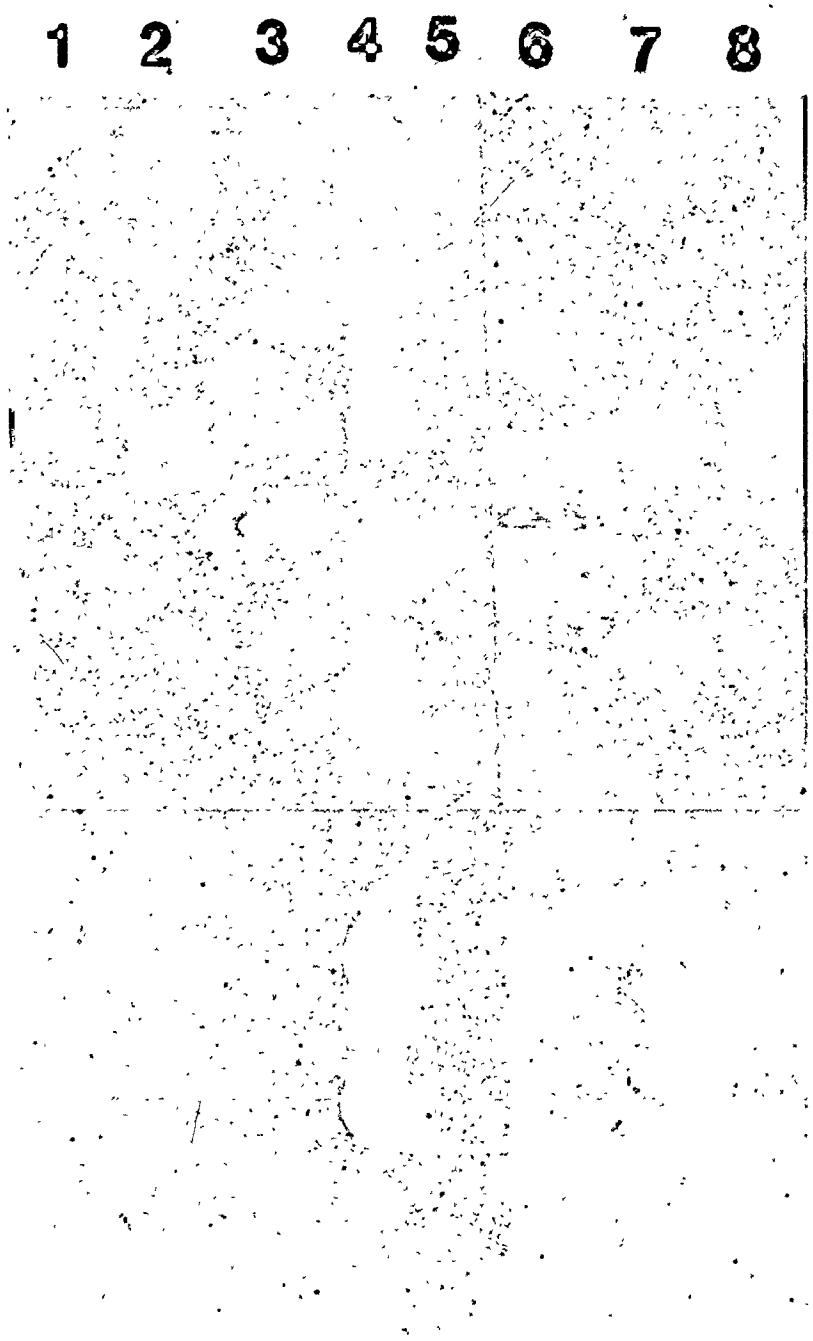


Fig. 15: Analysis by SDS polyacrylamide gel electrophoresis and fluorgraphy of protein synthesized in vitro by reticulocyte lysate in the presence of free polysomes or total RNA, and the products of immunoprecipitation. Details of the incubation are outlined in the text. Lanes 1-3 involve free polysomes: Lane 1, non-immune serum; lane 2, anti-beef heart mMDH antiserum: competition by 200 µg unlabelled beef heart mMDH denatured with the lysate; lane 3, anti-beef heart mMDH antiserum. Lanes 6-8 involve total RNA: lane 6, anti-beef heart mMDH antiserum; lane 7, competition by unlabelled beef heart mMDH; lane 8, non-immune serum. Lane 4, molecular weight markers. Lane 5, purified rat liver mMDH labelled with [^{14}C]formaldehyde. Exposure time was longer for total RNA than for free polysomes.

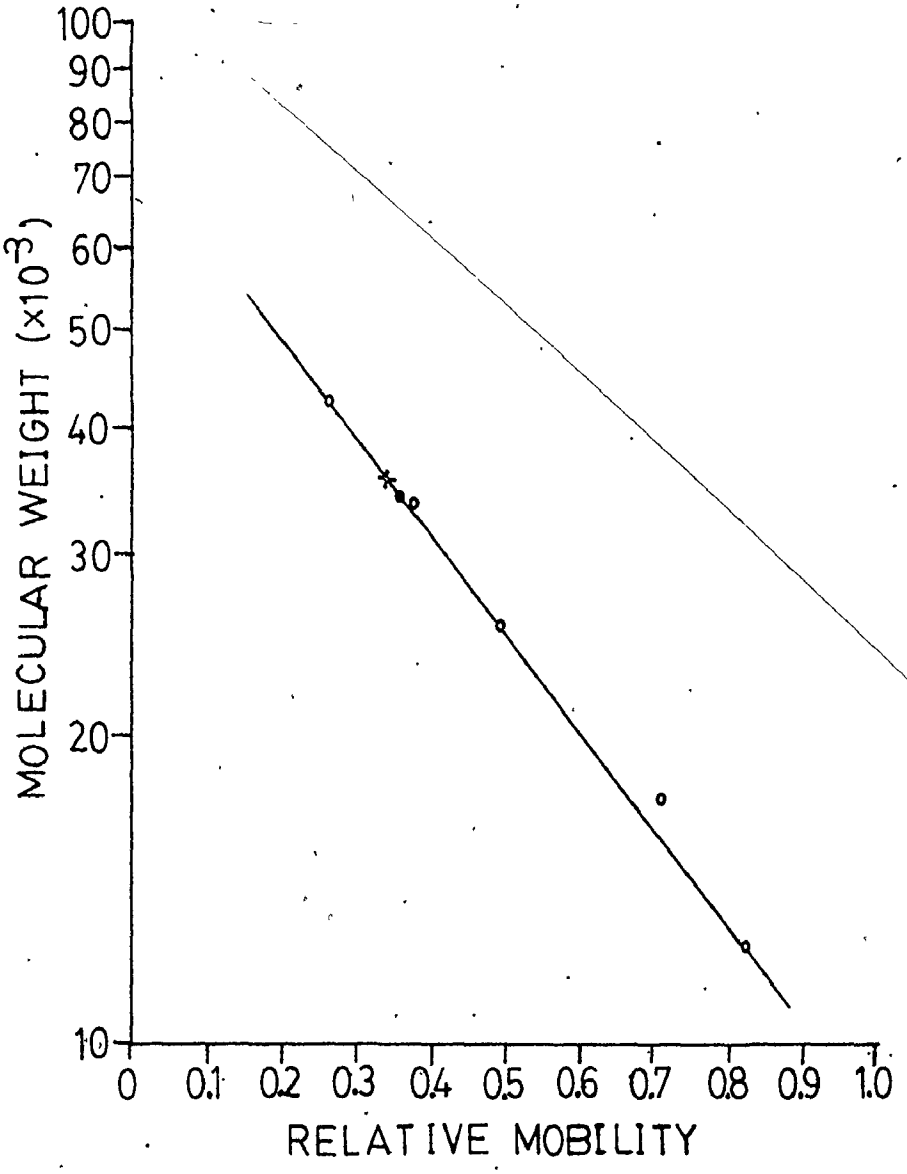


Fig. 16: Determination of the molecular weight of the major product of immunoprecipitation from the fluorogram of Fig. 15. Marker proteins (o): molecular weights 43,000, 34,000, 26,000, 17,000 and 12,500. Rat liver mitochondrial malate dehydrogenase (●): 34,500, major product of immunoprecipitation (X): 36,000.

V. DISCUSSION

If the free polysome fraction from rat liver is incubated in a cell-free protein synthesizing system derived from a reticulocyte lysate, there is efficient translation of exogenous mRNA. Total RNA containing a similar amount of mRNA which is translated in the same system yields less than one-third of the acid-precipitable counts produced by free polysomes (Fig. 14). Nontheless, translation of both sources of mRNA produces polypeptides of high molecular weight (free polysomes: Fig. 7, lane 1; total RNA: data not shown). When the translation products from either source are incubated with anti-mMDH antiserum, a protein of molecular weight 36,000 is immunoprecipitated and this protein is not precipitated when non-immune serum is used (Fig. 15, lanes 3 and 6 compared to lanes 1 and 8). As this antiserum was demonstrated to be highly specific for mMDH, it is probable that the 36,000 dalton protein contains antigenic determinants of mMDH and is possibly a higher molecular weight form of the enzyme. Mature rat liver mMDH has a molecular weight at 34,000-34,500 (Fig. 15, lane 6), being 1,500 to 2,000 smaller than the product of immunoprecipitation. Further evidence that this 36,000 dalton protein might be a higher molecular weight precursor comes from competition experiments. The

addition of excess unlabelled beef heart mMDH completely prevents the recovery of this protein (Fig. 15, lanes 2 and 7). The relationship could be confirmed by comparing proteolytic peptide maps of the two proteins as has been done with carbamyl phosphate synthetase and ornithine transcarbamylase from rat liver (Morita et al, 1979; Conboy and Rosenberg, 1981, respectively) and their precursors. Furthermore, pulse and pulse-chase experiments in cells might be used to demonstrate precursor-product kinetics. Carbamyl phosphate synthetase is the only rat liver mitochondrial protein for which these kinetics have been established (Raymond and Shore, 1979). Post-translational uptake and processing by isolated rat liver mitochondria has been demonstrated for pre-ornithine transcarbamylase (Conboy and Rosenberg, 1981) but similar experiments were not attempted here for rat liver pre-mMDH.

Three rat liver mitochondrial matrix enzymes have been shown previously to be synthesized by free rather than membrane-bound polysomes (carbamyl phosphate synthetase (Shore et al., 1979); δ-aminolevulinate synthetase (Yamauchi et al., 1980); ornithine transcarbamylase (Morita et al., 1981)). Evidence presented here would suggest that rat liver mMDH is also synthesized by the free polysome fraction. The intensity of the band at 36,000 daltons was found to be 10 times greater when immunoprecipitated from translation

products of free polysomes than when total RNA was used to prime synthesis. Conventionally, the separation of free and membrane bound polysomes (and a measurement of the degree of cross contamination) has been done in order to compare the translation products of each (Shore et al., 1979).

Demonstration that chicken liver aspartate aminotransferase (Sondregger et al., 1979) and now here that rat liver mMDH are synthesized as higher molecular weight precursors raises interesting questions about the role of the polypeptide extensions. Both of these enzymes from rat liver, in their mature form can be taken up into isolated mitochondria (Marra et al., 1980; Passerella et al., 1981). Although the relevance of these experiments to the in vivo situation has been questioned by Neupert and Schatz (1981b) due to the relatively large quantities of enzymes used, it is pertinent to note that the soluble isozymes of these two enzymes are excluded from mitochondria under the same conditions (Marra et al., 1980; Passerella et al., 1981). In model studies, Furuya et al. (1979) have shown that rat liver mitochondrial aspartate aminotransferase, but not the soluble isozyme is protected from proteolysis when incubated with phospholipid vesicles. Webster et al. (1979) have demonstrated that porcine heart mMDH was protected from inactivation by heat and thiol reagents by the presence of phospholipid vesicles, under conditions where the dimer is

dissociated into its subunits. Binding of these subunits was accompanied by a small conformational change. These experiments with aspartate aminotransferase and malate dehydrogenase suggest that the mature mitochondrial proteins (or their subunits) contain the information required for interaction with membranes and uptake into mitochondria. The apparent shift of these enzymes between intramitochondrial compartments in response to changing substrate concentrations (Waksman et al., 1980) further indicates an inherent ability to cross membranes.

If, then, the mature matrix enzymes can be taken up into isolated mitochondria, what is the role of the polypeptide extension and what allows these water-soluble proteins to cross the hydrophobic membrane barrier? (Similarly, how do hydrophobic membrane proteins travel through aquaeous compartments?) Wickner has proposed a model (termed "the membrane trigger hypothesis") which places emphasis on membrane-triggered conformational change in the migrating protein. In this model, interaction of the protein with the membrane causes spontaneous folding into or across the membrane and the peptide extension, when present, serves to alter the folding pathway. The relative inefficiency of uptake of mature proteins might be explained by such a mechanism. Similar models have been suggested by Webster et al. (1979) and Waksman et al. (1980). Models of

Blobel (1980) and Neupert and Schatz (1981) for uptake of mitochondrial protein favour a true "signal" - type role for the extra peptide. In these models, after organelle selection is made it is suggested that transport is achieved by a linear-type extrusion through a proteinaceous pore. There is no concrete evidence however, for any of these models.

No generalizations on the physical characteristics of the peptide extensions of mitochondrial precursors can be made, except for the large variation in size (2000-6000 daltons). The differences are seen among matrix enzymes (e.g. mMDH, 2000 and δ-aminolevulinate synthetase, 6000, Table 1), and among the subunits of oligomeric enzymes (e.g. F_1 ATPase, Table 1), so that the relevance of size to final localization is not clear. The variation among pre-secretory and pre-plasma membrane proteins is not as large (Blobel et al., 1979).

According to Anderson (1981) three mitochondrial precursor proteins have extra peptides that are highly basic. This characteristic is distinctly different from the secretory and plasma membrane proteins examined so far (Blobel et al., 1979; Inouye and Halegoua, 1980) and the precursor to the chloroplast protein ribulose-1,5-biphosphate carboxylase (Highfield and Ellis, 1978). It is interesting to speculate that these differences denote a role in organelle selection, but clearly, more sequence information is required

before any conclusion can be drawn.

Attempts have been made to locate the signal sequence of ovalbumin. This approach used proteolytic fragments to try to compete with nascent chains for membrane binding sites (Lingappa et al., 1979). A similar strategy might be applied for mitochondrial proteins. It might be determined if similar peptides are present in proteins such as the translocator and cytochrome c which are not synthesized as precursors, and mature rat liver matrix enzymes.

It seems that it would be highly optimistic to expect a general mechanism for the import of proteins into mitochondria. This is already obvious in yeast where both co- and post-translational transport have been demonstrated (Ades and Butow, 1980a, b; Maccheccini et al., 1979a). Mature mitochondrial matrix proteins from rat liver exhibit an unusual characteristic in their ability to be taken up into isolated mitochondria, a phenomenon that could not be demonstrated with the mature subunits of yeast F_1 ATPase (Maccheccini et al., 1979a). It would not be surprising if more new and interesting variations are found in the mechanisms of import of mitochondrial protein to further complicate the picture before it is finally clarified.

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