AN INVESTIGATION INTO THE BINDING OF SHEEP HEART PHOSPHOFRUCTOKINASE TO AN INTRACELLULAR COMPONENT.

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ABSTRACT

Phosphofructokinase (E.C.2.7.1.11) is an ambiquitous enzyme; ie it can be located either in a cytosolic or particulate form, depending on the metabolic status of the cell. Two indepedent methods were used to attempt to determine the particulate location of sheep heart PFK.

The first method involved an adaptation of the method of subcellular fractionation of liver, for use with heart tissue. A crude fractionation procedure was first used, in which the PFK was found to be located in a fraction which precipitated at 800g. Specific enzyme assays of acid phosphatase, succinic dehydrogenase and 5'-nucleotidase, were performed to determine the presence of lysosomes, mitochondria and plasma membrane respectively, in the fraction containing PFK. These assays revealed that most of the enzyme activity corresponding to the above organelles, had also precipitated in this fraction. Both mitochondria and myofibrils were purified to determine if PFK copurified with these organelles, but the results obtained were inconclusive.

The second method involved immunochemical localisation of PFK. Antibodies against purified sheep heart PFK were raised in rabbits, and an attempt was made to separate the anti-sheep heart PFK antibodies from the other antibodies by affinity chromatography. Both CDI-activated and cyanogen bromide activated sepharose columns were made and used. Successful separation was not achieved. Western blotting followed by staining with a second antibody labelled with alkaline phosphatase, revealed that the antibody preparation was sufficiently specific to proceed to immunochemical localisation. Sections of fresh sheep heart were embedded at low temperature in Lowicryl K4M resin, then incubated with the anti-sheep heart antibodies followed by secondary antibodies labelled with 15nm gold particles. Electron micrographs of the sections revealed the electron dense gold particles, and hence the location of the PFK.

By comparing the density of gold particles on the myofibrils with that of the background it was shown that the density on myofibrils was 4 to

4.5 times higher than on the background. Thus it was tentatively shown that the myofibrils was the site of localisation of PFK in sheep heart cells.

Investigations into the effect of metabolites on the solubilisation of PFK from the membrane, by including various salts in the extraction buffer, revealed that divalent anions, specifically sulphite, sulphate and thiosulphate ions, were effective in releasing PFK into the cytosol. The effect of fructose-2,6-bisphosphate on the solubilisation and activity of sheep heart PFK was uncertain, but it appeared to inhibit the activity of cytosolic PFK and have no effect on the solubilisation.

The possible cross reactivity of the anti-sheep heart antibodies with other sheep tissues and species was studied via polyacrylamide gel electrophoresis of immunoprecipitates, Micro-Ouchterlony plates and alkaline phosphatase staining of Western Blots. Cross reactivity was observed with sheep muscle and to a less extent, rat heart. Only minor cross reactivity was observed against liver PFK (rat or sheep), though this may be due to low levels of PFK in that tissue.

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LIST OF ABBREVIATIONS

ADP	Adenosine 5′-diphosphate
AMP	Adenosine 5′-monophosphate
ATP	Adenosine 5′-triphosphate
Bisacrylamide	N′N′-methylenebisacrylamide
BSA	Bovine serum albumin
CDI	1′1-carbonyldiimidazole
CMC	1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide-metho-ρ-toluene-sulphonate
CoQ	Coenzyme Q
DCPIP	2,4-dichlorophenolindophenol
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
F-2,6-P_2	Fructose-2,6-bisphosphate
α-GDP	α-glycerophosphate dehydrogenase
KCNS	Potassium thiocyanate
PBS	Phosphate buffered saline
PFK	Phosphofructokinase
Pι	Inorganic Phosphate
SDS	Sodium dodecyl sulphate
TEMED	N′N′N′N′-tetramethylethylenediamine
TPI	Triosephosphate isomerase
Tween 20	Polyoxyethylenesorbitan Monolaurate

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CHAPTER 1 GENERAL INTRODUCTION

Glycolysis has two main roles. The first is the degradation of glucose in order to generate ATP, and the second is to provide carbon skeletons for synthetic reactions. The process of glycolysis needs to be regulated to suit the needs of the cell for these components. Those enzymes which catalyse reactions which are virtually irreversible, tend to play a regulatory as well as catalytic function in biochemical pathways, and in glycolysis these enzymes are hexokinase (E.C.2.7.1.1), phosphofructokinase (E.C.2.7.1.11) and pyruvate kinase (E.C.2.7.1.40).

The most important regulatory enzyme in the glycolytic pathway, is phosphofructokinase (PFK), which catalyses the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate with the concomitant dephosphorylation of ATP to ADP. PFK, rather than hexokinase, is the main glycolytic control enzyme because it regulates the committing step of glycolysis. Glucose-6-phosphate can be used in glycogen synthesis, or oxidised by the pentose phosphate pathway as well as be converted to pyruvate. The conversion of fructose-6-phosphate to fructose-2,6-bisphosphate however, commits the 6 carbon skeleton to glycolytic catabolism.

The Discovery and Structure of PFK

PFK was first identified in 1935 by Dische (Dische, 1935), after it had been shown that the phosphorylation of fructose-6-phosphate to fructose-2,6-bisphosphate was an essential reaction of glycolysis in yeast (Harden and Young, 1908; Young, 1909). PFK was later identified in animal tissues (Ostern *et al*, 1936) and in plant tissue (Axelrod *et al*, 1952).

Catalytically active mammalian PFK is a tetramer of molecular weight 320,000 to 360,000, (Aaronson and Frieden, 1972; Mansour,1972), although polymers of up to $2x10^6$ have been reported (Mansour, 1970). Brennan *et al* (1974) determined a range of molecular weights of sheep heart PFK protomers of 80,000 to 85,000 by SDS gel electrophoresis. Peptide mapping of tryptic peptides was consistent with a 85,000 molecular weight form composed of identical subunits. Preliminary sequencing by Walker *et al* (1976) lead to the conclusion that rabbit muscle PFK was a tetrameric enzyme consisting of four similar (probably identical) subunits with a molecular weight of 85,000, and to the proposal that gene duplication could have occurred in the evolution of eukaryotic PFKs.

Rabbit skeletal muscle PFK and sheep heart PFK undergo association-dissociation reactions to catalytically active and inactive forms (Mansour and Ahlfors, 1968; Aaronson and Frieden, 1972); the active form being the polymer and the inactive form the dimer. Strong activators such as fructose-2,6-bisphosphate and fructose-1,6-bisphosphate stabilise the tetramer, whereas inhibitors such as citrate, depolymerise the active tetramer to inactive dimers. Fructose-6-phosphate modulates the effect of citrate by stabilising a mixture of aggregates of intermediate size and activity (Lad *et al*, 1973).

PFK is more active in those tissues with a high rate of glycolysis such as muscle and brain, rather than gluconeogenic tissues such as liver (Brock, 1969). However, regardless of its source PFK is controlled by the same allosteric effectors (Lowry and Passonneau, 1964). When cellular energy is low (indicated by a low ATP/AMP ratio), or the availability of cellular building blocks is limited (indicated by low citrate levels), then PFK is activated. If the cell is rich in energy and carbon skeletons (high ATP/AMP ratio and high citrate levels), then PFK is inactivated.

ATP is a potent inhibitor of PFK activity below pH7.5 and at low fructose-6-phosphate concentrations. The ATP-inactivated enzyme can be reactivated by cyclic 3',5'-AMP, 5'-AMP, ADP and inorganic phosphate, since these compounds antagonize this inhibition. ATP inhibition and cyclic AMP reactivation are not observed at high fructose-6-phosphate concentrations, or at an alkaline pH. (Mansour, 1963; Mansour, 1965). A review of the strucure and function of PFK is presented by Goldhammer and Dies (1979).

Regulation by pH Induced Dimerisation

Both Trevedi and Danforth (1966) and Ui (1966) independently demonstrated *in vitro*, the suppression of PFK activity as the assay pH was lowered. They suggested that this PFK inhibition was the cause of pH-induced glycolytic inhibition.

Bock and Frieden (1974) demonstrated the reduction of PFK activity in rabbit skeletal muscle as the pH was lowered. They later (Frieden *et al*, 1976a,b, and c) studied the kinetics of inactivation and reactivation as a function of pH and enzyme concentration at constant temperature. From the enzyme concentration dependence they concluded that the mechanism involved at least a protonation step followed by isomerisation to an inactive form, and then dissociation into a species one half the molecular weight. Ionisable groups control the inactivation process and the apparent pK for these groups is temperature dependent so that the enzyme is cold labile below pH 7.0. Reactivation of the inactive enzyme occurs by a kinetically different pathway. The inactive, dissociated form is protonated to a form which may either isomerise to another inactive form, or dimerise to the active enzyme. Frieden *et al*, (1976a,b and c), postulated a mechanism containing four species (two with four subunits and two with two subunits), each of which can be protonated or unprotonated. Inactivation or reactivation is induced by changes in pH or

temperature. They also concluded that substrates and allosteric effectors exert their effect on pH-dependent inactivation by binding preferentially to either the protonated or unprotonated forms, and thus shifting the apparent pK for the process.

Hand and Somero (1983) showed that the acidification which occurs when squirrels enter hibernation, acting synergistically with a fall in body temperature, promotes PFK inhibition. They showed that the loss of activity was due to a freely reversible conversion of the active, tetrameric form of the enzyme to the catalytically inactive, but structurally active, dimeric form of PFK, as proposed by Bock and Frieden (1976a,b and c). When the squirrels return to their euthermic state the tetrameric form is restored. Hand and Somero (1984) in a similar study to the above on thornbacked rays, showed that the particulate form of PFK had a marked reduction in pH induced sensitivity compared with the soluble form. They believed this reduced sensitivity may have been due to an aggregation of monomers. This particulate PFK non-sensitivity was demonstrated only *in vitro* however.

Hand and Carpenter (1986a and b), showed reversible dissociation and inactivation of PFK in ischemic rat hearts, and decided that the enzyme dissociation represented dimerisation of PFK tetramers, rather than dissociation into four protomers. This suggestion was based on several pieces of evidence. The sedimentation value for the inactive form was consistent with that of a two protomer species (80,000-90,000 Mwt each), and the dimer-dimer contact site appeared to be hydrophobic as compared with the protomer-protomer contact which is ionically bonded. Thirdly, both the inactive and active forms were immunoprecipitable, whereas PFK protomers are not. Hand and Carpenter (1986a and b) also showed that certain metabolites, such as fructose-2,6-bisphosphate, stabilized the enzyme against pH-induced dimerisation. Citric acid, on the other hand, enhances the inactivation process.

Regulation of PFK by Phosphorylation-Dephosphorylation

Incorporation studies by Brand and Soling (1975), showed that rat liver PFK was activated by a kinase catalysed phosphorylation, and inactivated by a phosphatase catalysed dephosphorylation. Brand and Soling (1976), also showed that *in vivo*, both active and inactive forms of PFK exist, and that the proportion of the two forms depends on the nutritional state of the animal. The sum of the two forms, however, remains constant. Hofer and Furst (1976), isolated the phosphorylated form of PFK from rabbit skeletal muscle and suggested that a possible role of the phosphate residue may be as an interconversion mechanism.

Tryptic digestion of the phosphorylated PFK, removed a peptide of less than 30 amino acids from the carboxyl terminus. This peptide contained a phosphoserine. It was found that the susceptibility of the terminal region to proteolysis was dependent on the conformational state of the enzyme (Riquelme and Kemp, 1980). Kemp *et al* (1981), found that rabbit skeletal muscle PFK was phosphorylated by the catalytic subunit of a cyclic AMP dependent protein kinase, and that the rate of phosphorylation was increased in the presence of allosteric activators eg AMP, and decreased in the presence of inhibitors eg ATP. The activators and inhibitors alter the V_{max} of phosphorylation but not the K_m. Kemp *et al* (1981) also determined that the exact site of phosphorylation was a serine residue, six amino acid residues from the carboxyl terminus.

The question of whether or not phosphorylation-dephosphorylation of PFK does actually play a regulatory role is debatable. Furuya and Uyeda (1980) suggest that the degree of phosphorylation determines the binding of activators and hence the activity of PFK would be directly controlled by intracellular metabolite levels. Since phosphorylated PFK is more susceptible to proteolysis (Riquelme and Kemp, 1980),

phosphorylation-dephosphorylation may act to limit the levels of activated PFK. Soling and Brand (1981), in their review of the covalent modification of PFK by phosphorylation-dephosphorylation suggest the possibility that PFK phosphorylation is involved in the spatial arrangement of the enzyme within the cell. Luther and Lee (1986) believe that phosphorylated PFK has a higher affinity for F-actin than the dephosphorylated form. They found that upon muscle contraction the enzyme become more phosphorylated and binding to the muscle matrix increased. However, the experimental evidence of Riquelme *et al* (1978), and Kemp and Foe (1983), suggests that phosphorylation has little effect on enzyme activity.

Intracellular Distribution as a Regulatory Mechanism

Until recently, research into enzyme-structure interactions has been limited to those enzymes which are strongly bound or integral components of membranes, and those which are compartmentalised in subcellular organelles. However, over recent years there has been study and discussion on the possibility of cytosolic enzymes interacting with the cell matrix, or being organised into multi-enzyme complexes.

Historically, the study of interactions between cytosolic components and cellular structure can be traced back to the investigations of Green *et al* (1965). Green and his co-workers described membrane fractions derived from erythrocytes and yeast cells, which were capable of catalysing glycolysis and put forward the postulate that all metabolic sequences may be membrane bound.

John E. Wilson coined the term 'ambiquitous' to describe those enzymes 'whose distribution between soluble and particulate forms may vary with the metabolic status of the cell as reflected in the levels of certain metabolites capable of influencing that distribution' (Wilson, 1978).

Brain hexokinase is the prototype ambiquitous enzyme (Wilson, 1980). Association of hexokinase with brain mitochondria was first reported many years ago by Crane and Sols (1953) and Johnson (1960). They found that about 80% of the total hexokinase activity in brain homogenates was particulate. Rose and Warms (1967) found that the mitochondria-bound enzyme could be solubilised by the addition of glucose-6-phosphate or ATP. Wilson (1968) reported that a change in soluble-particulate distribution was a factor in the regulation of hexokinase activity *in vivo*. Brain hexokinase has been shown to definitely exhibit ambiquitous behaviour *in vivo*, by Knull *et al* (1973, 1974) in their study of chick and mouse brains. Their data indicated that an increased glycolytic rate due to ischaemia or insulin treatment resulted in a rapid and reversible shift to increased proportions of the particulate enzyme. Felgner *et al* (1979) have isolated a protein from the outer mitochondrial membrane, which they believe is the binding site for hexokinase.

Aldolase is another ambiquitous enzyme which has been well studied. Arnold and Pette (1968) first noted that aldolase bound to the particulate fraction in rabbit muscle. In rat brain it was found that one isoenzyme of aldolase preferentially bound to the microsomes, whereas another isoenzyme was located primarily in the cytosol (Clarke *et al*, 1970). Since then aldolase has been shown to bind to F-actin and troponin-tropomyosin complexes in bovine and rabbit muscle (Arnold and Pette, 1970; Arnold *et al*, 1971; Clarke *et al*, 1974; Morton *et al*, 1977). Walsh *et al* (1977) showed that the binding of aldolase to F-actin-tropomyosin-troponin filaments produced major alterations in the kinetic parameters of aldolase. Aldolase has also been shown to bind to other cell components depending on the tissue being studied. Using immunohistochemical techniques, Foemmel *et al* (1975) demonstrated the association of aldolase with endoplasmic reticulum, and Strapazon and Steck (1976, 1977) have shown that rabbit muscle aldolase binds to Band 3, the predominant polypeptide of the human erythrocyte membrane.

Aldolase can be specifically released from the membrane by relatively low levels of its substrate, fructose-1,6-bisphosphate, but not by any other common metabolite (Arnold and Pette, 1970; Strapazon and Steck, 1977).

In 1974, Craven and Basford found that PFK bound to purified rat brain mitochondria in the presence of ADP, and to a certain extent in the presence of ATP. Inorganic phosphate had a slight effect on the solubilisation of PFK, whereas fructose-6-phosphate had no effect.

Later Karadsheh and Uyeda (1977) noted a specific interaction between PFK and the inner surface of erythrocyte membrane, and that this binding to the membrane was associated with a change in the allosteric properties of the enzyme. The membrane bound enzyme was apparently not inhibited by ATP or 2,3-diphosphoglycerate, and its fructose-6-phosphate curve reverted to a non-sigmoidal shape. It seemed that the interaction had reduced the conformational flexibility of the enzyme. Their results implied that the regulatory role of PFK may be influenced by adsorption to cellular structure. Jenkins *et al* (1985), showed that the polyacidic amino-terminal sequence of Band 3 of erythrocyte membranes, bound electrostatically to the polybasic adenine nucleotide-activation site of PFK. ADP and NADH displaced PFK from the membrane by binding to this adenine nucleotide activation site. From their results, it was concluded that Band 3 protein binds PFK dimers with a higher affinity than the tetramers, thus shifting the cytosolic tetramer-dimer equilibrium towards the inactive dimer, and so resulting in a decrease in PFK activity.

By applying subcellular fractionation techniques to sheep heart homogenates, Choate *et al* (1985), showed that PFK appeared to be associated with myofibrils. Their results also indicated that PFK binding to the particulate fraction was enhanced when the pH was lowered, and solubilised by increased pH or ATP concentrations. Choate *et al* suggested that the shifting of the equilibrium of PFK

from soluble to particulate may be a mechanism to regulate PFK activity. Binding to the particulate fraction during acidosis may prevent irreversible inactivation of the enzyme, and ensure continued production of fructose-1,6-bisphosphate.

The main aim of this thesis is to determine the particulate location of PFK by two independent means. The first method involved adapting the subcellular fractionation techniques used for liver tissue (Fleischer and Kervina, 1974), for use with sheep heart. Once the range of possible locations for PFK have been narrowed by crude cell fractionation, the location could be pinpointed by more refined fractionation methods. Procedures for heart fractionation are not as well developed as those for liver, but sheep heart was chosen for study, since large supplies of highly purified sheep heart PFK could be readily obtained. Having a supply of pure PFK was essential for the second part of the study, in which antibodies specific against sheep heart PFK are raised, for use in determining the location of PFK by Immunogold Localisation and Electron Microscopy (Robertson *et al*, 1985).

CHAPTER 2 SUBCELLULAR FRACTIONATION

2-1 INTRODUCTION

This section describes the attempts made to determine the location of PFK within the sheep heart cell by subcellular fractionation. Very little work has been done previously on the fractionation of heart cells. The methods were developed from those used for the fractionation of liver tissue, which has been extensively studied by others (Fleischer and Kervina, 1974).

Crude cell fractionation was first performed on the sheep heart tissue to narrow the range of possible subcellular locations for the PFK. More refined purification procedures were then carried out on the possible candidates for PFK attachment. PFK was assayed in all relevant fractions to determine if it copurified with the organelle under investigation. Consequently, the organelle could either be eliminated or subjected to further study.

According to published reports there are two likely locations for PFK, and these were studied by refined purification techniques and enzyme assays. One possible location was the mitochondria. Hexokinase has been conclusively shown to attach to the mitochondria; Wilson (1980), Masters (1981), Bustamante *et al* (1981), and Casio and Bustamante (1984). Craven and Basford (1974) have stated that PFK can be induced to bind to brain mitochondrial membrane in the presence of ADP.

Another likely location for PFK was the myofibril (Choate *et al*, 1985). However, Choate *et al*, seem to base their conclusion on the assumption that the 9000g precipitate consists entirely of myofibrils. Probable contamination of the pellet with nuclei etc. has not been

investigated. Luther and Lee (1985), suggest that phosphorylated PFK has a high affinity for F-actin, a component of myofibrils, and Starr and Offer (1982) discuss a method for the purification of PFK, based on the possibility that PFK is attached to myosin.

2-2 MATERIALS

Sheep tissue was obtained from Waitaki Freezing Company, Feilding. All general chemicals were reagent grade and obtained from May and Baker Ltd, Dagenham, England, Serva, New York, British Drug Houses or Ajax Chemicals, Sydney. Biochemicals were obtained from Sigma Chemical Company, St. Louis, U.S.A. All water was deionised and distilled.

2-3 METHODS

2-3.01 CRUDE CELL FRACTIONATION

A procedure was developed which was based upon methods for rat liver subcellular fractionation described by Fleischer and Kervina (1974).

All steps were carried out at 0 - 4^{o}. 20 grams of sheep heart tissue was chopped finely into 60ml of homogenisation buffer (0.25M sucrose, 10mM tris-HCl, pH 7.5), and passed through a tissue press. It was then homogenised in a Potter-Elvehjem homogeniser using half-maximal speed and a plunger with a clearance of 0.4mm. The suspension was centrifuged at 800g for ten minutes. The precipitate was saved as Fraction 1, and the supernatant recentrifuged at 35,000g for ten minutes. The second precipitate was saved as Fraction 2. The supernatant was ultracentrifuged at 100,000g for one hour, and the

precipitate saved as Fraction 3, and the final supernatant as Fraction 4. (see figure 2.01).

Prior to assay for PFK activity each precipitate was mixed with 10ml partial extraction buffer (20mM Tris-HCl, 50mM $MgSO_4$, 0.5mM EDTA, pH 8.6), and 5 ml of the final supernatant was diluted by a half with partial extract buffer containing 100mM $MgSO_4$, to give 50mM. These fractions were homogenised in a hand held glass homogeniser (0.4mm clearance), heated to 40^{o} for five minutes and then centrifuged at 12,000g for ten minutes. The supernatants were assayed for PFK activity according to the assay described in 3-3.02.

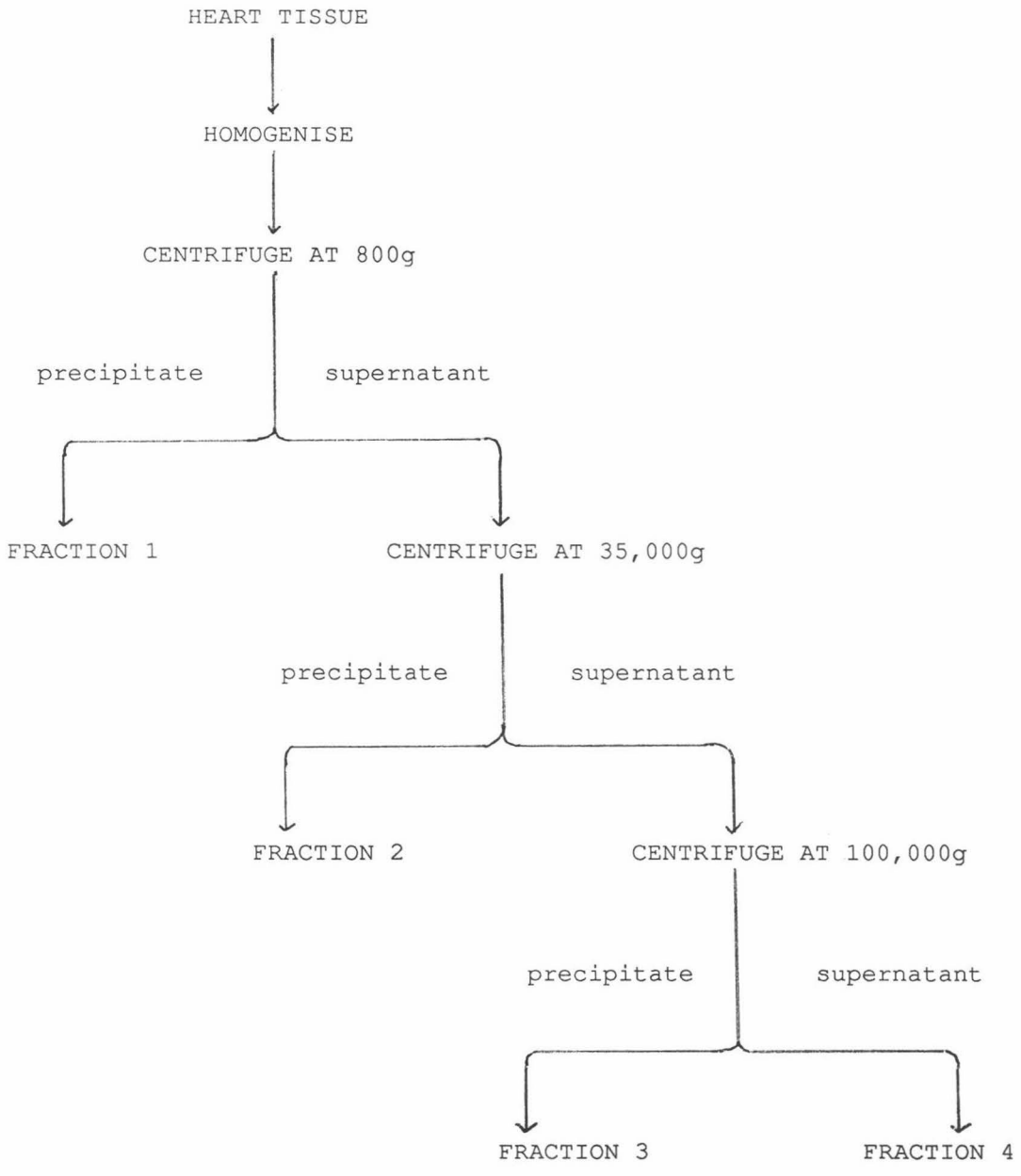
Crude cell fractionation was also attempted using minor modifications to the above technique. In one case, the fractions containing the partial extract buffer, were homogenised in a glass homogeniser with 0.8mm clearance (method B), and in another, all homogenisation was done using an UltraTurrax (method C). Both modifications were tried in order to decrease the amount of unbroken cells left after homogenisation.

Each fraction was also assayed for marker enzymes in order to determine the purity of the fractions for particular organelles.

Acid Phosphatase assay (Trouet, 1974):
Acid phosphatase is specific for lysosomes, which according to Fleischer and Kervina, should be present predominantly in Fraction 2. The assay relies on the conversion of β-glycerophosphate to inorganic phosphate and glycerol by acid phosphatase in the sample. The inorganic phosphate released is measured colorimetrically. Each precipitated fraction was resuspended in a minimum amount of partial extract buffer (20mM Tris-HCl, 50mM $MgSO_4$, 0.5mM EDTA, pH 8.6), and homogenised in a glass homogeniser. Fraction 4 was diluted with an equal volume of buffer. A 1ml aliquot of each fraction was added to the assay medium (0.1ml 1M acetate buffer, pH 5.0, 0.1ml 2% Triton-X-

Figure 2.01

THE METHOD OF CRUDE CELL FRACTIONATION



100, 0.6ml water), and the reaction was started by the addition of 0.2ml 0.5M β-glycerophosphate. The reaction mix was incubated at 37° for 30 minutes. 10mls of 8% TCA was added to stop the reaction and the fractions were filtered through Whatman No42 paper to remove denatured proteins. An aliquot of the filtrates was assayed for inorganic phosphate concentration (1ml). A blank was prepared by adding β-glycerophosphate to the approprate fraction after the TCA.

Inorganic Phosphate Determination (Fiske and Subbarow, 1925):
One ml of sample or standard (0 - 10 mg Pi), was mixed with 1ml 5N H_2SO_4, 1ml 2.5% ammonium molybdate, 0.4ml Fiske-Subbarow reducing solution (0.19% 1-amino-2-naphthol-4-sulphonic acid, 1.15% anhydrous sodium bisulphite, 1.15% anhydrous sodium sulphite), and 6.6ml of water. After 10 minutes the absorption was read at 660nm, and the phosphate concentrations determined from the standard curve.

5′ Nucleotidase Assay (Segal and Brenner, 1960):
This assay was used to determine the distribution of plasma membrane in the fractions. The plasma membrane was expected to occur in the first fraction. Enzyme samples were prepared as for the acid phosphatase assay, and 0.1ml of each fraction was incubated at 30° for 10 minutes with 150μM Tris-HCl, pH 7.5, 20μM $MgCl_2$, 2μM 5′AMP, in a total of 2mls. The reaction was stopped with the addition of 2.0ml 10% TCA. Denatured proteins were removed by centrifugation for 20 minutes in an International bench centrifuge. Inorganic phosphate assays were done on 1ml samples of the supernatant (see method above).

Succinic Dehydrogenase Assay (Bonner, 1955):
This enzyme occurs on the inner mitochondrial membrane, and thus indicated the presence of mitochondria (intact or broken), in the fractions. Fraction 2 was expected to contain the highest levels. Fractions were prepared as for the acid phosphatase assay, and 0.1ml of each fraction was mixed with 0.1ml 1M potassium phosphate buffer, pH 7.0, 0.04ml 0.1% DCPIP, 0.02ml 0.04% CoQ (dissolved in 95%

ethanol), and 0.69ml of water, to give a total of 0.95ml. This reaction mix was placed in a 1ml cuvette and incubated at 37^{o} in a Cecil recording spectrophotometer. The absorbance was monitored at 600nm and when a steady trace was obtained 0.05ml of 0.5M sodium succinate was added to begin the reaction. The decrease in absorbance per minute was calculated for each fraction.

2-3.02 THE SEDIMENTATION PATTERN OF SHEEP HEART MITOCHONDRIA

Sheep heart was chopped finely into homogenisation buffer (0.25M sucrose, 10mM Tris-HCl, pH 7.5), (100g tissue per 300ml buffer), and then passed through a tissue press. It was then homogenised with an UltraTurrax homogeniser, and separated into 20ml volumes. Individual aliquots were centrifuged at one of the following; 30, 121, 270, 480, 755, 1085, 1475, and 1935g for ten minutes. The precipitate was resuspended in a small volume of buffer, and both the resuspended precipitate and supernatant were assayed for O_2 uptake to determine the presence of mitochondria (see 2-3.03). Both fresh and frozen sheep heart was used.

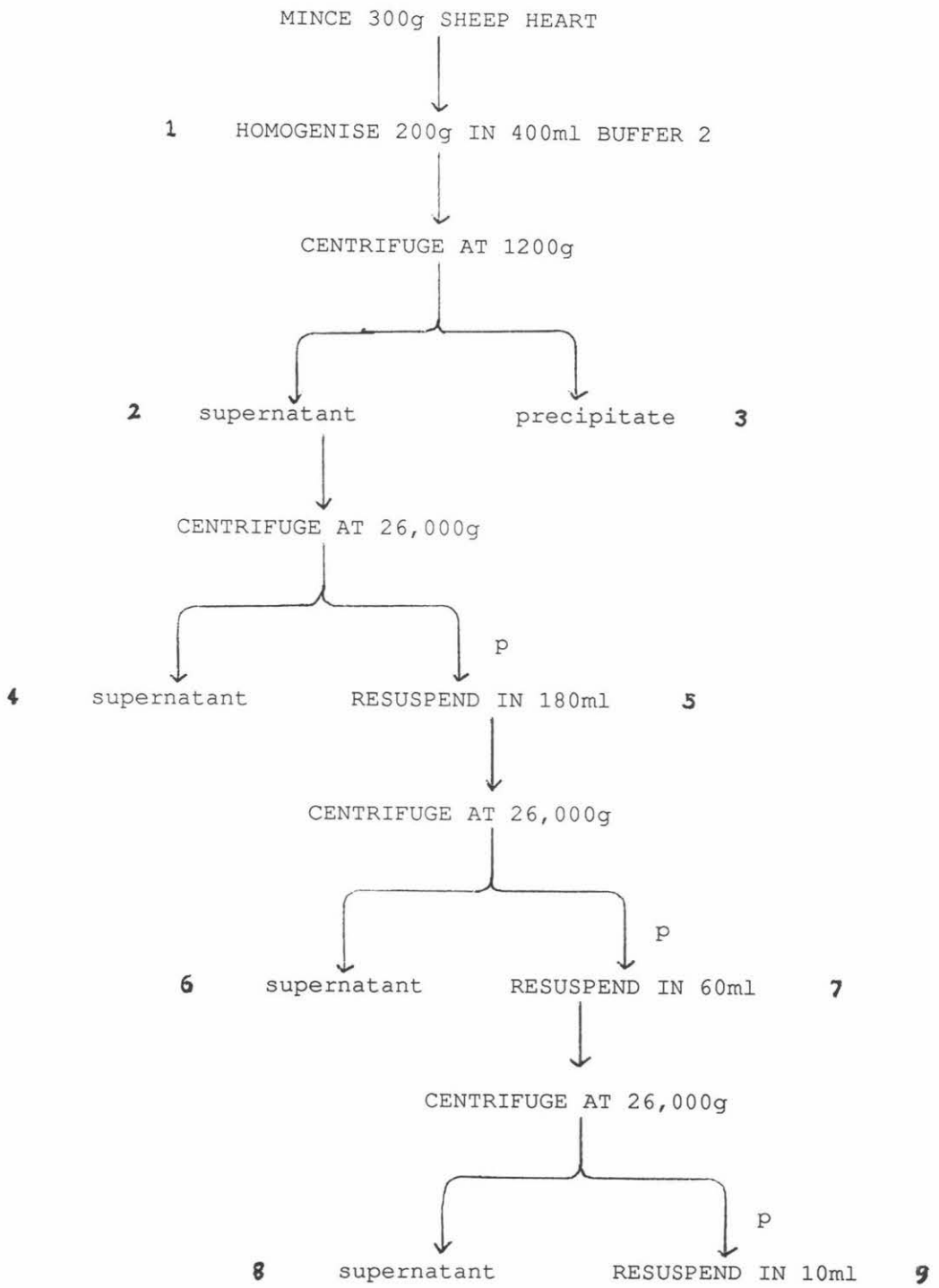
2-3.03 THE PREPARATION OF SHEEP HEART MITOCHONDRIA

The preparation of sheep heart mitochondria was based on methods for the small scale preparation of mitochondria from slaughterhouse material by Smith (1967). (see figure 2.02).

Freshly obtained sheep hearts were trimmed of fat and connective tissue, and small pieces put through a meat grinder. The mince was suspended in buffer 1 (0.25M sucrose, 0.01M Tris-HCl, pH 7.8), (300g per 400ml buffer), and the pH quickly adjusted to 7.5 with 6M KOH. The neutralised mince was then placed in a double layer of cheesecloth and squeezed free of buffer 1. The mince was then suspended in buffer 2 (0.25M sucrose, 0.01M Tris-HCl, 0.2mM EDTA, pH 7.8), (200g per 400ml buffer), and homogenised briefly in a glass homogeniser (0.4mm

Figure 2.02

THE METHOD OF MITOCHONDRIAL PREPARATION



clearance). The pH was readjusted to 7.8 and the homogenate centrifuged at 1200g for 20 minutes. The supernatant was filtered through cheesecloth to remove fat and the pH adjusted to 7.8 if necessary. The suspension was centrifuged for a further 15 minutes at 26,000g. The resulting pellet consisted of three layers: a light layer consisting of broken mitochondria, which was removed by swirling with a few mls of supernatant and decanting; a dark layer of intact mitochondria; and a brown pellet. The dark layer was resuspended in 10mls of buffer 2 , leaving behind as much of the brown pellet as possible. This mitochondrial suspension was homogenised in a glass homogeniser (0.2mm clearance), and the volume adjusted to 180mls before centrifugation at 26,000g for 15 minutes. The dark pellet was collected as before, and the volume adjusted to 60ml. The suspension was centrifuged again and the pellet finally resuspended in 10 mls of buffer 2.

At most stages, samples were taken for assays either for PFK (see 3-3.02), or O_2 uptake.

Oxygen Uptake by Mitochondrial Fractions:

Oxygen uptake was monitored with a Sekonic Strip Chart Recorder using a Yellow Springs Instrument Model 53 Biological Oxygen Monitor System. The apparatus was assembled and calibrated according to the relevant instruction booklet. The salt solution and distilled water were equilibrated by bubbling with air while being held at 30°. The assay medium consisted of 2ml of sample (either supernatants or precipitates resuspended in an amount of buffer), mixed with 0.6ml salt solution (45mM KH_2PO_4, 60mM Tris-HCl, 5.5mM $MgCl_2$, 2.5mM EDTA, 55mM KCl, pH 7.4), and 1.0ml water in a glass chamber maintained at 30°. When a steady trace was observed, 0.2ml of 0.1M sodium succinate was added and the oxygen uptake monitored. The results were expressed as µmoles O_2 utilised per minute by the total fraction, and also as a percentage of the total (ie homogenate).

2-3.04 THE PREPARATION OF SHEEP HEART MYOFIBRILS

Sheep heart myofibrils were prepared according to Knight and Trinick (1982). All steps were carried out at 4^{o}. Sheep heart was minced and 100g was blended at high speed for 30 seconds in 500ml of buffer (0.1M KCl, 5mM EDTA, 39mM boric acid, pH 7.1). The homogenate was centrifuged at 2000g for 5 minutes. The precipitate was resuspended in 1l of buffer, and recentrifuged. The precipitate was resuspended and centrifuged a further 3 or 4 times, and the final precipitate was resuspended in 270ml of buffer. If the myofibril preparation was not used immediately, it was stored in 50% glycerol at -20^{o}. Samples of both supernatants and resuspended precipitates were saved at various stages in the preparation for subsequent assaying. (see figure 2.03).

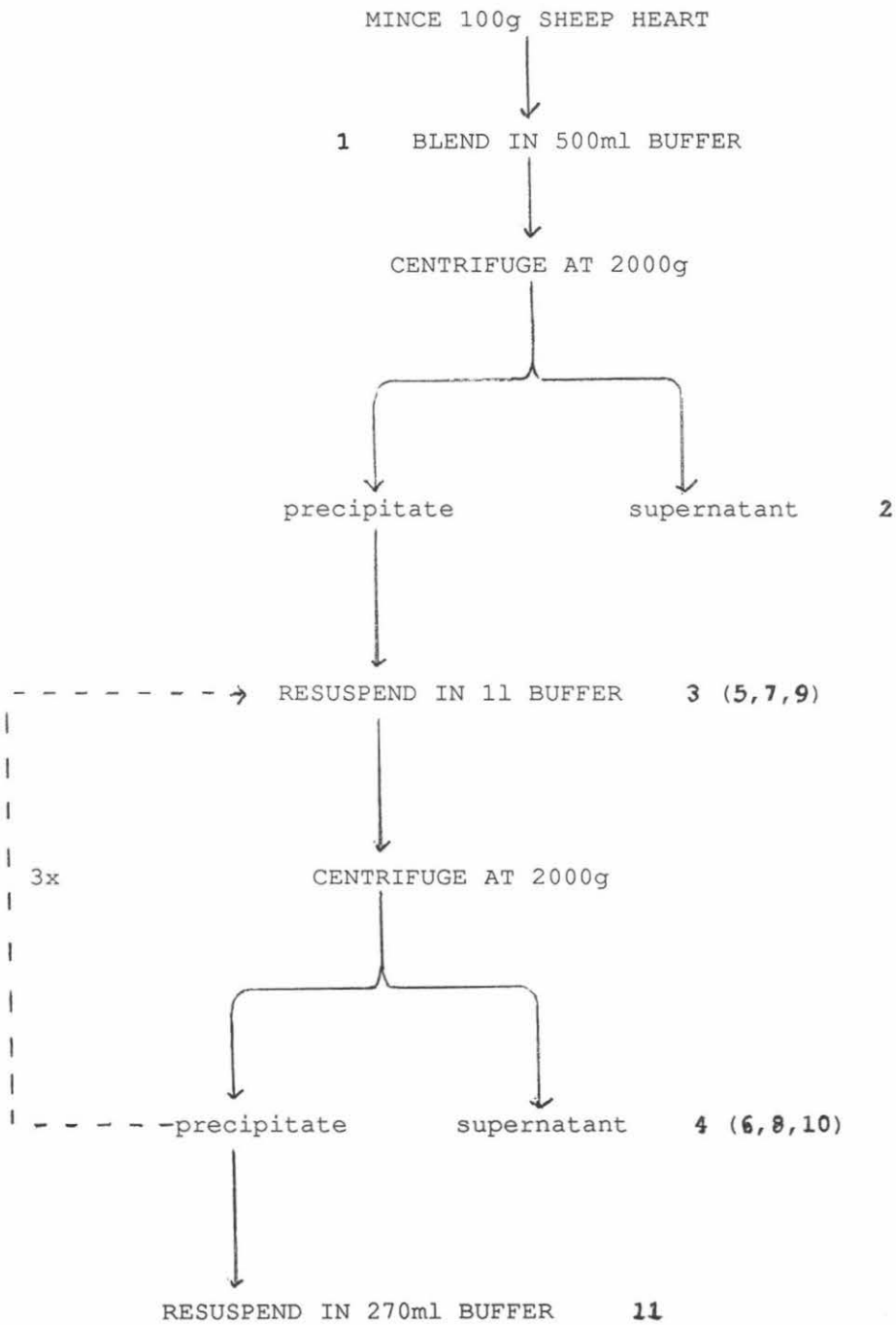
In some preparations either 5mM 2-mercaptoethanol or 1mM DTT was included in the buffer, in order to maintain the activity of PFK during the preparation.

Samples (supernatants or resuspended precipitates) were diluted with an equal volume of 5ml wash buffer (10mM Tris-HCl, 2mM EDTA, pH 8.0), homogenised in an UltraTurrax, and centrifuged at 12,000g for 10 minutes. The precipitates were resuspended in 1ml of partial extract buffer (20mM Tris-HCl, 50mM $MgSO_4$, 0.5mM EDTA, pH 8.6), heated to 40^{o} for five minutes and recentrifuged at 12,000 g for ten minutes. The supernatants were assayed for PFK activity (3-3.02).

Samples of the original homogenate and the final precipitate were observed under a light microscope (magnification 250x), for the presence of myofibrils and contaminating organelles (especially nuclei). A small sample was placed on a slide, diluted with water to give a thin smear and air dryed. The slides were either observed using phase contrast, or stained with Leishman Stain.

Figure 2.03

THE METHOD OF MYOFIBRIL PREPARATION



2-3.05 THE USE OF A SUCROSE GRADIENT TO ELIMINATE NUCLEI FROM THE MYOFIBRIL PREPARATION

According to Tata (1974), a rat heart homogenate ultracentrifuged on a 2.4M sucrose gradient, should result in a pellet of nuclei free from contamination with cytoplasmic particles, cell debri and erythrocytes, since nuclei are the only organelle with sufficient density to penetrate the 2.4M sucrose. A small portion (about 1g) of the initial homogenate and the final precipitate were resuspended in both 2.4M sucrose, and 2.4M sucrose plus 3mM $MgCl_2$. The samples were centrifuged at 50,000g for 45 minutes. After centrifugation, slides were prepared of the precipitate (if present), and the layer which accumulated at the top of the sucrose.

2-4 RESULTS AND DISCUSSION

2-4.01 CRUDE CELL FRACTIONATION

PFK Assays:

Table 2.01. As can be seen from the table, about 78 - 79% of the PFK activity was found in the first fraction. When fractionating liver tissue, this fraction is expected to contain plasma membrane, nuclei and cell debris. As method B did not give reproducible results it was discontinued.

Acid Phosphatase Assays:

Table 2.02. 80 - 84% of the acid phosphatase was found in fraction 4, which should contain only the cytosolic components. The presence of this enzyme in fraction 4, was most probably due to the disruption of the lysosome membrane with the consequent release of the acid phosphatase. For this reason it was ignored in relation to the distribution of the lysosomes. It was apparent that the majority of

Table 2.01

THE DISTRIBUTION OF PFK IN CRUDE CELL FRACTIONS

	% TOTAL PFK ACTIVITY IN EACH FRACTION		
FRACTION	METHOD A	METHOD B	METHOD C
1	79	62	78
2	14	21	17
3	5	10	5
4	2	7	0

Method A: Homogenisation using a glass homogeniser with 0.4mm clearance only

Method B: Homogenisation using a glass homogeniser with 0.4mm clearance followed with one with 0.8mm clearance.

Method C: Homogenisation using an UltraTurrax.

Table 2.02

THE DISTRIBUTION OF ACID PHOSPHATASE IN CRUDE CELL FRACTIONS

	% TOTAL ACID PHOSPHATASE IN EACH FRACTION	
FRACTION	METHOD A	METHOD C
1	13	18
2	1.4	0.4
3	1.1	2.1
4	84	80

Method A: Homogenisation using a glass homogeniser with 0.4mm clearance only

Method C: Homogenisation using an UltraTurrax.

the unbroken lysosomes were located in fraction 1. When liver is fractionated by this procedure, the lysosomes are generally found in fraction 2 (Fleischer and Kervina, 1974).

5′ Nucleotidase:

No activity of this enzyme could be demonstrated in any fraction. Although Kidwa (1974), in his description of the purification of smooth, skeletal and heart muscle membrane, states that 5′-nucleotidase activity should be concentrated in the plasma membrane fraction, this may not neccessarily be the case with cardiac tissue. Kidwa also states that the preservation of plasma membrane enzymic activity requires fast and mild fractionation procedures; conditions which may not have met with the crude cell fractionation procedure used. If further experimentation was required to determine the distribution of plasma membrane, other marker enzymes, such as alkaline phosphodiesterase I (Aronson and Towster, 1974), may prove more useful.

Succinic Dehydrogenase:

Table 2.03. There was a large proportion of this enzyme activity in the cytosolic fraction (71-77%). Succinic dehydrogenase is easily removed from the inner mitochondrial membrane by mechanical forces, such as homogenisation. By ignoring the 'mitochondria-free' enzyme, it can be seen from the table that the majority of the unbroken mitochondria were present in fraction 1. During liver cell fractionation the mitochondria are expected to precipitate in fraction 2.

As seen in Figure 2.04, the PFK is predominantly found in Fraction 1. It appears that unlike liver fractionation the majority of cell organelles (ie mitochondria, lysosomes, nuclei, plasma membrane, cytoskeleton) are also precipitating in Fraction 1. This is probably due to two major reasons.

Table 2.03

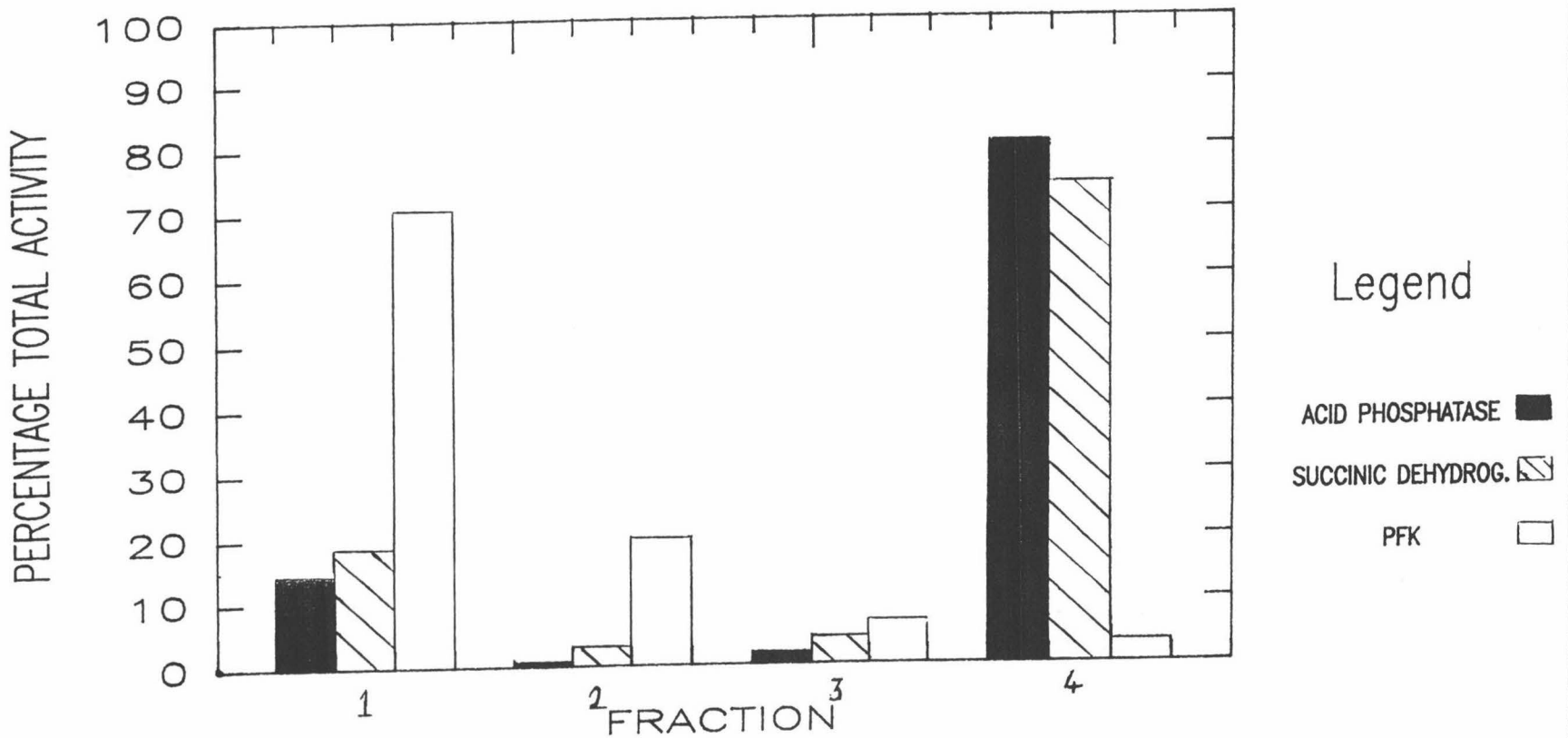
THE DISTRIBUTION OF SUCCINIC DEHYDROGENASE IN CRUDE CELL FRACTIONS

	% TOTAL SUCCINIC DEHYDRO - GENASE IN EACH FRACTION	
FRACTION	METHOD A	METHOD C
1	18	20
2	4	3
3	7	0.5
4	71	77

Method A: Homogenisation using a glass homogeniser with 0.4mm clearance only

Method C: Homogenisation using an UltraTurrax.

Figure 2.04
DISTRIBUTION OF MARKER ENZYMES
IN CRUDE CELL FRACTIONS



This first reason relates to the fact that efficient fractionation requires a balance between sufficient mechanical shear to separate the organelles, which have partially contiguous membranes (Palade, 1964), and keeping the force to a minimum so as not to destroy the integrity of the organelles. Cardiac tissue contains an extensive endomysium (the capillary-rich connective tissue which surrounds individual muscle cells), as well as the usual connective tissue which surrounds striated muscle (perimysium and epimysium). This high degree of cardiac connective tissue requires the application of greater mechanical force to disrupt the cells. This greater force probably destroys the integrity of the cell organelles, and consequently alters their sedimentation properties.

The second reason is due to the different size and shape of cardiac organelles compared with those of the liver. The liver organelles provide a wider range of sedimentation rates, thus aiding the efficiency of separation. On the other hand, most heart organelles (nuclei, lysosomes, mitochondria and plasma membrane) have similar sedimentation rates, even when intact and complete, adding to the problem of efficient separation.

The results obtained suggest that the sarcoplasmic reticulum can be eliminated as a site of binding for PFK with a high degree of certainty. Sarcoplasmic reticulum upon fractionation, is generally disrupted to form vesicles or microsomes, which can be precipitated only with ultracentrifugation (Meissner, 1974). These microsomes are found in Fraction 3. Activity in Fraction 3 of the three enzymes being studied is probably due to the presence of membranes from mitochondria, lysosomes or other organelles, present in the microsome fraction.

2-4.02 THE SEDIMENTATION PATTERN OF SHEEP HEART MITOCHONDRIA

A force of 800g is sufficient to separate liver mitochondria from those denser organelles expected to precipitate (eg nuclei). However, it is obvious from the graphs (Figures 2.05 and 2.06), that 800g is totally inadequate for the fractionation of heart muscle. The use of differential centrifugation to fractionate heart mitochondria from nuclei and plasmalemma cannot be used as the mitochondria precipitate at very low centrifugal forces. This is probably due to larger size and more densely packed cristae of heart mitochondria (Leeson and Leeson, 1981), compared with those of other organs.

2-4.03 THE PREPARATION OF SHEEP HEART MITOCHONDRIA

Table 2.04. This method of purifying mitochondria results in a small (0.03% of original amount) amount of pure mitochondria. Only about 5% of the mitochondria remain in the 1st supernatant for further purification. It appears from this result that PFK is copurifying with the mitochondria. However, these results were not conclusive as the majority of PFK was precipitated during the first centrifugation, and the PFK present in the mitochondrial fractions may only be present as a contaminant. Also, a large proportion of the PFK activity was lost with each centrifugation. For a more conclusive result, a variety of mitochondrial purification procedures should be performed, each with PFK assays carried out on relevant fractions.

2-4.04 THE PREPARATION OF SHEEP HEART MYOFIBRILS

Table 2.05, demonstrates clearly that the PFK is precipitating with the myofibril fraction. Only 2-3% of the PFK was present in the first supernatant. A large proportion of the activity of the PFK was lost with each centrifugation. The addition of 5mM 2-mercaptoethanol or 1mM DTT helped to stabilise the enzyme for the first few centrifugations, but activity was rapidly lost thereafter. Large

Figure 2.05
THE SEDIMENTATION PATTERN OF
MITOCHONDRIA IN FRESH HEART

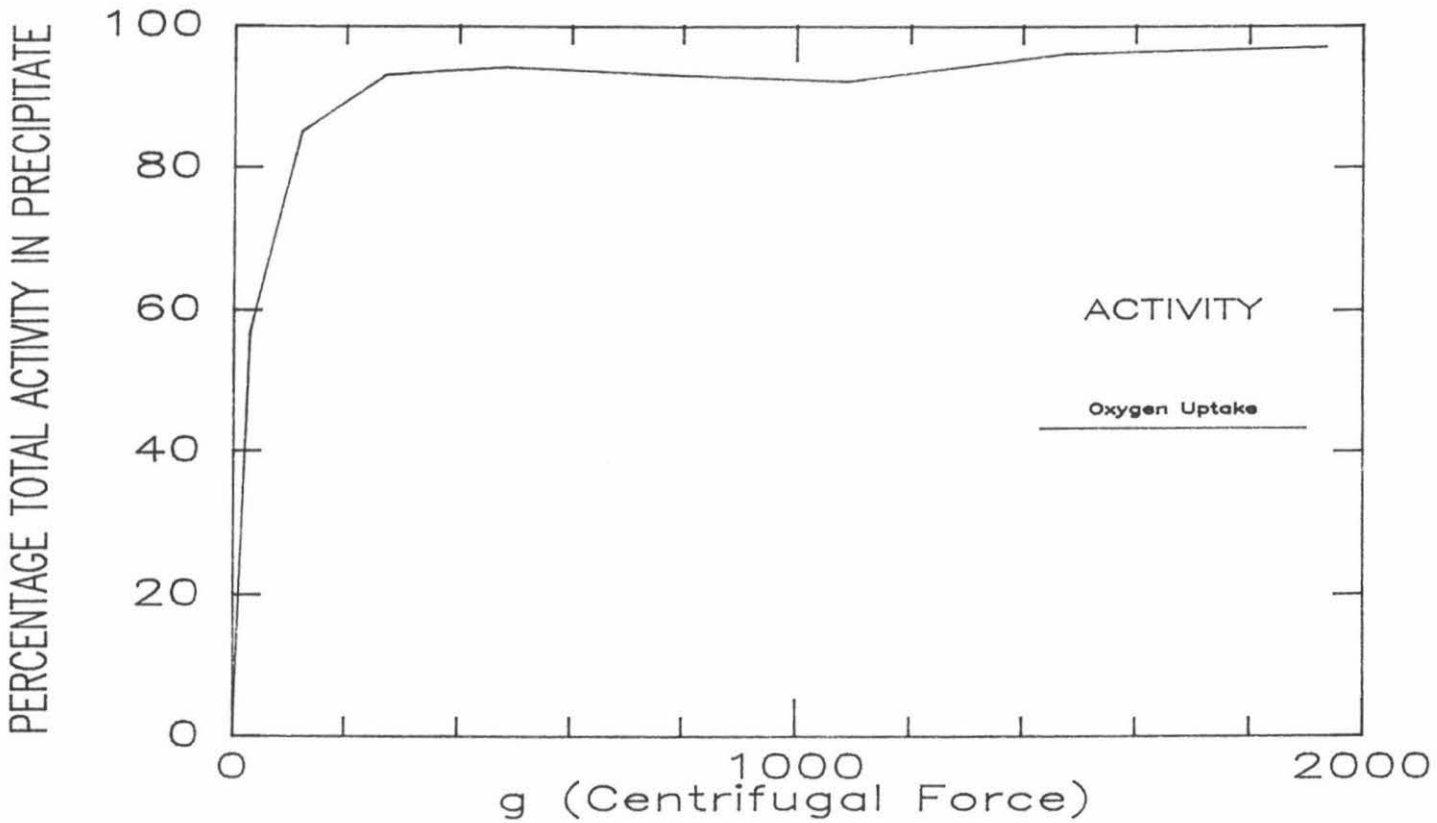


Figure 2.06
THE SEDIMENTATION PATTERN OF
MITOCHONDRIA IN FROZEN HEART

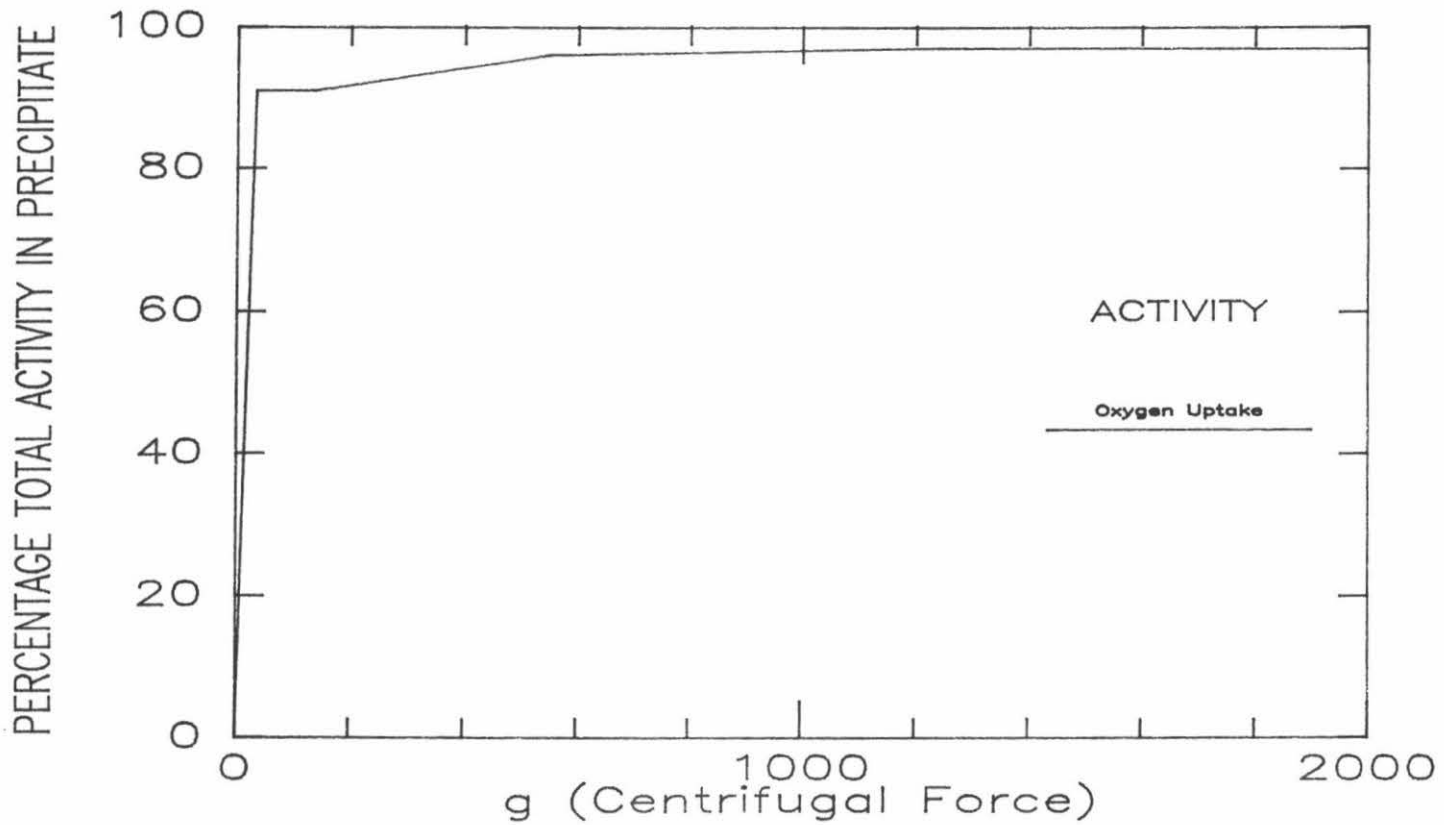


Table 2.04

THE % PFK ACTIVITY IN, AND THE % O_2 UPTAKE BY MITOCHONDRIAL FRACTIONS

FRACTION	% PFK ACTIVITY	% O_2 UPTAKE
1	100	100
2	31	5
3	70	82
4	25	4
5	6	1
6	0.6	0.7
7	0.3	0.3
8	0.2	0.16
9	0.05	0.03

Table 2.05

THE DISTRIBUTION OF PFK IN MYOFIBRIL FRACTIONS

	% TOTAL PFK ACTIVITY IN EACH FRACTION		
FRACTION	NO ME* OR DTT	5mM ME*	1mM DTT
1	100	100	100
2	2	2	3
3	65	100	100
4	3	0	6
5	47	3	100
6	0	0	6
7	25	1	6
8	0	0	0
9	10	0	2
10	0	0	0
11	0	0	0

* 2-mercaptoethanol

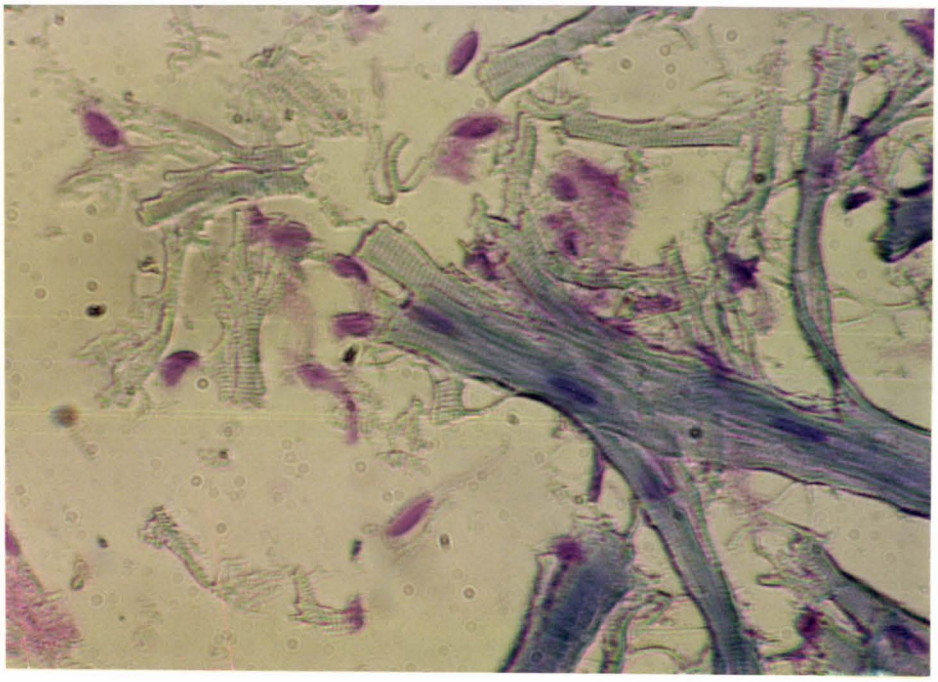
volumes of buffer (1l), were used at each stage to resuspend the precipitate, and the PFK may have become too dilute to retain its activity. Wenzel *et al* (1976), state that, in the case of erythrocyte PFK, a decrease in specific enzyme activity occurs with decreasing enzyme concentration. It appears that a decreasing enzyme concentration causes a shift in association equilibrium towards the inactive dimeric form of the enzyme. Also each wash of the precipitate will be washing away stabilising cofactors, such as fructose-6-phosphate, which displace the equilibrium in favour of the active polymeric forms, (Wenzel *et al*, 1976). Luther *et al* (1985), in their study of the subunit interactions of rabbit muscle PFK, suggest that the addition of fructose-6-phosphate to a solution containing a low PFK concentration, should result in a significant perturbation of the monomer-tetramer equilibrium. Thus, the addition of 1-10mM fructose-6-phosphate (depending on the amount of endogenous ATP) to the myofibril extraction buffer, should help retain the activity of the enzyme during myofibril extraction. It is also possible that the shift from polymer to dimer, caused by the removal of stabilising factors, could result in dissociation from the membrane.

According to the results of the crude cell fractionation, a centrifugal force of 800g was sufficient to precipitate the majority of cell organelles from the sheep heart. The myofibril preparation involved centrifugal forces of 1200g, and thus the precipitates would be expected to be heavily contaminated with organelles other than myofibrils. According to Knight and Trinick (1982), the successive washes should have removed the majority of nuclei and other organelles. Photomicrographs (250x) were taken after staining with Leishman stain, of the initial homogenate and the final precipitate (Figure 2.07). This enabled an estimate to be made of the contamination of the myofibrillar preparation by other organelles. Both photomicrographs show clearly the crossbanded myofibrils. The purple, elongated nuclei are also clearly evident on both slides. However, the homogenate shows a greater number of separated nuclei,

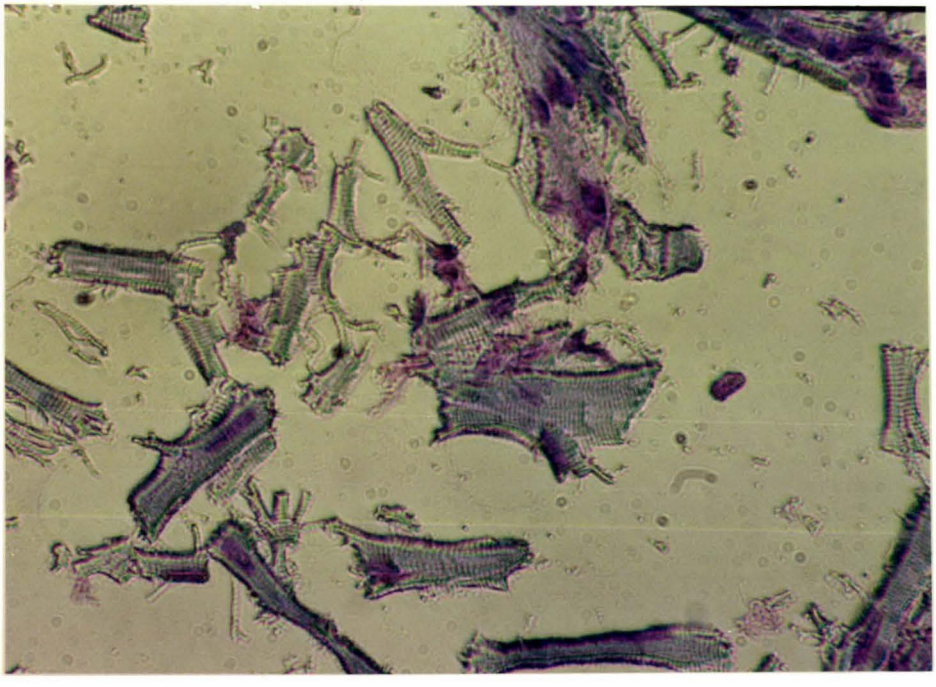
Figure 2.07

PHOTOMICROGRAPHS (250x) OF THE INITIAL HOMOGENATE AND FINAL PRECIPITATE FROM A MYOFIBRIL PREPARATION

INITIAL HOMOGENATE (Leishmans Stain)



FINAL PRECIPITATE (Leishmans Stain)



compared with the final precipitate of myofibrils in which they are mostly present associated with the myofibrils. The other organelles are difficult to visualise.

2-4.05 THE USE OF A SUCROSE GRADIENT TO ELIMINATE NUCLEI FROM THE MYOFIBRIL PREPARATION

It is obvious that there is still a large degree of contamination of the myofibrillar preparation with nuclei. Thus the PFK could be attached to the myofibrils or nuclei (and possibly to the mitochondria or lysosomes which may also be contaminating the preparation). An attempt was made to remove nuclei from the myofibrillar preparation by centrifuging the final precipitate through a 2.4M sucrose gradient. According to Widnell *et al* (1967), the nuclei should be the only organelle dense enough to precipitate through sucrose of this density (2-3.05).

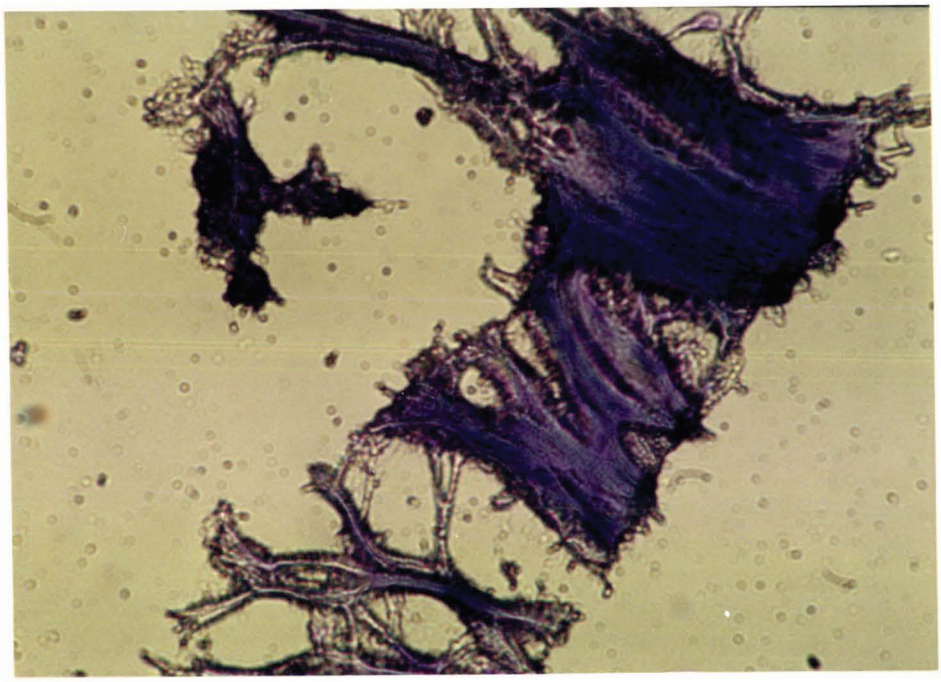
Widnell and Tata (1964), state that the presence of Mg^{2+} ions cause the nuclear membranes to aggregate, and therefore affecting the sedimentation properties of the nuclei. A sheep heart homogenate was ultracentrifuged through a sucrose gradient both with and without 3mM $MgCl_2$.

Following ultracentrifugation a small white precipitate of nuclei was expected at the bottom of the 2.4M layer. This did not occur in any of the attempts made to separate the nuclei from the myofibrils. Instead a clearly defined, dense layer was observed floating on top of the sucrose solution. A small sample of this layer was observed using photomicroscopy. Figure 2.08 shows Leishman stained photomicrographs of the layer taken from the initial homogenate following ultracentrifugation through the sucrose gradient with and without $MgCl_2$. Figure 2.09 shows similar photomicrographs resulting from the final precipitate.

Figure 2.08

PHOTOMICROGRAPHS (250x) OF THE INITIAL HOMOGENATE FOLLOWING ULTRACENTRIFUGATION THROUGH A 2.4M SUCROSE GRADIENT

WITH 3mM $MgCl_2$ (Leishmans Stain)



WITHOUT 3mM $MgCl_2$ (Leishmans Stain)

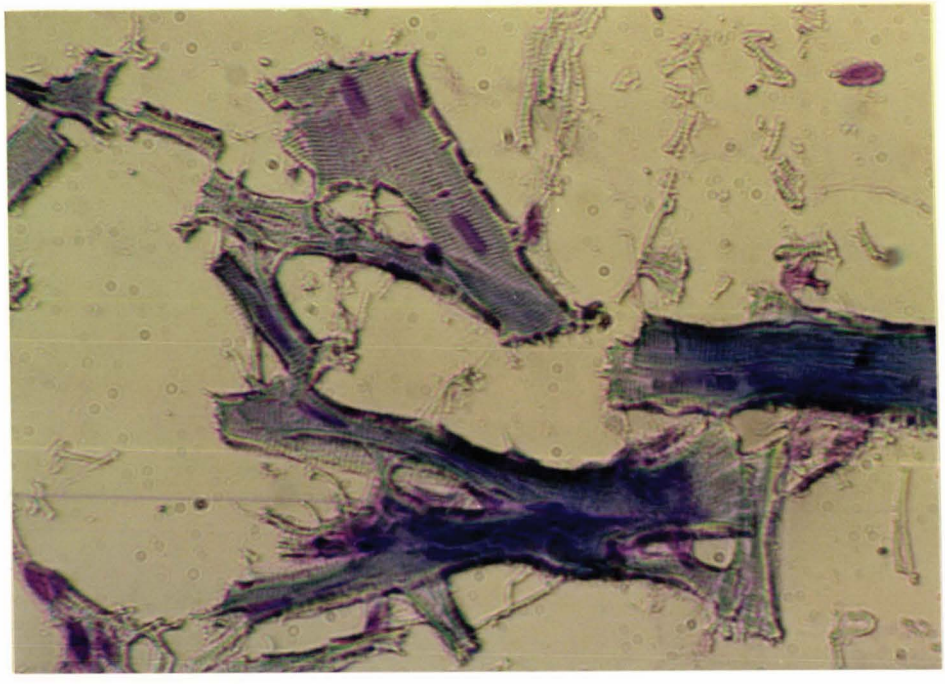
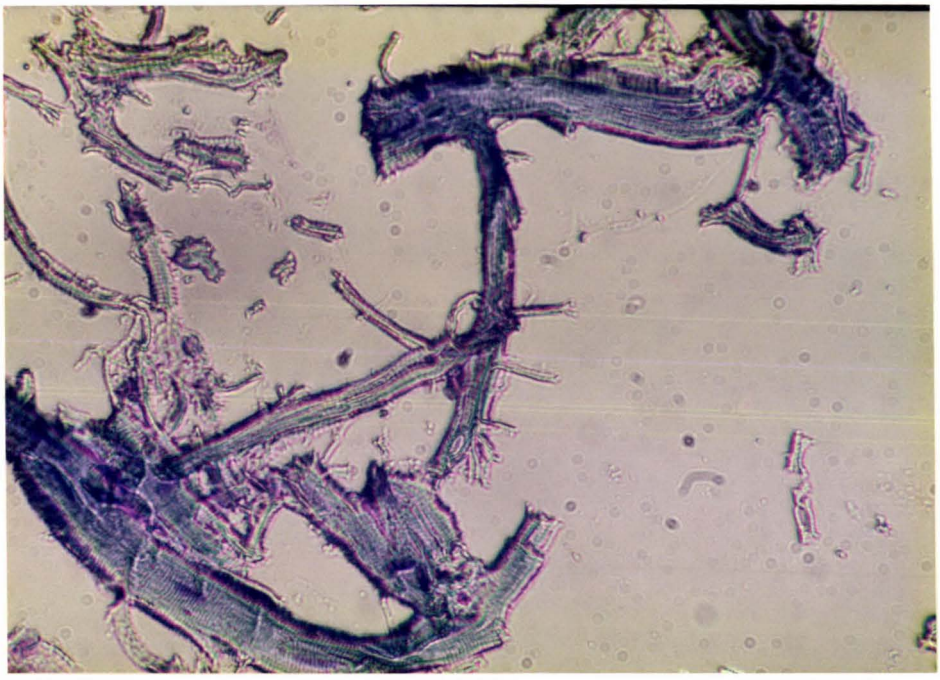


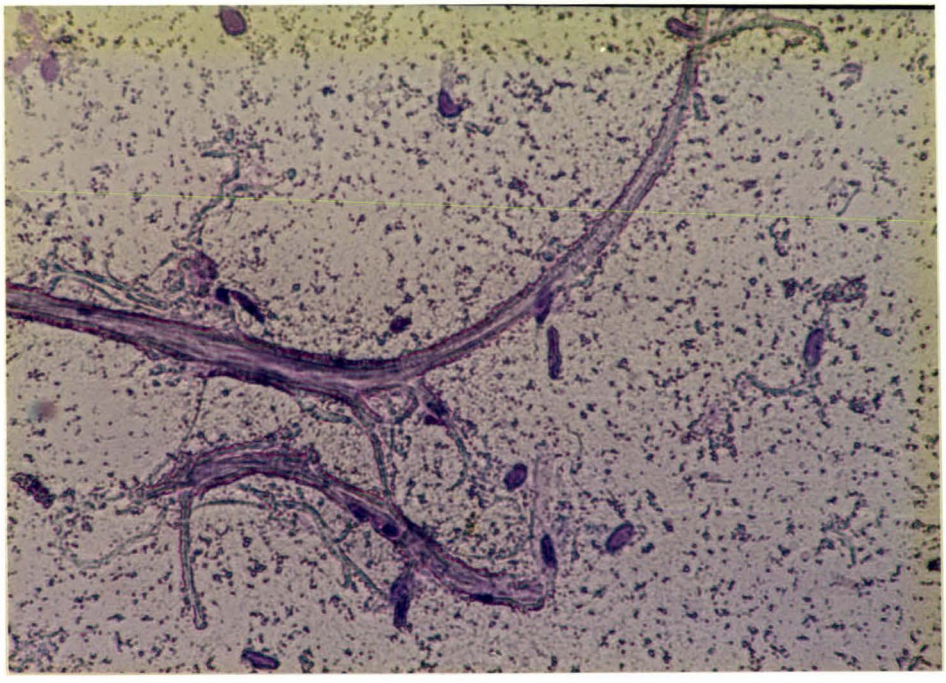
Figure 2.09

PHOTOMICROGRAPHS (250x) OF THE FINAL PRECIPITATE FOLLOWING ULTRACENTRIFUGATION THROUGH A 2.4M SUCROSE GRADIENT

WITH 3mM $MgCl_2$ (Leishmans Stain)



WITHOUT 3mM $MgCl_2$ (Leishmans Stain)



It can be seen that the nuclei are part of this floating layer and have not sedimented through the sucrose. Photomicrographs of samples of sucrose solution taken from various levels in the centrifuge tubes indicate the presence of only a few nuclei. It can also be seen that the inclusion of $MgCl_2$ in the sucrose solution has no apparent effect on the sedimentation of the nuclei.

CHAPTER 3 IMMUNOCHEMICAL LOCALISATION

3-1 INTRODUCTION

This section describes the attempts to determine the location of PFK within the cell, by immunogold localisation (Robertson *et al*, 1985), and electron microscopy. With these techniques, slices of embedded sheep heart tissue are incubated with monospecific rabbit antibodies to PFK. After removal of excess antibody by washing, the slice is then incubated with gold-labelled goat anti-rabbit secondary antibodies. These antibodies should bind specifically to the primary antibody bound to the PFK in the tissue. After further washing to remove excess secondary antibody, the tissue is examined by transmission electron microscopy. The presence of the electron-dense gold particles can be correlated with the ultrastructure of the tissue.

The success of this tissue technique depends primarily on two factors. The first is the preparation of the tissue slice in such a way that the ultrastructure of the tissue is preserved and fixed without destroying the antigenicity of the proteins in the tissue. The second factor is the high specificity required for the interaction between the antigen and the antibody.

The tissue was embedded in Lowicryl K4M resin at low temperatures (Wells, 1985). This method results in well maintained ultrastructure and antigenicity compared with conventional techniques (Roth *et al*, 1981; Carlemalm *et al*, 1982). The high specificity of the antibodies was determined by the Western Blot Technique (Towbin *et al*, 1979).

3-2 MATERIALS

Sheep hearts were obtained from Waitaki Freezing Company, Feilding. Aldolase (E.C.4.1.2.13), α-glycerophosphate dehydrogenase (E.C.1.1.1.8), triosephosphate isomerase (E.C.3.3.1.1.), Tris, EDTA, ATP, Fructose-1,6-bisphosphate, Fructose-6-phosphate, Sepharose-6B, Dithiothreitol, NADH, BSA, Bisacrylamide, SDS, Tween 20, Anti-rabbit gamma globulin alkaline phosphatase labelled secondary antibody, Fast Violet B, Naphthol As-mx phosphate, CDI, γ-aminocaproic acid, and CMC were obtained from the Sigma Chemical Company, St. Louis, Missouri. Freunds Complete Adjuvant was from from Difco Laboratories, Detroit, Michigan. Coomassie brilliant blue R-250 was obtained from Serva, New York. Lowicryl K4M was supplied by Chemische Werke Lowi, and the 15nm gold-labelled goat anti-rabbit antibodies by Janssens under the brand name 'Auroprobe'. The BSA used in the immunogold localisation ws obtained from Sigma and was 98-99% pure, and free from essential fatty acids and globulins. All other chemicals were reagent grade and obtained from May and Baker Ltd, Dagenham, England, Serva, New York, British Drug Houses or Ajax Chemicals, Sydney. Water was deionised and distilled before use.

3-3 METHODS

3-3.01 THE PURIFICATION OF PFK

PFK was purified from sheep heart according to methods modified from those of Kemp (1975), and Hussey *et al* (1977). The final steps in the preparation of the first batch involved a 'high salt' Sephacryl 300 column, followed by a 'low salt' Sepharose 6B column. For subsequent batches these two steps were replaced with the more efficient Cibacron Blue Column (Kasten *et al*, 1983).

All steps were carried out at 4^{o} except where stated.

One kilogram of sheep heart was minced and blended in a Waring Blendor with 2.9l of wash buffer (10mM Tris-HCL, 2mM EDTA, pH 8.0) for 30 seconds at low speed, and 30 seconds at high speed. The homogenate was centrifuged at 12,000g for ten minutes. The supernatant was discarded and the precipitate was resuspended in 1.8l of extraction buffer (20mM Tris-HCL, 50mM $MgSO_4$, 5mM 2-mercaptoethanol, 0.5 mM ATP, 0.5mM EDTA, pH 8.6), and blended for 15 seconds at low speed. The process was repeated with a second kilogram of sheep heart, and the final homogenates combined.

The pH of the suspended precipitate was adjusted to 8.0 with saturated tris. The temperature of the suspension was raised to 57^{o}, and maintained at this level for three minutes. The suspension was then cooled rapidly to 0^{o} in a methanol bath, and centrifuged at 12,000g for 15 minutes. All subsequent steps were performed at 0^{o}. Solid ammonium sulphate was added until 38% saturation was reached. Then the solution was allowed to equilibrate for 20 minutes, and then centrifuged at 12,000g for ten minutes. Ammonium sulphate was added to the supernatant until 55% saturation was reached, and the solution was allowed to equilibrate for 20 minutes. Following centrifugation at 12,000g for ten minutes, the white precipitate was resuspended in 100ml of dialysis buffer (20mM phosphate, 5mM 2-mercaptoethanol, pH 8.0), and dialysed against 5l of dialysis buffer overnight, at 4^{o}. The dialysate was centrifuged at 24,000g for ten minutes, and the supernatant filtered to remove fat. The pH was lowered to 6.1, and the precipitate collected by centrifugation at 9,000g for ten minutes. Subsequent treatment of the precipitate depended on the final steps chosen for the purification.

Gel Filtration at high and low ionic strength:

The precipitate was dissolved in a minimum volume of 'high salt' buffer (50mM Tris-phosphate, 1mM 2-mercaptoethanol, 1mM EDTA, 1M ammonium sulphate, pH 8.0), and passed through a Sephacryl 300 column. The fractions containing the highest absorbance at 280nm (A^{280}) were pooled, and ammonium sulphate added to 55% saturation. The precipitate was collected by centrifugation at 9,000g for 15 minutes, and then resuspended in a minimum volume of 'low salt' buffer (50mM Tris-phosphate, 5mM 2-mercaptoethanol, 1mM EDTA, 0.2mM fructose-1,6-bisphosphate, pH 8.0). The solution was then dialysed against 2x 200ml low salt buffer for three hours. The sample was centrifuged at 12,000g for 12 minutes, and the supernatant was passed through a Sepharose 6B column, equilibrated with low salt buffer. The fractions containing the highest A^{280} readings were pooled.

Chromatography on Cibacron Blue Sepharose:
The acid precipitate was resuspended in 100ml of equilibrium buffer (50mM Tris-phosphate, 0.1mM EDTA, 0.05mM fructose-1,6-bisphosphate, 1.0mM Dithiothreitol, pH 8.0) and loaded onto a Cibacron Blue Sepharose column, equilibrated with the same buffer. The column was washed with equilibrium buffer plus 0.15mM ADP, until the A^{280} readings reached base line. The PFK was eluted with equilibrium buffer plus 2mM ATP, and 2mM fructose-6-phosphate. The fractions were assayed for PFK activity, according to the method outlined in 3-3.02, and the fractions containing the highest activities were pooled.

PFK was stored as a suspension in 55% ammonium sulphate at 4^{o}.

Before use, an appropriate amount of PFK suspension was centrifuged in a Microcentrifuge for three minutes, and the precipitate resuspended in a suitable volume of PFK dilution buffer (10mM Tris-HCl, 7mM 2-mercaptoethanol, pH 8.0).

3-3.02 THE CHARACTERISATION OF PFK

PFK activity was measured by coupling the PFK reaction to the oxidation of NADH to NAD^+. Coupling was achieved by the addition of aldolase, triose phosphate isomerase, and α-glycerophosphate dehydrogenase to the assay solution. (see Figure 3.01).

The assay solution consisted of 1ml of reaction mixture (55mM Tris-HCL, 11mM $MgCl_2$, 4mM 2-mercaptoethanol, 0.011% BSA, 2mM fructose-6-phosphate, 1mM ATP, 0.22mM NADH, 75 units per ml triose phosphate isomerase, 7.5 units per ml α-glycerophosphate dehydrogenase, 0.5 units per ml aldolase, pH 8.0) in a quartz cuvette, which was first equilibrated at 30°. Ten microlitres of sample was added to the reaction mix, and the rate of decrease in absorbance was monitored at 340nm, in a Cecil recording spectrophotometer or Unicam SR-800. In this assay, phosphorylation of 1 mole of fructose-6-phosphate, catalysed by PFK, results in the oxidation of 2 moles NADH.

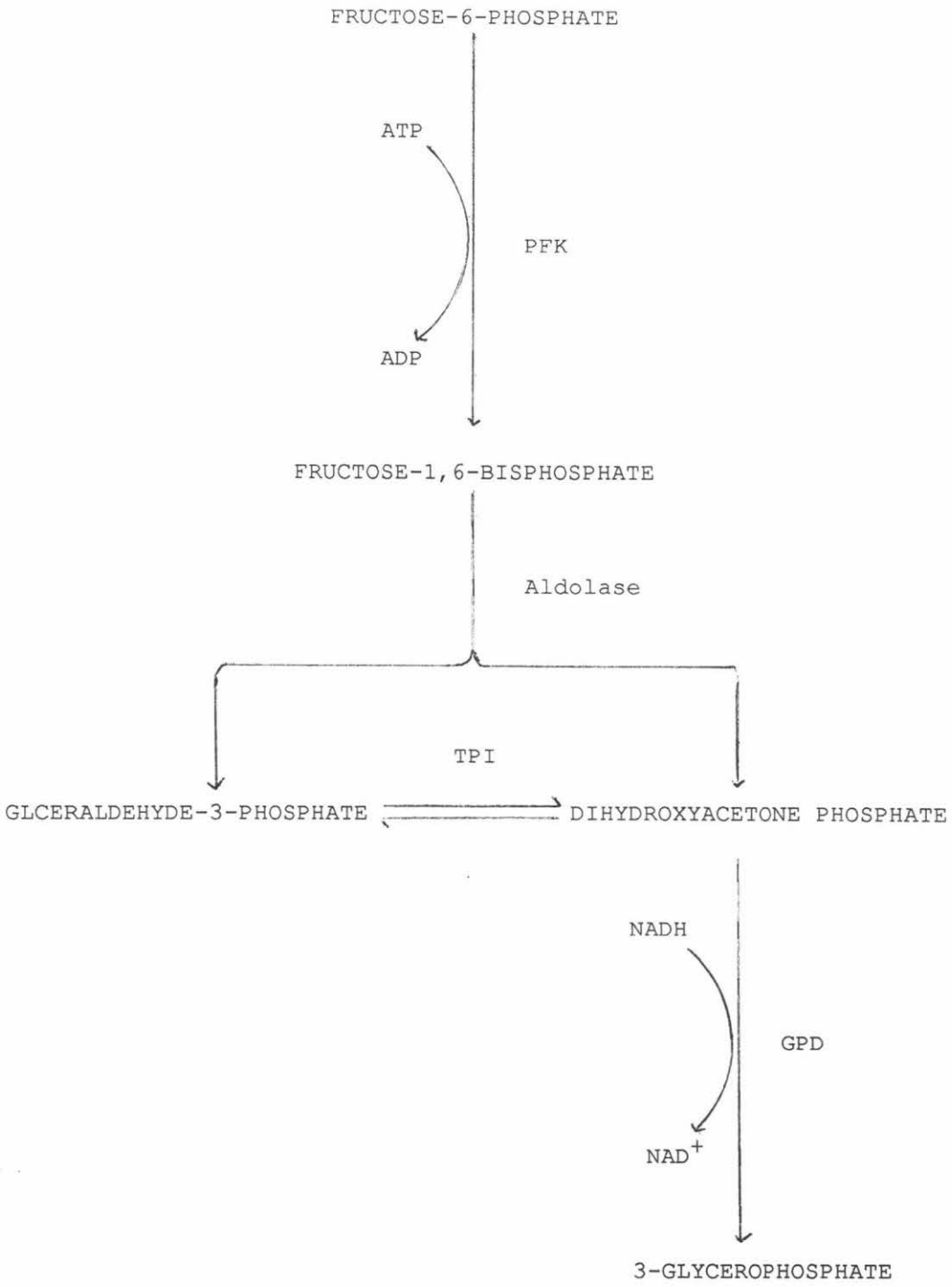
Protein concentration was estimated by the Coomassie blue method (Bradford,1976),using bovine serum albumin as the standard. Absorbances were read at 595nm in the Cecil spectrophotometer.

Homogeneity of the purified enzyme was determined by SDS Orstein-Davis discontinuous gel electrophoresis (Laemmli, 1970)

A slab gel was first sealed with 15ml of running gel (8% acrylamide, 0.26% bisacrylamide, 0.1% v/v TEMED, 0.1% SDS, 0.375M Tris, 0.06M HCL, pH 8.9), plus 0.13% ammonium persulphate. When the sealing gel had polymerised, the remainder of the running gel, plus 0.086% ammonium persulphate, was poured over this to within 2 cms of the top of the gel plates. Following running gel polymerisation, stacking gel (5% acrylamide, 0.5% bisacrylamide, 0.1% v/v TEMED, 0.1% SDS, 0.06M HCL, 0.061M Tris, 0.1% ammonium persulphate, pH 6.7), was applied and a sample slot comb inserted before polymerisation.

Figure 3.01

THE REACTION PATHWAY FOR THE PFK ASSAY.



The polymerised gel was transferred to an electrophoresis tank containing reservoir buffer (10mM Tris, 77mM Glycine, 0.1% SDS, pH 8.3) in both upper and lower chambers. A few drops of a 0.05% bromophenol blue solution was added so that the solvent front could be followed.

The protein solution to be electrophoresed was mixed with an equal volume of denaturing solution (6M urea, 10% SDS). Since PFK contains disulphide bonds, 1% 2-mercaptoethanol was also included. This mixture was heated for two minutes in a boiling water bath. Samples containing 2.5 - 15 µg of protein were applied to the wells, and the gel electrophoresed at 10mA until the solvent front had migrated about two thirds down the gel.

The gels were stained by immersion in staining solution (0.125% Coomassie brilliant blue R-250 in acetic acid/methanol/water 1:9:10 v/v/v), for 4-16 hours at room temperature, and then destained with several changes of destaining solution (staining solution minus the Coomassie blue).

3-3.03 IMMUNIZATION PROTOCOL

Two female rabbits (New Zealand White), labelled 'A' and 'B', were immunised according to Crowle (1973). On days 1 and 7, 1mg of antigen was dissolved in 1ml of PBS (10mM K_2HPO_4, 0.15M NaCl, pH 7.2), and emulsified in an equal volume of Freunds Complete Adjuvant. 0.5ml was injected intradermally along the back, and 0.5ml was injected intramuscularly into a hind leg. On days 21, 22, and 23, 0.1, 0.5, and 2mg ,respectively, of antigen, was dissolved in 0.5ml of PBS and injected subcutaneously along the back. The rabbits were bled from a marginal ear vein at weekly intervals for seven weeks following the last injection. At each bleeding 30-40mls of blood was collected.

The blood obtained from the immunised rabbits was allowed to clot for one hour at room temperature, and overnight at 4°. The clot was precipitated by centrifugation at 35,000g for ten minutes. The supernatant (serum) was heated to 54° for ten minutes to inactivate the complement. The denatured proteins were removed from the serum by centrifugation at 35,000g for ten minutes. The serum was stored at -20°.

Antibody titres of the sera were obtained in order to pool those with the highest concentrations of anti-PFK antibodies. The assay involved a set amount of antigen (PFK), mixed with a varying amount of antibody. The mixture was then incubated for one hour at 37°. The immunoprecipitate was removed by centrifugation, and the supernatant assayed for PFK activity. The remaining PFK activity was calculated as a percentage of the total PFK activity. Those sera containing the highest antibody titres (indicated by the lowest levels of PFK activity) were pooled and subjected to ammonium sulphate precipitation.

Ammonium sulphate fractionation was performed to separate the gamma globulins from the serum (Palmiter *et al*, 1971). The serum was diluted with an equal volume of PBS. Saturated ammonium sulphate solution was added until the serum was 40% saturated, and then allowed to stand for 30 minutes at 4°. The precipitate was collected by centrifugation at 12,000g for ten minutes, and the pellet was resuspended in 2.5 volumes of PBS (relative to the original serum volume). Saturated ammonium sulphate was again added until the serum was 40% saturated. The solution stood for a further 30 minutes at 37°, and centrifuged as before. The pellet was resuspended in a volume of buffer equivalent to one half the serum volume, and dialysed overnight in PBS at 4°. The gamma globulin solutions were stored at -20°.

3-3.04 PREPARATION OF CRUDE PFK EXTRACTS

A crude PFK preparation was obtained using the following proportions. One gram of tissue was homogenised in 2ml of wash buffer (10mM Tris-HCl, 2mM EDTA, pH 8.0), using an UltraTurrax. The homogenate was centrifuged at 12,000g for ten minutes. The precipitate was resuspended in 2ml of partial extract buffer (20mM Tris-HCl, 50mM 2-mercaptoethanol, 0.5mM EDTA, pH 8.6), and blended. The suspension was heated to 40^{o} for five minutes and then centrifuged at 12,000g for ten minutes. The supernatant was saved as the crude PFK extract.

3-3.05 THE MICRO-OUCHTERLONY TECHNIQUE

The specificity of the immunoglobulins, previously purified by ammonium sulphate fractionation, was roughly determined using a two dimensional double diffusion technique, developed simultaneously by Ouchterlony (1949), and Elek (1948). A circular well pattern was used, with the antigen placed in the central well, and the antibodies in the surrounding wells. The two reactants diffused into the gel, and immunoprecipitates formed at the points of equivalence for each antigen-antibody pair.

Agarose was dissolved in PBS by heating to obtain a 1% solution. 0.2ml of this solution was diluted to 2ml with warm water, and 1ml was pipetted onto a clean microscope slide. After being spread evenly, the slide was baked at 100^{o} for 15 minutes. 2.5ml of the 1% agarose solution was pipetted on to the slide, and spread evenly. It was left to set at room temperature (10-15 minutes). Once set, the slide was placed in a moist container, and left for one hour at 4^{o}. Two sets of wells were punched into the agar on each slide.

A crude PFK preparation was obtained from 5 grams of sheep heart tissue according to 3-3.04. A pure preparation of PFK was diluted,

with PFK dilution buffer, to an equivalent concentration (8.12 units per ml). Small amounts of each PFK preparation were applied to a centre well in two plates. Ammonium sulphate fractionated antibodies from rabbit 'A', were placed in the outsides wells of Plate One, and Antibodies from rabbit 'B', were placed in the outside wells of Plate Two.

3-3.06 PREPARATION OF A CDI ACTIVATED SEPHAROSE COLUMN.

Preparation of Activated Sepharose:

Prepared according to Bethell *et al*, (1981a and b). 20 grams of moist Sepharose-CL-6B-200 was washed with water to remove impurities, and then solvent exchanged to degassed dimethylformamide. The gel was then activated with CDI (1,1-carbomyldiimidazole). Since PFK is a large molecule (Mwt 85,000 per monomer), a low degree of activation was chosen to minimise protein-protein interactions. CDI (0.25g/10g of Sepharose) was reacted with the gel in dimethylformamide for 75 minutes at room temperature. The gel was then solvent exchanged back to water and dried in a Buchner funnel. A small portion was stored in 5ml 0.1M NaOH for later titration to determine the degree of activation. γ-aminocaproic acid was added next as a spacer arm. Two grams of the amino caproic acid was dissolved in 50ml of 0.1M $NaHCO_3$ buffer, pH 11.0, and allowed to was react with the activated gel overnight at room temperature.

Degree of CDI Activation:

The sample, which had been set aside, was bubbled through with nitrogen gas to replace the carbon dioxide naturally present in the buffers. The imidazole group of the activated gel, was titrated with 1M NaOH using an automatic titrator, to determine the degree of CDI acivation. Results were recorded as meq imidazol group per gram of Sepharose.

Degree of γ-aminocaproic acid substitution:

A sample of gel was washed extensively with distilled water, 0.1M NaOH (to remove excess caproic acid and CDI), and distilled water again to remove excess base. The acid group was titrated with 1M NaOH using the automatic titrator. Results were expressed as meq of caproic acid per gram of Sepharose, or µmoles caproic acid per ml of gel.

Coupling of PFK to the Column:

The gel was thoroughly washed with distilled water and the pH adjusted to 4.7 with HCl. 10ml of a 6% solution of CMC (1-cyclohexyl-3-[2-morpholinoethyl]-carbodiimide-metho-ρ-toluene sulphonate), was added to the Sepharose over ten minutes (both were precooled to 4^{o}). The CMC - Sepharose solution was left for 30 minutes on ice, with the pH maintained at 4.7. The pH was then shifted to 7.0 with KOH, and 2ml of a 2% PFK solution added. The Sepharose and PFK solution were placed in a screw topped bottle, and left for two days on a slowly rotating wheel at 4^{o}. The Sepharose was then washed with 0.1M phosphate, 0.5M NaCl, to remove unbound protein. These washings were assayed for protein levels (Coomassie Blue method), to estimate the degree of PFK binding. A Pharmacia column, with two adjustable ends, was packed with the Sepharose and equilibrated with degassed 0.1M phosphate buffer, pH 7.0. The column was stored in 0.1% Thiomersal at 4^{o} when not in use.

3-3.07 PREPARATION OF A CYANOGEN BROMIDE ACTIVATED SEPHAROSE COLUMN

Prepared according to methods by Porath (1970), and Pharmacia. Sepharose 6B (5g) was washed with distilled water and suspended in 15ml of 2.0M phosphate buffer, pH 12. Cyanogen Bromide (1.5g) was dissolved in 2 ml of acetonitrile, and added to the Sepharose. The temperature of the Sepharose-Cyanogen bromide mixture was maintained at 15^{o} for 15 minutes, and the reaction stopped by filtering with ice cold water. 30mg of PFK was dissolved in 7.5ml of coupling buffer (0.1M $NaHCO_3$, 1.0M $(NH_4)_2SO_4$, 0.05M 2-mercaptoethanol, pH 8.0), and reacted with the activated Sepharose, by rotation on a wheel, for two

hours at room temperature. Excess protein was removed by washing the gel with coupling buffer, and the washings collected for later protein estimations. The excess active groups on the Sepharose were then blocked by rotating the gel with 10ml of blocking buffer (1.0M ethanolamine, 0.1M Na_2CO_3, pH 9.0),for two hours at room temperature. The Sepharose was then washed alternatly with washing buffer 1 (0.1M Tris-HCl, 0.5M NaCl, pH 8.3), and washing buffer 2 (0.2M glycine-HCl, pH 2.9), finishing with buffer 1. Finally the Sepharose was equilibrated with 0.1M phosphate buffer, pH 7.0, degassed, and packed into a Pharmacia double-ended column. The column was stored in 0.1% Thiomersal at 4^{o} when not in use.

3-3.08 ELUTION OF ANTIBODIES FROM THE AFFINITY COLUMNS.

The non-specific antibody solutions were diluted to about 0.7%, with 0.1M phosphate buffer, pH 7.0. The solution was applied carefully to the top of the column (10ml to the CDI-column, and 4 ml to the Cyanogen Bromide column).

Two sets of buffer systems were tried, in order to elute the specific and non-specific antibodies. Absorbance readings at 280nm were automatically monitored.

Method 1:
0.1M phosphate buffer, pH 7.0, at 5 mls per hour, until the A^{280} readings reached baseline
0.1M sodium acetate, 0.5M NaCl, pH 4.0 at 20 mls per hour
0.1M Tris-HCl, 0.5M NaCl, pH 8.3 at 20 mls per hour
0.1M sodium acetate, 0.5M NaCl, pH 4.0 at 20 mls per hour
0.2M glycine-HCl, pH 2.7 at 20 mls per hour, run in the reverse direction.

The eluted samples were collected in 5ml tubes containing 2.5mls of 5M sodium/potassium phosphate to neutralise them.

Method 2:

0.1M phosphate buffer ,pH 7.0, at 5 mls per hour, until the A^{280} readings reached baseline

0.01M phosphate, 0.15M NaCl, pH 7.2, at 20 mls per hour

0.1M Tris-HCl, 0.5M NaCl, pH 8.3, at 20 mls per hour

3M potassium thiocyanate in 0.01M phosphate, 0.15 NaCl, pH 7.2, at 20 mls per hour, run in the reverse direction.

3-3.09 DETERMINATION OF THE SPECIFICITY OF ANTIBODY PREPARATIONS BY IMMUNOPRECIPITATION FOLLOWED BY POLYACRYLAMIDE GEL ELECTROPHORESIS

Preparation of Samples:

A crude PFK sample was prepared from 7g sheep heart (and sheep liver) according to the method outlined in section 3-3.04. The supernatants, plus those obtained from the first centrifugation, were ultracentrifuged at 100,000g for one hour to remove all particles.

Preparation of Immunoprecipitates:

Immunoprecipitates were prepared using the following proportions. 100 µls of sample was mixed with 200 µls of buffer (0.1M phosphate, 1% Triton-x-100, 1% Deoxycholate, pH 7.0), and 50 µl of antibody solution. These mixtures were incubated at 37° for one hour. Following a 3 minute precipitation in a Microcentrifuge, the precipitates were resuspended in 200 µl of the same buffer, and left on ice for 15 minutes. The centrifugation, resuspension and the 15 minute wait on ice was repeated 3 times, in order to extensively wash the immunoprecipitate.

Preparation of Samples for the Gel:

The samples to be electrophoresed were prepared as follows;

Immunoprecipitates - 250 µl of prepared crude PFK sample

25 µl of denaturing solution (10% SDS, 6M Urea)

1 µl 2-mercaptoethanol

Original ultracentrifuged supernatants - 15 µl of sample
25 µl of denaturing solution
1 µl 2-mercaptoethanol

Pure PFK - 15 µl of 0.05% PFK solution (15µgs total, 3 units)
25 µl of denaturing solution
1 µl 2-mercaptoethanol

All samples were heated in a boiling water bath for 3 minutes.

An 8% polyacrylamide gel was prepared and run according to the method outlined in 3-3.02.

The completed gel was stained with Coomassie stain as described in 3-3.02, and then with the following more sensitive silver stain. (described below).

Silver Staining of polyacrylamide gels (Merril *et al*, 1981):
The gel was left in 40% Methanol, 10% Acetic acid for one hour, and then in 10% ethanol, 5% Acetic acid for another hour (with two changes of fixative). It was then oxidised in oxidiser solution (0.1% potassium dichromate, 0.143% v/v Nitric acid) for ten minutes, followed by 4 x 10 minute washes with distilled water to remove yellow colour. A 0.1% solution of silver nitrate was mixed with the gel for 30 minutes, and the gel then rinsed with distilled water for one minute. Developer (3% sodium carbonate, 0.0185% v/v formaldehyde) was added for one minute and then rinsed away with distilled water. More developer was added and rinsed away until sufficient black colour had appeared. Development was stopped with 5% Acetic acid.

3-3.10 A DETERMINATION OF THE SPECIFICITY OF THE ANTIBODY SOLUTIONS BY WESTERN BLOTTING.

The Polyacrylamide Gel:

Samples of crude PFK were prepared according to the procedure for ultracentrifuged supernatants, outlined in 3-3.04 and 3-3.09. Sheep muscle, rat heart, and rat liver samples were also prepared by this method. An appropriate volume of each of the prepared samples was mixed with 10% SDS/6M Urea and 1% 2-mercaptoethanol applied to an 8% SDS polyacrylamide gel, which was then run in the usual way.

The Western Blot:

Method 1:

Two Scotch Brite pads and six layers of Whatman No1 filter paper were presoaked in transfer buffer (25mM Tris, 192mM glycine, 20% v/v methanol, pH 8.3), for 30 minutes. A small piece of nitrocellulose (equivalent in size to the polyacrylamide gel) was also presoaked in transfer buffer, by capillary action. After electrophoresis was complete the gel was placed on top of three layers of filter paper, taking care not to trap any air bubbles. The nitrocellulose was placed on top of the gel, followed by the remaining filter paper. The Scotch Brite pads were placed on either side of the nitrocellulose-gel sandwich, and finally the whole assembly was clamped between two plastic holders. The sandwich was inserted into a LKB Trans Blot cell, previously filled with transfer buffer. The sandwich was orientated so that the nitrocellulose was closest to the anode. The Western Blot was run overnight at 60V, 0.17Amps, at 4^{o}.

Method 2:

As above except transfer buffer contained 0.1% SDS, and the voltage was adjusted frequently to maintain 60V.

Blocking and Staining the Blot:

Method 1:

Based on methods by Knecht and Dimond (1984), and Blake *et al*, (1984). The nitrocellulose was carefully removed from the gel, and placed in several changes of blocking/washing solution (59mM Tris-HCl, 203mM NaCl, 0.1% BSA, 0.1% Tween 20, pH 7.4) for one hour. The gel was stained with Coomassie Blue gel stain to determine the degree of

protein transfer (3-3.02). The nitrocellulose was then sealed into a plastic bag and mixed with 1 ml of undiluted primary antibody solution for one hour. Excess antibody was removed by washing the blot with five changes of washing buffer, over 30 minutes. Anti-rabbit gamma globulin alkaline phosphatase secondary antibody (diluted 1/1000), was then incubated with with the blot for one hour at 37°. Excess antibody was removed as above. The nitrocellulose was then reacted with staining solution (0.2M Tris-HCl, 1mM $ZnCl_2$, 1mM $MgCl_2$, 0.3% Fast Violet B, 0.05% Naphthol As-mx phosphate, pH 8.2), for about 15 minutes, or until sufficient colour developed. Excess stain was rinsed away with distilled water.

Method 2:

As above except using blocking buffer (59mM Tris-HCl, 203mM NaCl, 3% BSA, pH 7.4), and washing buffer (59mM Tris-HCl, 203mM NaCl, 0.1% Tween 20, pH 7.4)

3-3.11 IMMUNOGOLD LOCALISATION AND ELECTRON MICROSCOPY

Low Temperature Embedding:

The embedding procedure, slightly modified from that of Wells, (1985), was begun on the tissue within minutes of it being excised from the sheep. 1mm cubes of tissue were placed in a fixative (0.5M sodium cacodylate, 2.5% glutaldehyde, pH 7.2), for two hours at room temperature. The tissue was then dehydrated by immersion in the following:

30% ethanol for 1 hour at room temperature
50% ethanol for 1 hour at -20°
75% ethanol for 1 hour at -35°
95% ethanol for 1 hour at -35°
100% ethanol for 1 hour at -35°
100% ethanol for 1 hour at -35°

followed by infiltration with Lowicryl K4M resin

50% resin in ethanol for 1 hour at -35°
75% resin in ethanol for 1 hour at -35°

100% resin in ethanol for 1 hour at -35°

100% resin in ethanol overnight

100% resin in ethanol for 8 hours at -35°

The tissue was then placed in fresh resin in beem capsules. The resin was polymerised by irradiation with diffuse ultraviolet light (360nm) for 24 hours at -20°, followed by irradiation for a further 24-48 hours at room temperature.

The resin blocks were cut using a Reichert-Jung Ultracut E Microtome with a diamond knife. The sections averaged 100nm thickness. They were floated into water and the thinnest sections (80mm), indicated by a pale gold colour, were saved. The sections were mounted on carbon stabilised Formyar support films on 200 mesh nickel grids.

Immunogold Localisation:

The antibody solution was diluted 1:10, 1:100 and 1:1000 with dilution buffer (0.8% NaCl, 0.115% Na_2HPO_4 anhydrous, 0.02% KH_2PO_4, 1% Tween 20, 1% BSA, pH 7.4). In a moist petri dish, the nickel grids were dropped onto 50µl drops of the undiluted and diluted antibody solutions, and incubated at 37° for one hour. The grids were then washed thoroughly with washing buffer (0.8% NaCl, 0.115% Na_2HPO_4 anhydrous, 0.02% KH_2PO_2, 0.1% Tween 20, pH 7.4), and water. The grids were then dropped into 20µl drops of Auroprobe (diluted one tenth), and incubated at 37° for one hour. The grids were washed as above.

Lead Citrate Staining :

The nickel grids were soaked in saturated uranyl acetate in 50% ethanol, for 1-3 minutes and then washed in 3 times in 50% ethanol. They were then soaked in lead citrate staining solution (Venable and Coggeshall, 1965) for 90 seconds and washed with water.

Electron Microscopy:

Using a Philips 201c Transmission Electron Microscope the grids were observed and photographed at either 15,000 or 30,000x magnification.

Using four of the low powered micrographs, the gold labels (visible as distinct black dots) were counted, and the density of the gold particles on the myofibrils and background was determined and compared.

3-4 RESULTS AND DISCUSSION

3-4.01 THE PURIFICATION OF PFK

Gel Filtration at high and low ionic strength:
The precipitate which had been resuspended in 'high salt' buffer, was passed through a Sephacryl 300 column, and 50 5ml fractions collected. Each fraction was measured at 280nm absorbance to determine the protein content, and the results plotted (Figure 3.02). Fractions 28 to 36, which contained the highest protein concentrations were pooled.

These pooled fractions were then treated as described in 3-3.01, and passed through a Sepharose 6B column. Forty six fractions were collected, each containing about 10 mls. Absorbance readings at 280nm were recorded and the results plotted (see Figure 3.03).

Fractions 37 to 45 were pooled to give a total volume of 51 mls. The absorbance at 280nm for this pooled solution was 0.98. Since 1 absorbance unit at 280nm is approximately equal to 1mg per ml of PFK, then the solution contained a total of 50mg protein (presumably PFK).

Chromatography on Cibacron Blue Sepharose:
Thirty fractions containing about 10 mls each were collected from the Cibacron Blue column. Due to the presence of ATP in the eluate, A^{280} readings could not be measured accurately. PFK activity readings were thus determined, although there is some enzyme inhibition due to ATP.

Figure 3.02
PROTEIN CONTENT OF FRACTIONS FROM
'HIGH SALT' COLUMN

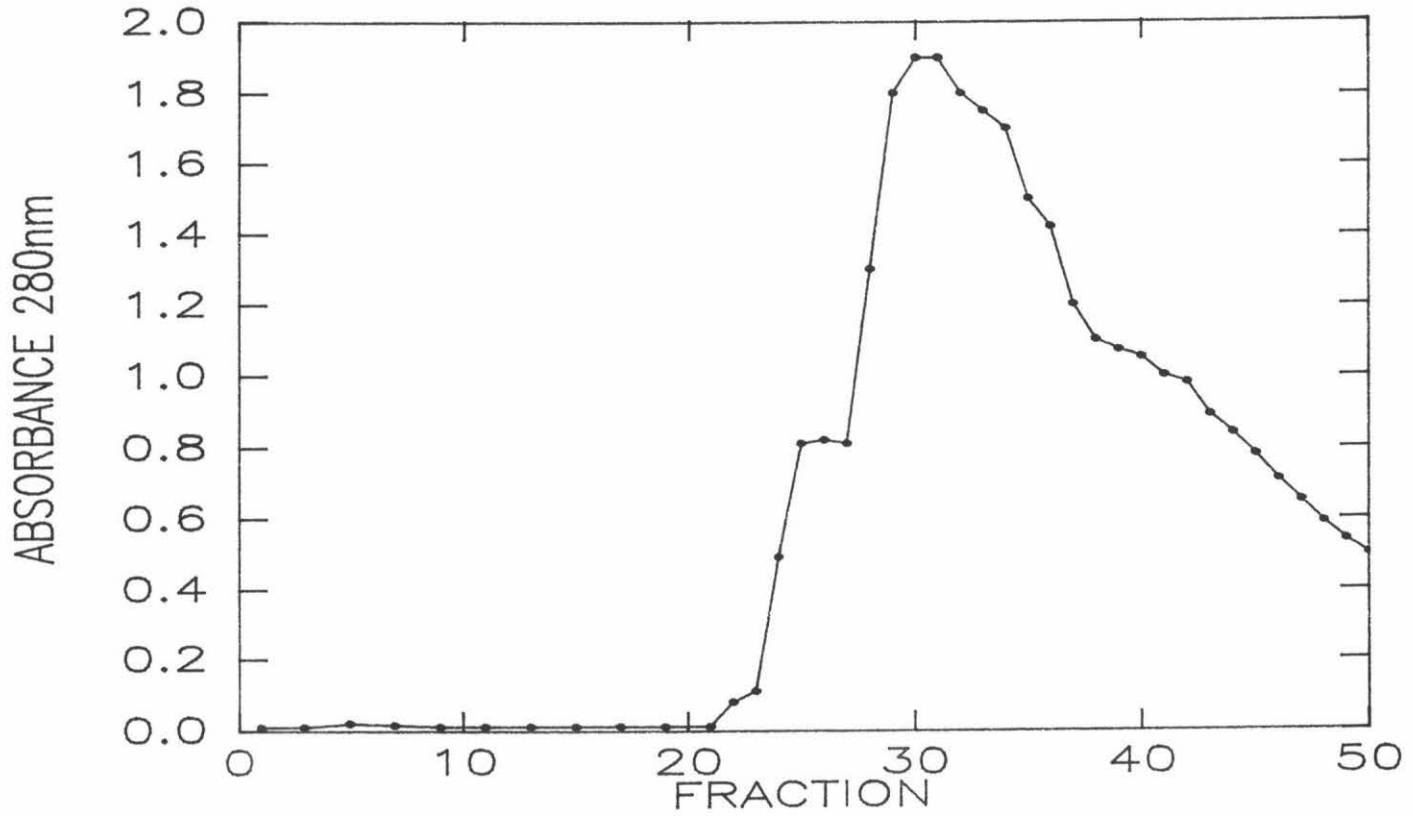


Figure 3.03
PROTEIN CONTENT OF FRACTIONS FROM
'LOW SALT' COLUMN

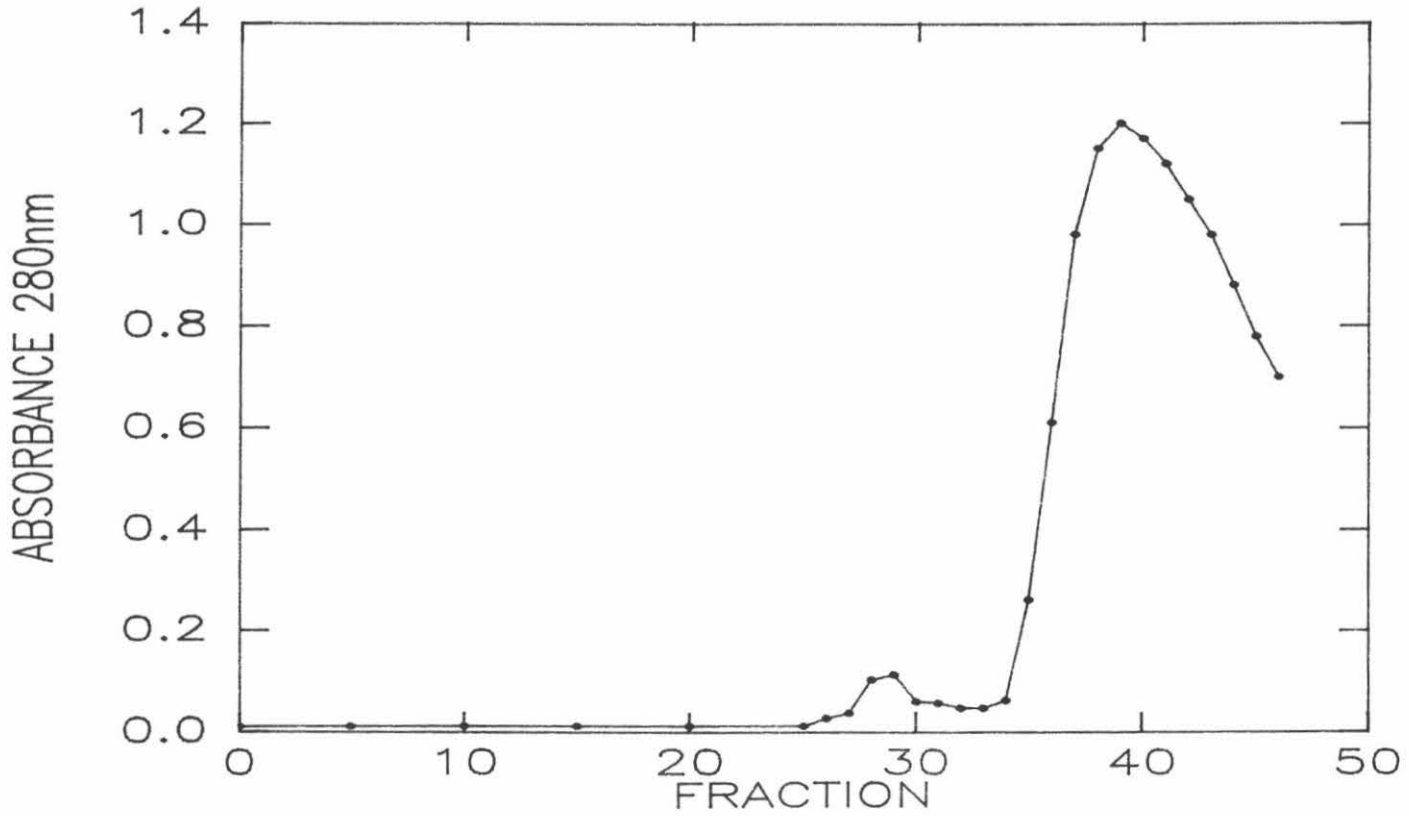


Figure 3.04 shows a typical elution profile from a Cibacron Blue column. The PFK is eluted abruptly in a short, sharp peak. The appropriate fractions were pooled.

This method resulted in 80-100mg of protein ie 1.5-2 times the amount collected from the gel filtration method.

3-4.02 THE CHARACTERISATION OF PFK

Gel Filtration at high and low ionic strength:
The activity of the PFK solution was determined as 10.55 units per ml. Thus the specific activity of the batch of PFK produced by this method was 107.5 units of PFK activity per mg of protein.

A polyacrylamide gel, which was overloaded with PFK (3-17µg), was run to determine the purity of the sample (Figure 3.05). No bands other than that of PFK was evident, indicating that the enzyme obtained was reasonably pure.

Chromatography on a Cibacron Blue column:
The PFK solution obtained by this method, generally resulted in specific activities of almost twice that obtained by the gel filtration method. Typical specific activities obtained were about 210 units of activity per mg protein.

3-4.03 ANTIBODY TITRES OF SERA

Antibody titres were determined for the sera obtained from the 1st, 3rd, 5th, and 7th bleeds from each rabbit. 40µl of a PFK solution (0.5mg PFK per ml of PFK dilution buffer - 10mM Tris-HCL, 7mM 2-mercaptoethanol, pH 8.0),was mixed with 0, 5, 10, 20, 40, and 80 µl of serum, and diluted to 200µl with PFK dilution buffer. Antibody titres were then determined according to 3-3.03. Percentage activity was plotted against volume of serum used (Figures 3.06 and 3.07).

Figure 3.04
PFK ACTIVITY IN FRACTIONS FROM
CIBACRON BLUE COLUMN

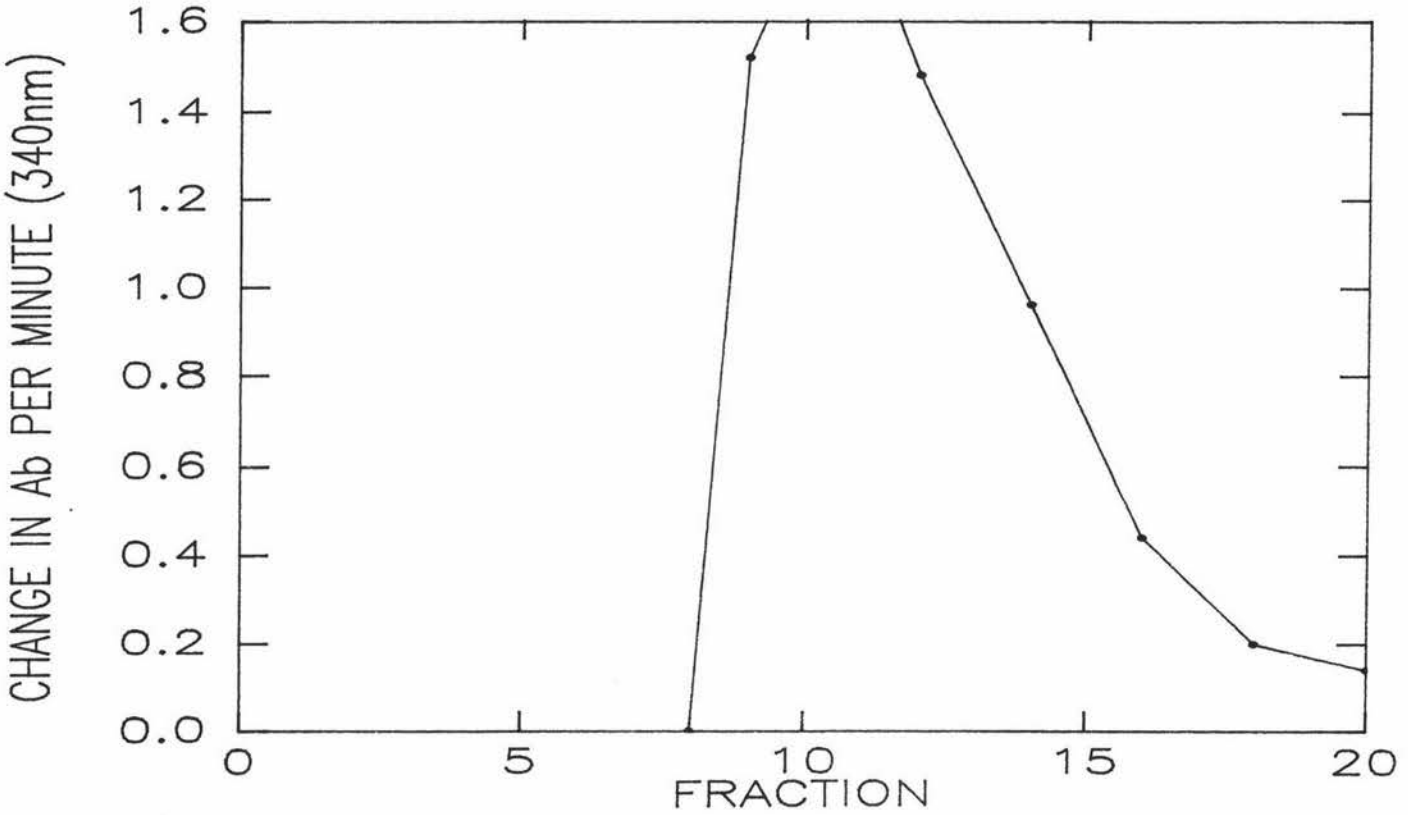


Figure 3.05

SDS GEL (8%) ANALYSIS OF PFK OBTAINED FROM GEL FILTRATION PURIFICATION PROCEDURE

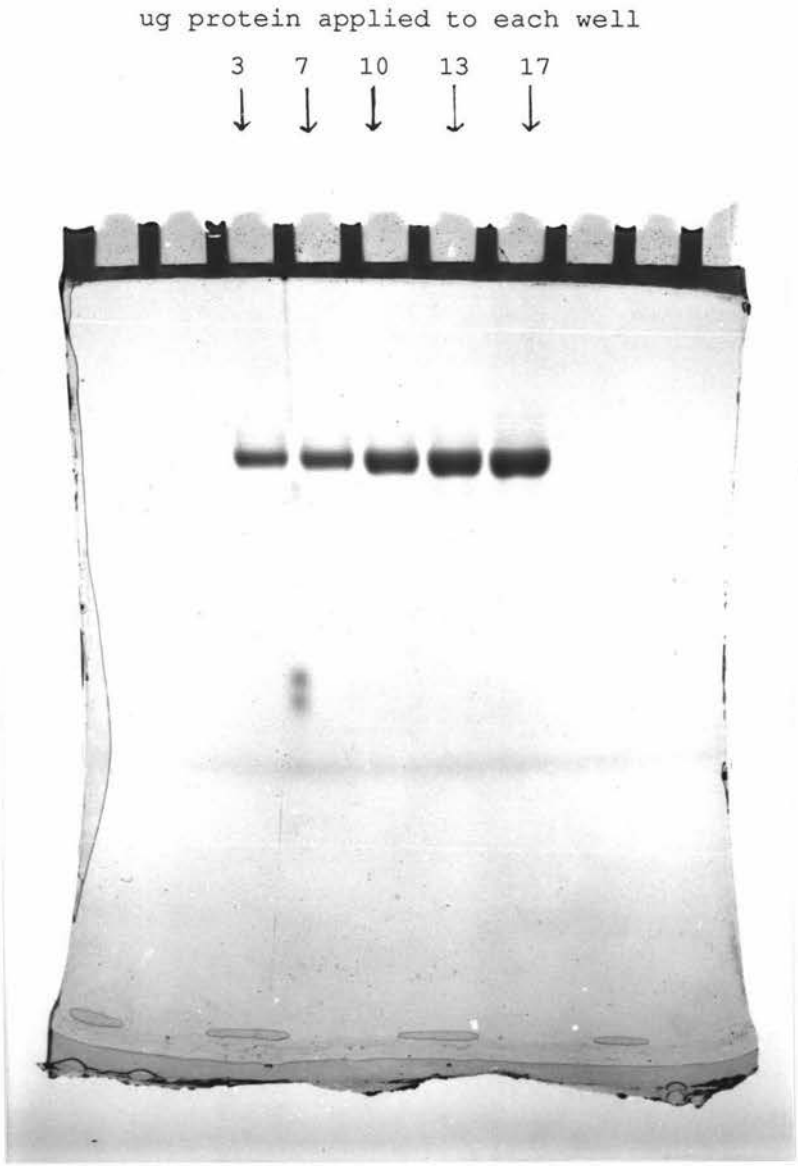


Figure 3.06
RABBIT A
PERCENTAGE TOTAL PFK ACTIVITY versus SERUM VOLUME

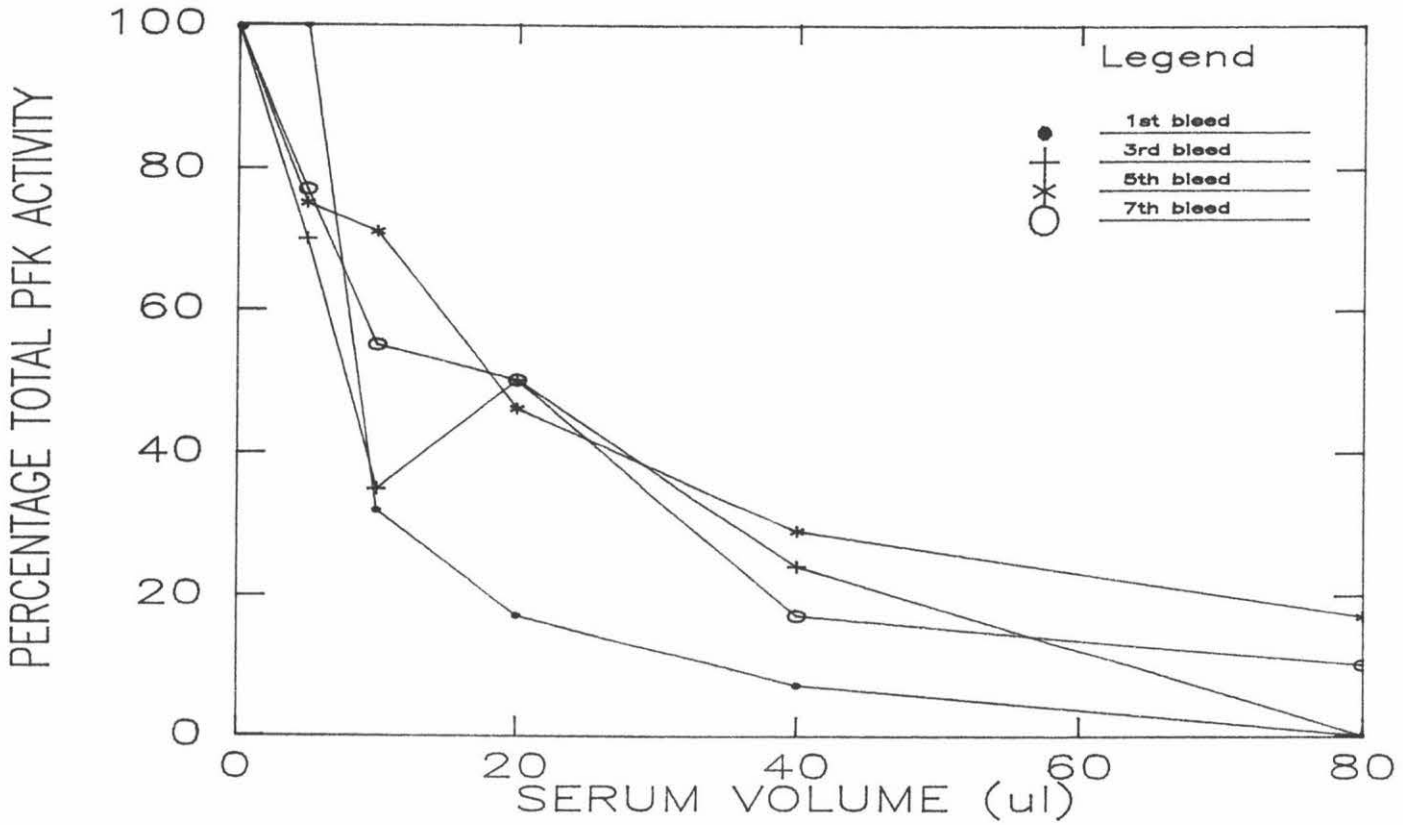
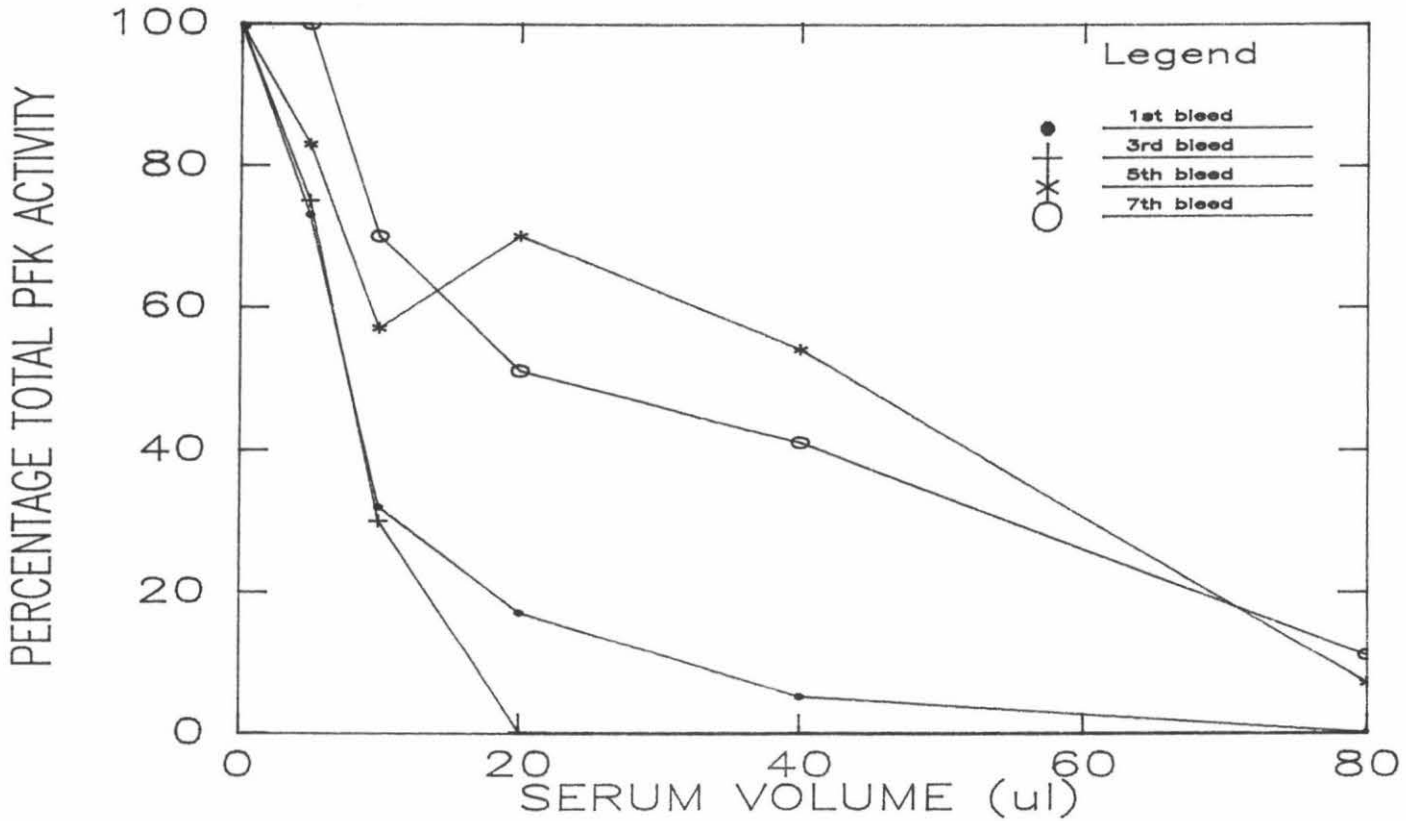


Figure 3.07
RABBIT B
PERCENTAGE TOTAL PFK ACTIVITY versus SERUM VOLUME



For rabbit A, weeks 1, 2 and 3, and for rabbit B, weeks 1, 2, 3 and 4 were pooled. These pooled fractions were subjected to ammonium sulphate treatment.

3-4.04 PREPARATION OF CRUDE PFK EXTRACTS

The supernatant containing the PFK, contained 8.12 units of PFK per ml.

3-4.05 MICRO-OUCHTERLONY PLATES

Refer to Figure 3.08. Single bands only appeared on each of the plates. This indicated that there was a reaction occuring between the anti-PFK antibodies and the PFK present in the centre well. There were no obvious cross reactions between the antibodies and any other antigen present in the crude extract. Thus, it is probable that the only anti-sheep heart antibodies present in the antibody solution were those against PFK. This method was not sensitive enough to accurately determine the purity of the antibodies, and so was used as a rough but quick indication of specificity.

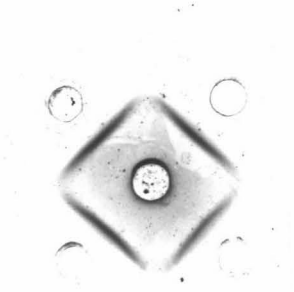
3-4.06 CDI ACTIVATED SEPHAROSE

Three columns were made. The first had a very low degree of activation for both the CDI (0.15 meq/g) and the amino-caproic acid (0.082 meq/g). It was discovered that the dimethylformamide had to be degassed, as it contains a gas, dimethylamine, which effectively competes with the γ-amino-caproic acid.

The following two columns gave activation degrees of 0.89 and 0.87 meq CDI per gram of sepharose, and 13 and 18.5 µmoles amino-caproic acid per ml of sepharose.

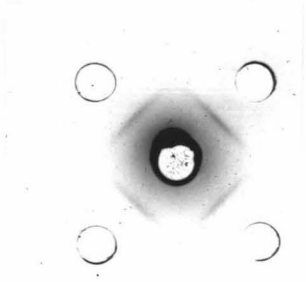
Figure 3.08

OUCHTERLONY IMMUNODIFFUSION ANALYSIS OF RABBIT GAMMA GLOBULIN



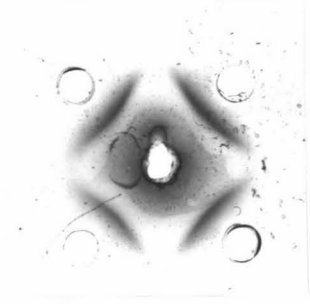
Centre Well - Pure Sheep Heart PFK

Outside Wells - Antiserum from Rabbit A



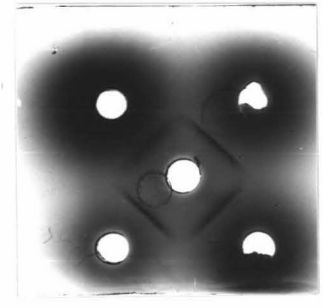
Centre Well - Crude Sheep Heart PFK Extract

Outside Wells - Antiserum from Rabbit A



Centre Well - Pure Sheep Heart PFK

Outside Wells - Antiserum from Rabbit B



Centre Well - Crude Sheep Heart PFK Extract

Outside Wells - Antiserum from Rabbit B

About 40mg of protein was added to each of the two last columns, and the washings were assayed to determine the amount of protein not attached to the column. It was assumed any protein not accounted for would be attached to the sepharose. The first column indicated that 77% of the PFK had attached to the column, but with the second column no protein had apparently attached. It was decided to try elution with both columns, in case the protein assays had been incorrect.

3-4.07 CYANOGEN BROMIDE ACTIVATED SEPHAROSE

Accurate protein assays could not be made on the washings, either by Coomassie Blue protein assay, or the PFK activity assay. This was probably due to the presence ofinhibitory components in the washings. Elution procedures were carried out on the assumption that PFK had attached to the column.

3-4.08 ELUTION OF ANTIBODIES FROM THE AFFINITY COLUMNS

The elution profile expected is shown in Figure 3.09. Non-specific antibodies should not attach to the PFK on the column, and thus should wash through with phosphate buffer. The combination of relatively low pH and high ionic strength of the next buffer (0.1M Sodium phosphate, 0.5M NaCl, pH 2.7), should remove semi-specific antibodies, or ionically bound antibodies. The final buffers (0.1M glycine-HCl, pH 2.7 or 3M KCNS, pH 7.2), should disrupt the antigen-antibody bonds, and thus the specific anti-PFK antibodies should be eluted.

CDI Coupled Column:
Figures 3.10 and 3.11, show typical elution profiles from the column, using both pH 2.7 and 3M KCNS as the final buffer.

The pH 2.7 buffer was originally used, but it was thought that the high acidity could have been denaturing the PFK on the column. With each successive elution from the same column, the elution profiles

Figure 3.09
EXPECTED ELUTION PROFILE FROM AN AFFINITY COLUMN

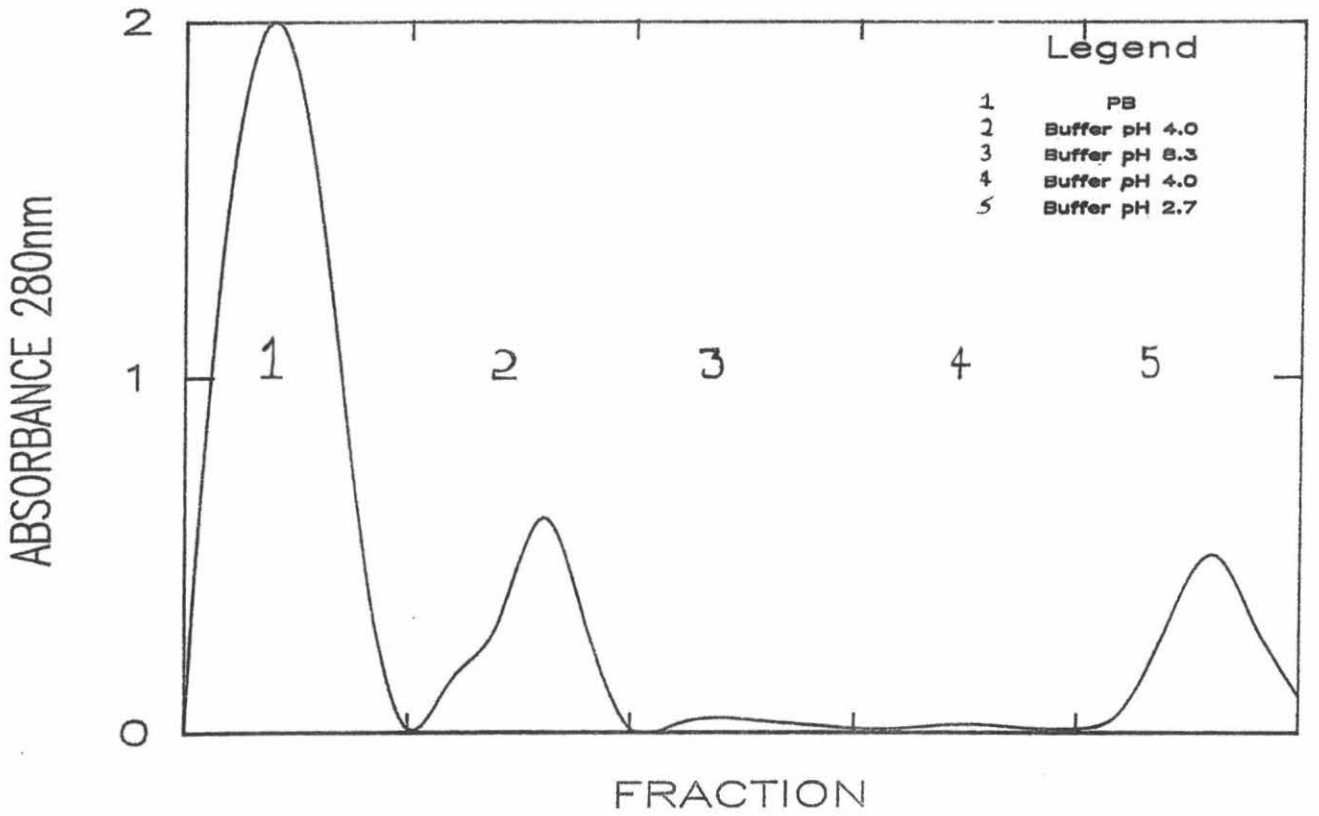


Figure 3.10
ELUTION PROFILE FROM CDI–COLUMN – Method 1

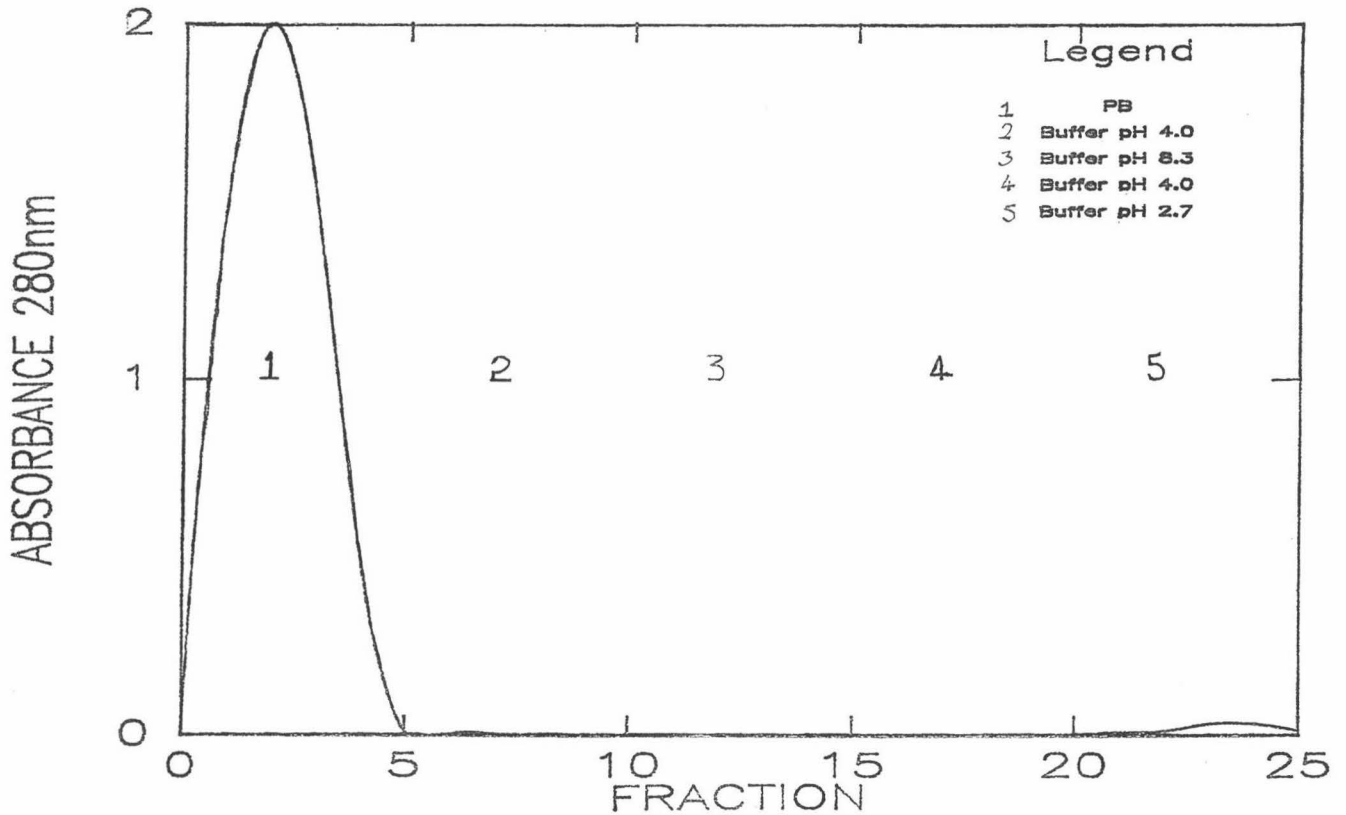
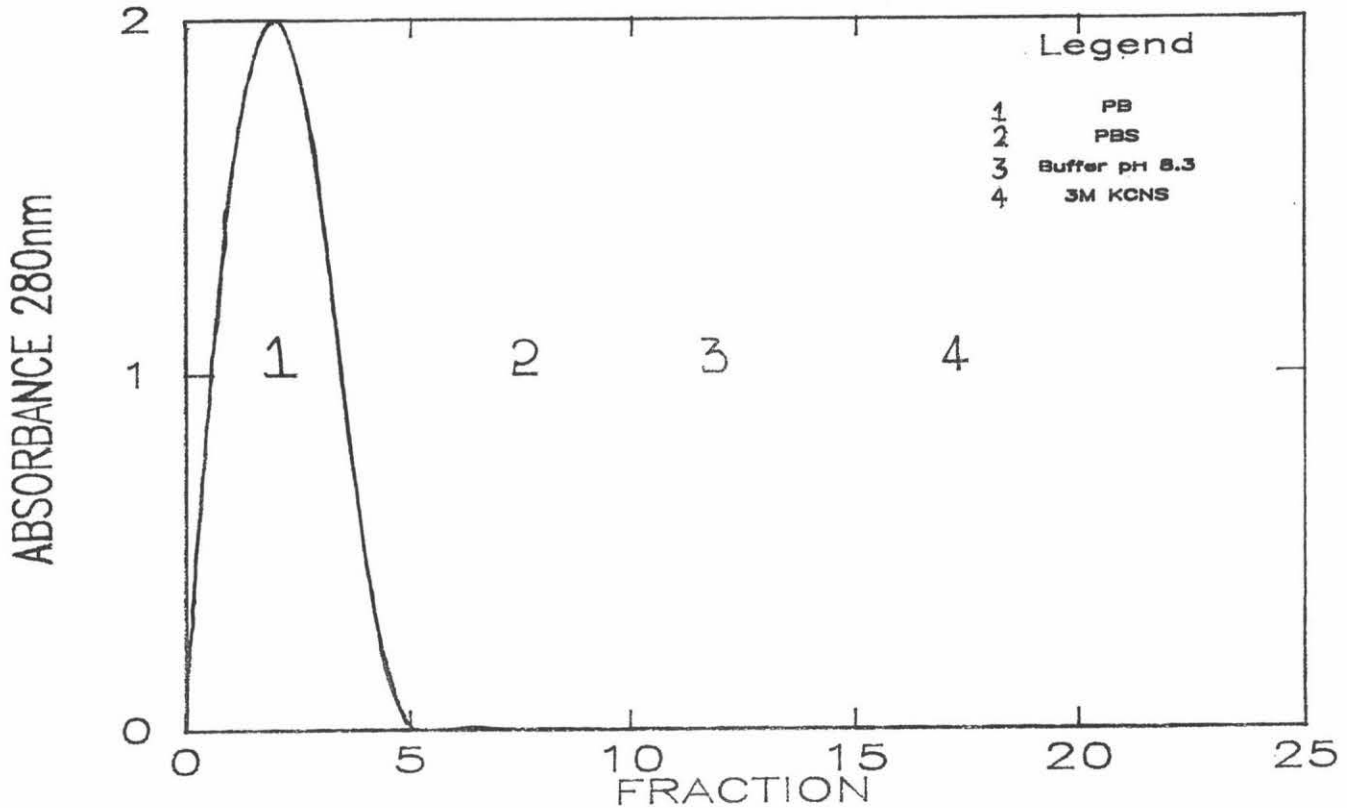


Figure 3.11
ELUTION PROFILE FROM CDI–COLUMN – Method 2



became less similar to the expected profile. The 3M KCNS, while effective in disrupting the antigen-antibody bond, was less likely to have this effect.

However, even with the change in buffer, and repeated elution attempts, effective separation of the non-specific and specific antibodies could not be achieved. Micro-Ouchterlony plates of the various fractions indicated the presence of specific antibodies in the phosphate buffer fraction. Protein assays on the eluted fractions indicated that about 95% of the antibody was eluted on the first elution, and up to 100% of the antibodies were eluted with successive elutions from the same column. Thus it is most likely that the specific antibodies were not attaching to the PFK on the column, rather than being too strongly bound, and unable to be eluted with the buffers used.

It was not known why the antibodies were not attaching to the column, but it was probably due to the PFK losing its antigenicity during the coupling to the resin.

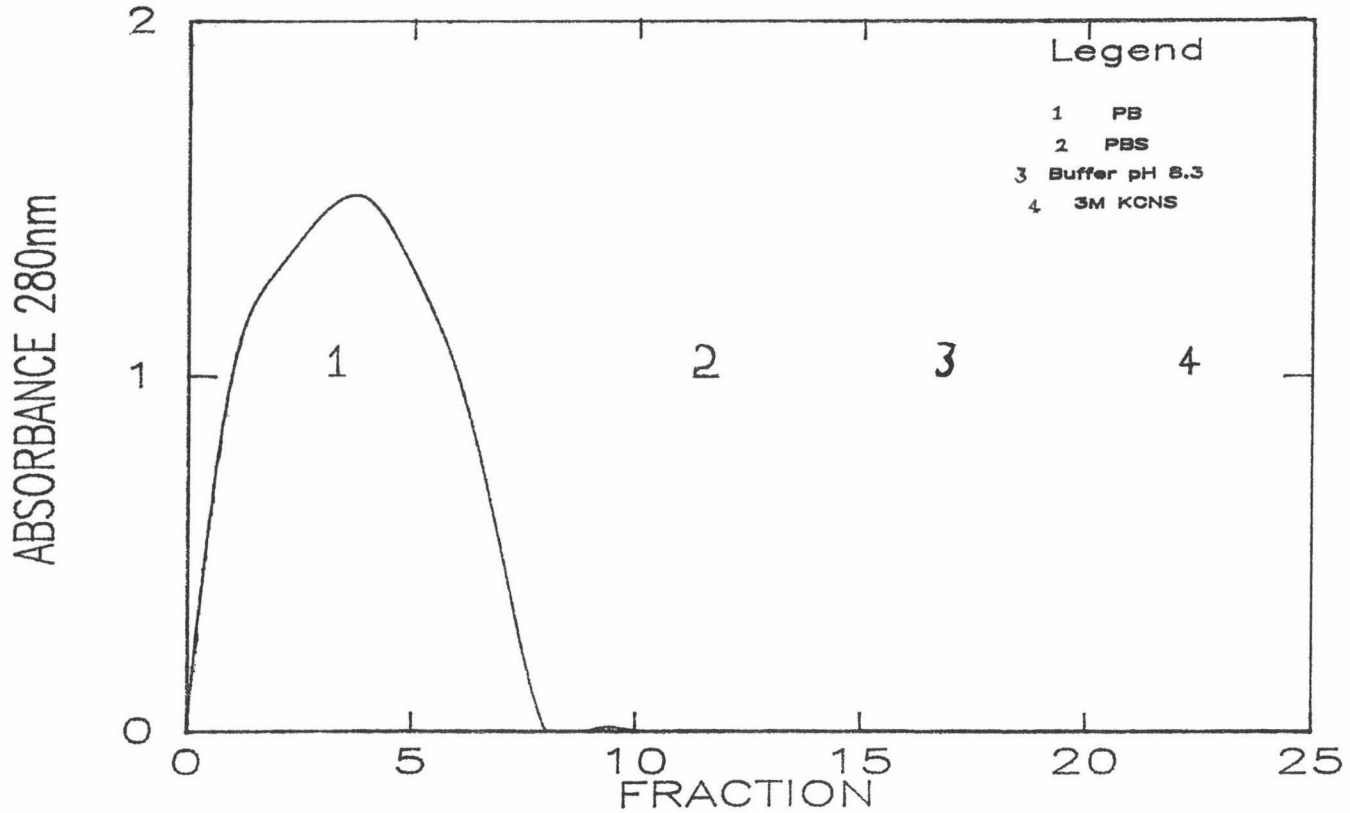
Cyanogen Bromide Coupled Column:
A similar elution profile (Figure 3.12), was produced with this column. Again, the specific antibodies were eluted with the non-specific antibodies in the first fraction. Either the PFK had failed to attach and had passed straight through the column, or the PFK had attached, but had lost its antigenicity.

3-4.09 THE SPECIFICITY OF ANTIBODY PREPARATIONS BY IMMUNOPRECIPITATION FOLLOWED BY POLYACRYLAMIDE GEL ELECTROPHORESIS

25µl of immumoprecipitate, 20µl of original supernatant, and 40µl of pure PFK was applied to the gel. The samples were labelled as follows:

Lane 1 - Sheep liver was homogenised in wash buffer and

Figure 3.12
ELUTION PROFILE FROM CYANOGEN BROMIDE–COLUMN – Method 2



centrifuged. The precipitate was resuspended in extract buffer, heat treated and centrifuged. The supernatant was applied to the gel - L4.

Lane 2 - Sheep liver was homogenised in wash buffer and centrifuged. The supernatant was applied to the gel - L3.

Lane 3 - The immunoprecipitate of the supernatant used for lane 2 - L1

Lane 4 - The immunoprecipitate of the supernatant used for lane 1 - L2

Lane 5 - The immunoprecipitate of pure sheep heart PFK

Lane 6 - The immunoprecipitate of the supernatant used for lane 9 - H2

Lane 7 - The immunoprecipitate of the supernatant used for lane 8 - H1

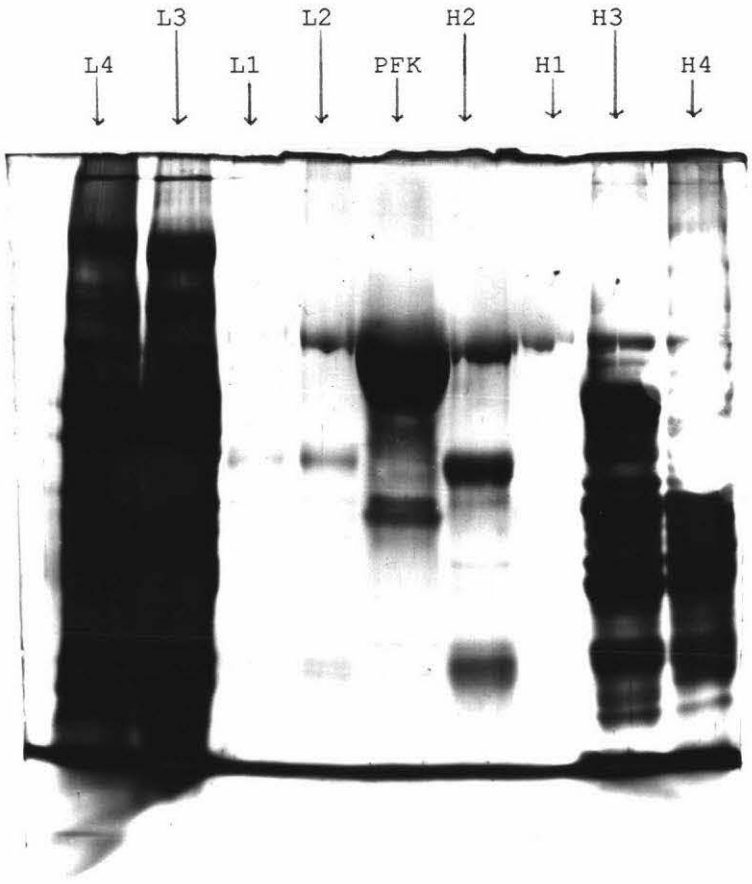
Lane 8 - Sheep heart was homogenised in wash buffer and centrifuged. The supernatant was applied to the gel - H3.

Lane 9 - Sheep heart was homogenised in wash buffer and centrifuged. The precipitate was resuspened in extract buffer, heat treated and centrifuged. The supernatant was applied to the gel - H4.

See Figure 3.13 for the silver stain of the gel. Lanes 1-4 concern sheep liver and are explained in section 5-4.02. Lanes 8 and 9 (H3 and H4 respectively), show a smear of proteins as would be expected from homogenates. Lanes 5,6 and 7 (pure PFK, H2 and H1 respectively) show three major bands corresponding to PFK, and the heavy and light chains of the immunoglobulin. The bands are very light in H1, indicating that there is not much PFK present in the wash supernatant. On the other hand, the bands for H2 are quite dark indicating that most of the PFK is present in the extract supernatant. The silver stain shows the presence of other proteins in lanes 5,6 and 7, thus the antibodies are reacting with proteins other than PFK. However the antibodies to these proteins must be present in low concentrations, as

Figure 3.13

SDS GEL (8%) ANALYSIS OF IMMUNOPRECIPITATES OBTAINED FROM SHEEP HEART



KEY

L1 - Immunoprecipitate of Crude Sheep Liver Wash - 25µl

L2 - Immunoprecipitate of Crude Sheep Liver Extract - 25µl

L3 - Crude Sheep liver Wash - 20µl

L4 - Crude Sheep Liver Extract - 20µl

H1 - Immunoprecipitate of Crude Sheep Heart Wash - 25µl

H2 - Immunoprecipitate of Crude Sheep Heart Extract - 25µl

H3 - Crude Sheep Heart Wash - 20µl

H4 - Crude Sheep Heart Extract - 20µl

PFK - Pure Sheep Heart PFK - 40µl

the immunoprecipitates of the contaminating antibodies can only be visualised using the very sensitive silver stain

3-4.10 WESTERN BLOTTING

Western Blot 1:

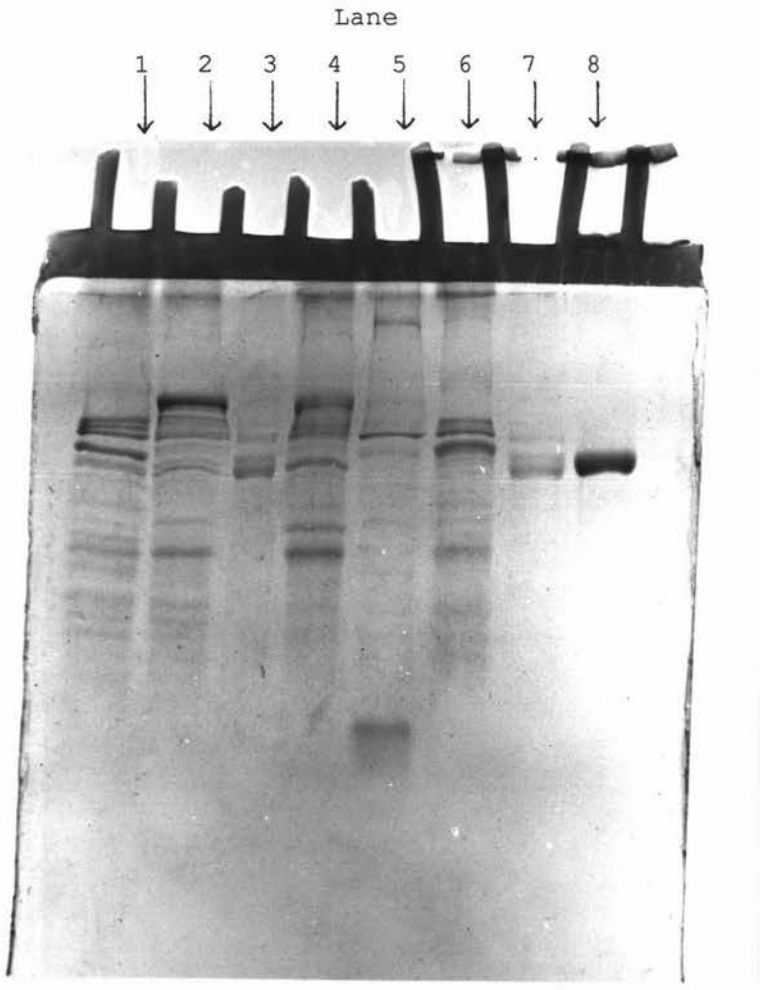
Applied to the gel was 25µl of crude rat heart, crude rat liver, crude sheep liver, crude sheep muscle and crude sheep heart sample, plus 20µl of pure sheep heart PFK and pure sheep liver PFK. Massive overheating was encountered during the blotting of this gel. The voltage and current had increased with time. The Coomassie Blue stained gel (Figure 3.14), indicated poor transfer of the high molecular weight proteins, and efficient transfer of the low molecular weight proteins. The nitrocellulose blot (Figure 3.15), showed a high background level. However, the track of interest ,6 (crude sheep heart), showed a single band at the level of PFK (compare with tracks 3 and 7, pure sheep heart PFK), with only very minor contaminant bands. Refer to section 5-4.03 for explanations of the reactions of the other samples. This blot was evidence in itself that the antibody solution was 'pure' enough to use for the immunogold experiments, but another Western Blot was performed, slightly modified, in order to alleviate some of the problems.

Western Blot 2:

Applied to this polyacrylamide gel was 25µl of crude sheep heart, crude sheep muscle and crude sheep liver extracts, plus 25µl of pure sheep heart PFK. A constant voltage was maintained during this blot, and overheating did not result. Coomassie blue staining of the gel, following blotting, revealed that 100% of the protein was transfered to the nitrocellulose. The nitrocellulose blot (Figure 3.16), indicated a much lower background staining than the previous blot. A distinct band was revealed in lane 5 (crude sheep heart), with no evident contaminating bands, supporting the conclusions determined from the previous blot. Lanes 4 and 6 (pure sheep heart PFK), show

Figure 3.14

SDS GEL (8%) ANALYSIS OF PROTEINS REMAINING AFTER WESTERN BLOTTING



KEY

Lane 1 - 25µl crude rat heart extract

Lane 2 - 25µl crude rat liver extract

Lane 3 - 20µl pure sheep heart PFK

Lane 4 - 25µl crude sheep liver extract

Lane 5 - 25µl crude sheep muscle extract

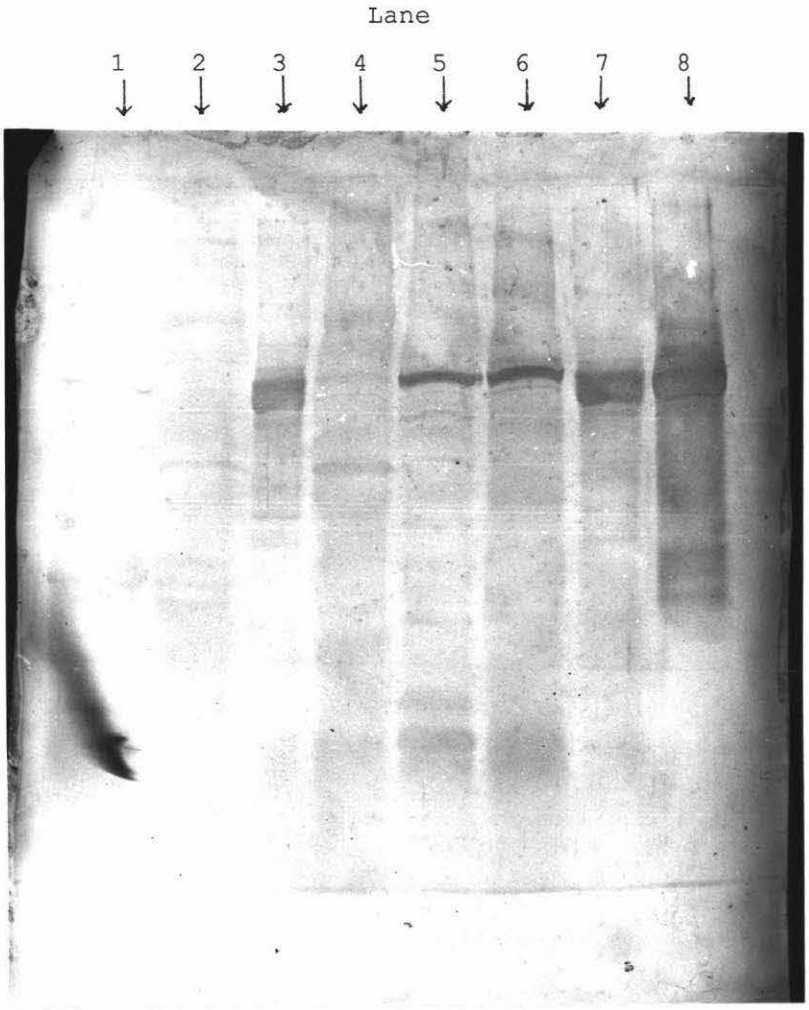
Lane 6 - 25µl crude sheep heart extract

Lane 7 - 20µl pure sheep heart PFK

Lane 8 - 20µl pure sheep liver PFK

Figure 3.15

NITROCELLULOSE BLOT OBTAINED FROM WESTERN BLOTTING OF AN SDS GEL (8%) - Method 1



KEY

Lane 1 - 25µl crude rat heart extract

Lane 2 - 25µl crude rat liver extract

Lane 3 - 20µl pure sheep heart PFK

Lane 4 - 25µl crude sheep liver extract

Lane 5 - 25µl crude sheep muscle extract

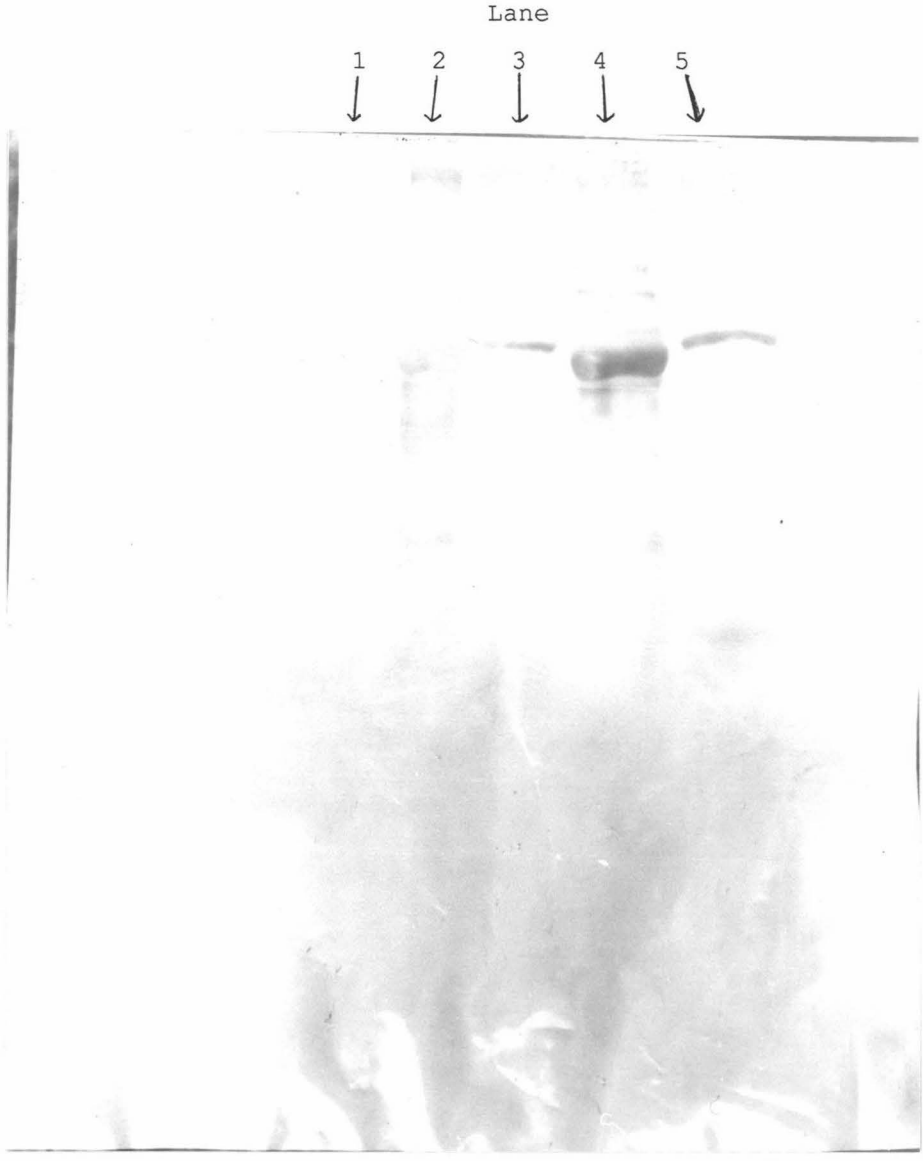
Lane 6 - 25µl crude sheep heart extract

Lane 7 - 20µl pure sheep heart PFK

Lane 8 - 20µl pure sheep liver PFK

Figure 3.16

NITROCELLULOSE BLOT OBTAINED FROM WESTERN BLOTTING OF AN SDS GEL (8%) - Method 2



KEY

Lane 1 - 25µl crude sheep liver extract

Lane 2 - 25µl pure sheep heart PFK

Lane 3 - 25µl crude sheep heart extract

Lane 4 - 25µl pure sheep heart PFK

Lane 5 - 25µl crude sheep muscle extract

slight smears of stain around the PFK band. These unexpected reactions were probably due to reactions of the antibody solution with PFK breakdown products, as the PFK samples were about six months old. Refer to section 5-4.03 for explanations of the other lanes.

3-4.11 IMMUNOGOLD LOCALISATION AND ELECTRON MICROSCOPY

Low Temperature Embedding:

Several grids were stained with the lead citrate in the absence of the antibodies or gold label, to check the quality of the cell slices before continuing with immunogold localisation. These slices showed that the cell structure was poorly preserved except for the myofibrils. This organelle destruction was probably due to the tissue freezing during embedding. The freezing could have resulted from an incomplete transfer from 30% to 50% ethanol, and 50% to 75% ethanol during the temperature drop stage (personal communication from A.Craig). A protocol suggested by A Craig for future immunogold studies of the sheep heart tissue was as follows:

30% ethanol for 1 hour at room temperature
50% ethanol for 1 hour at room temperature
50% ethanol for 1 hour at -20^{o}
75% ethanol for 1 hour at -20^{o}
75% ethanol for 1 hour at -35^{o}
95% ethanol for 1 hour at -35^{o}
100% ethanol for 1 hour at -35^{o}
100% ethanol for 1 hour at -35^{o}

Since the myofibrils were intact and a probable site for PFK location, immunogold localisation was carried out.

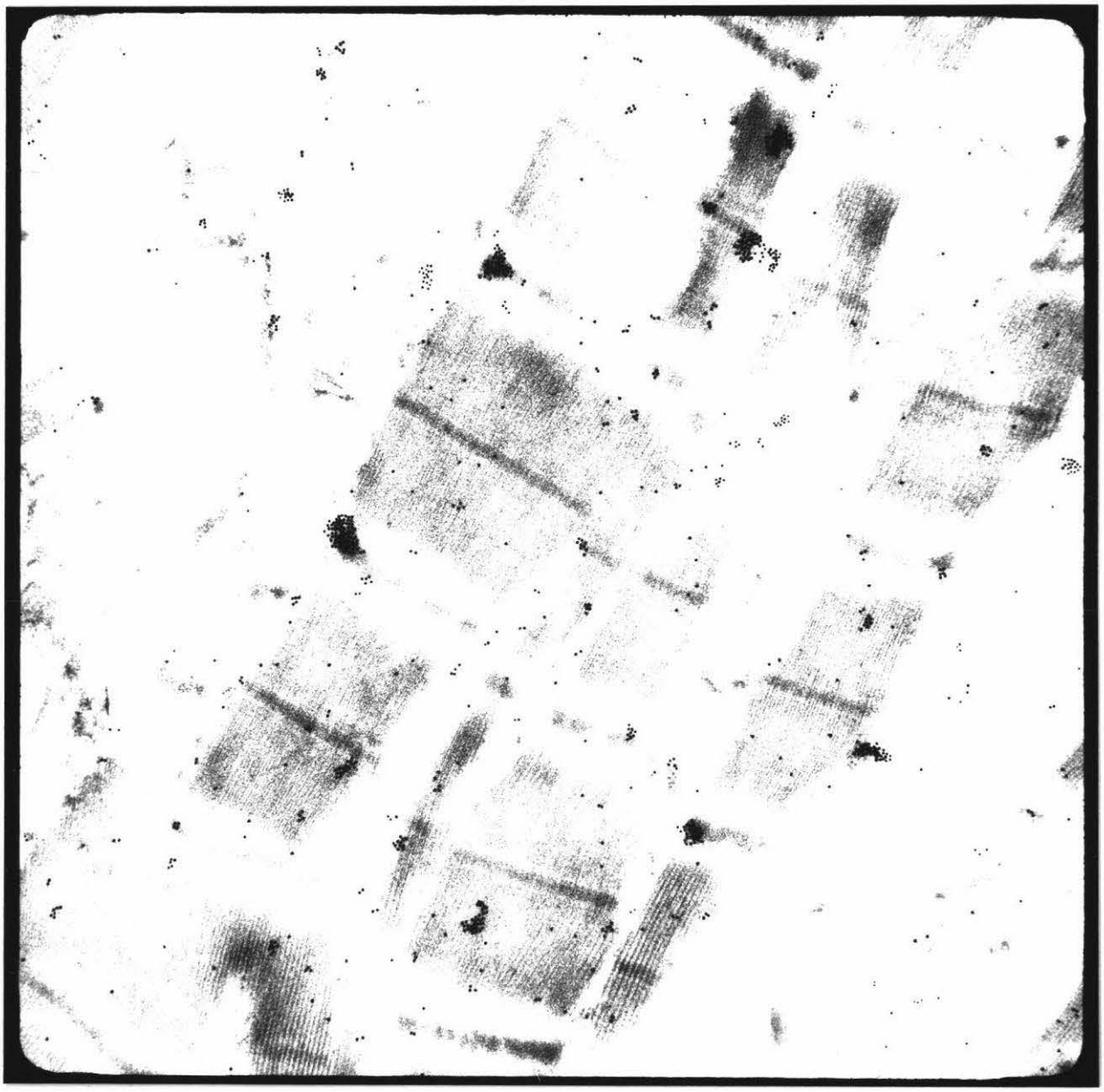
Electron Microscopy:

The grids incubated with the antibody which had been diluted by one hundredth, showed the greatest clarity and so were photographed, and used for determining the density of the gold labels. Two examples of the photographs, each with a magnification of 25,000x are shown

(Figures 3.17 and 3.18). The photomicrographs used for the counting of gold particles had a magnification of 31,500x and thus it could be determined that the total area of the electron micrographs was $38.688\mu m^2$. According to Table 3.01, the average staining density of the myofibrils with gold particles was about 4 to 4.5 times higher than that of the background. Figure 3.19 is an example of a photomicrograph (25,000x) obtained from the control grids (prepared in the same way as the other grids except for the absence of primary antibody solution), and this indicates that the gold labelled secondary antibody will only bind to the primary antibody, and thus there is very little non-specific binding of the labelled antibody to the heart tissue.

Figure 3.17

ELECTRON MICROGRAPH OF SHEEP HEART TISSUE STAINED WITH IMMUNOGOLD (25,000x)



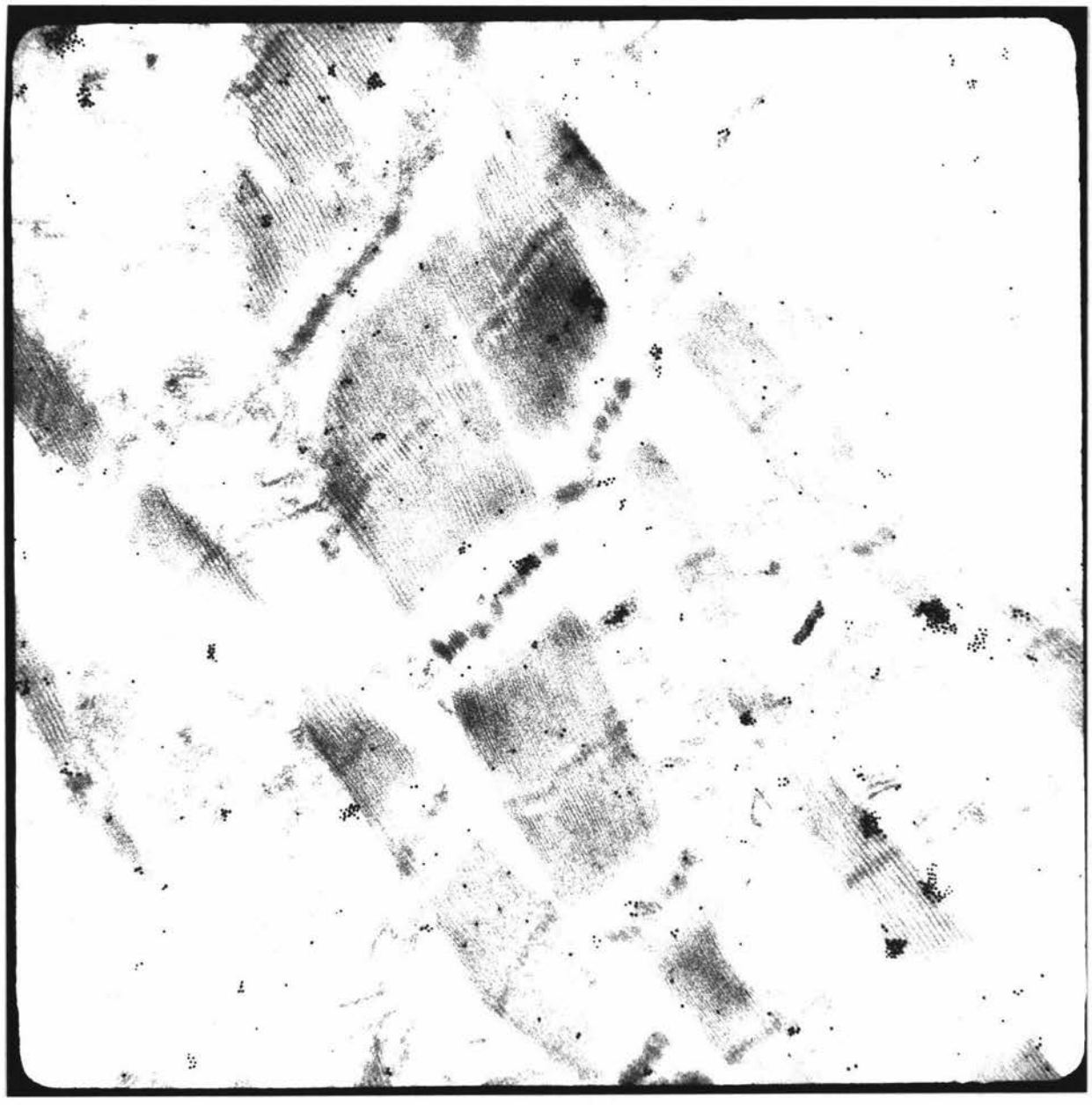
Staining Density of the Myofibrils - 36.3 gold particles per μm^2

Staining Density of the Background - 8.1 gold particles per μm^2

Staining Density of Myofibrils:Staining Density of Background 4.5:1

Figure 3.18

ELECTRON MICROGRAPH OF SHEEP HEART TISSUE STAINED WITH IMMUNOGOLD (25,000x)



Staining Density of the Myofibrils - 29.3 gold particles per μm^2

Staining Density of the Background - 9.6 gold particles per μm^2

Staining Density of Myofibrils:Staining Density of Background 3.0:1

Table 3.01

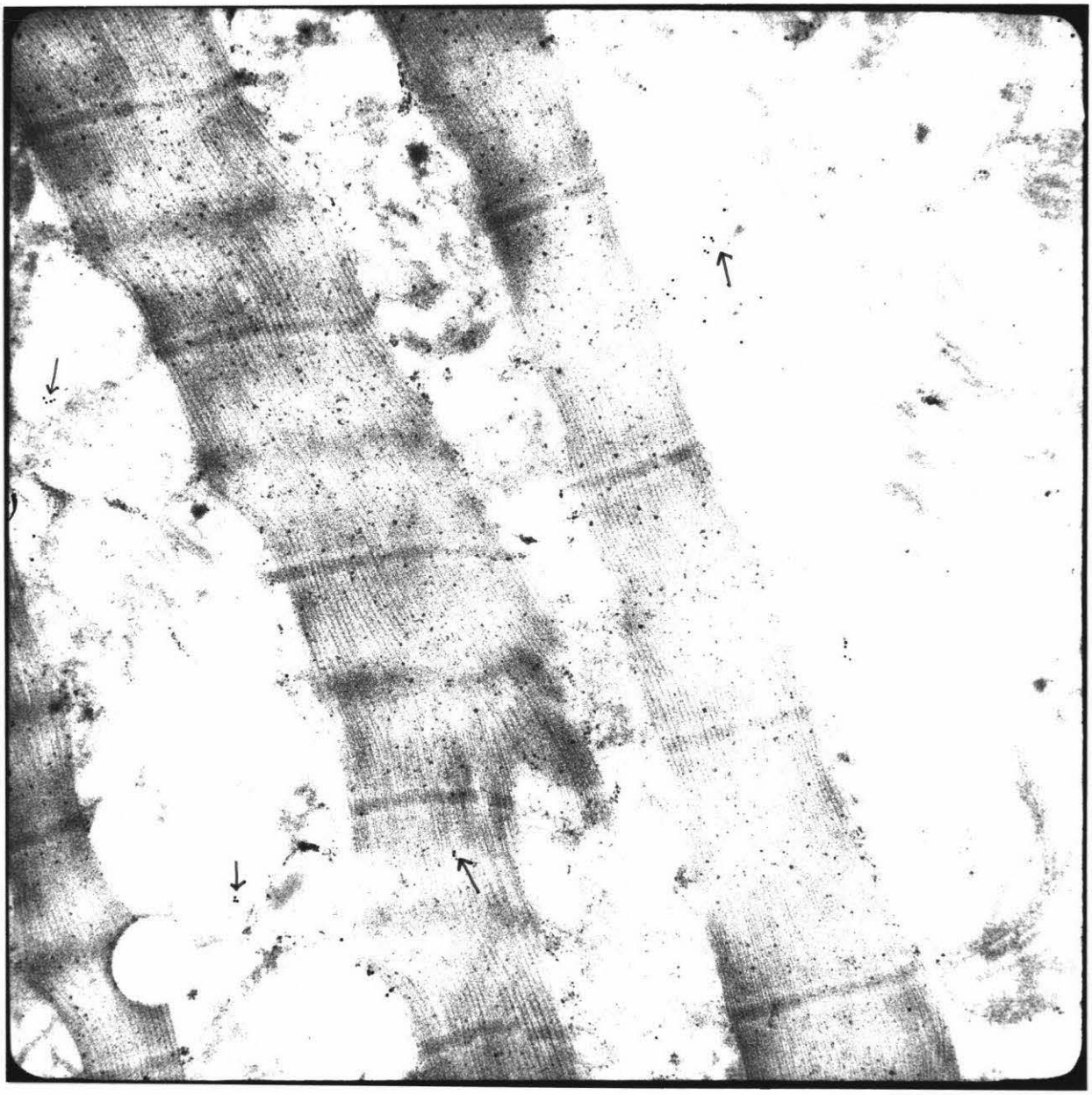
THE STAINING DENSITY OF GOLD LABELLED ANTIBODIES ON SHEEP HEART TISSUE

ELECTRON MICROGRAPH	1*	2	3	4
MYOFIBRILS				
% Area	29	30	52	41
No of Gold Particles	382	399	730	464
Gold Particles per μm^2	34.0	34.4	36.3	29.3
BACKGROUND				
% Area	66	70	48	59
No of Gold Particles	222	180	150	219
Gold Particles per μm^2	8.7	6.7	8.1	9.6
DENSITY OF MYOFIBRILS / DENSITY OF BACKGROUND	3.9	5.1	4.5	3.1

* 5% of this EM was stained excessively with lead stain and therefore could not be included

Figure 3.19

ELECTRON MICROGRAPH OF SHEEP HEART TISSUE STAINED WITH IMMUNOGOLD IN THE ABSENCE OF PRIMARY ANTIBODY (25,000x)



Although the Electron Micrograph appears to be heavily stained with gold particles, the majority of these black dots are in fact particles of lead stain. There are a few gold particles present and these are indicated by distinct, perfectly circular dots (indicated by arrows).

CHAPTER 4 THE EFFECTS OF METABOLITES ON THE BINDING OF PFK

4-1 INTRODUCTION

Mansour *et al* (1966), have reported that PFK can be extracted from a sedimentable fraction of skeletal muscle homogenates after incubation with ATP and $MgSO_4$. The initial steps in the purification of PFK (3-3.01), are based on these findings. This section describes attempts to determine whether or not the degree of solubilisation of the enzyme was related to the concentration of ATP or $MgSO_4$.

Mansour *et al* (1966), also reported that agents other than ATP were effective in solubilising the enzyme. These effectors included ADP, AMP, cyclic AMP, and high concentrations of phosphates and sulphates. Massey and Deal (1973), suggested that Mg^{2+} ions inhibit the solubility of PFK indirectly, by sequestering phosphate containing metabolites such as ATP and fructose-6-phosphate. They also report that other divalent cations such as Mn^{2+}, Ca^{2+} and Zn^{2+} inhibit solubilisation. Tarui *et al* (1972), state that erythrocyte PFK activity is inhibited by 2,3-diphosphoglycerate and this inhibition can be relieved by inorganic phosphate but not by ATP or ADP. Further solubilisation experiments to verify and expand this list of effectors, are described in this section.

While studying the effects of glucagon on rat hepatocytes, Van Schaftingen *et al* (1980a), observed the presence of a low molecular weight stimulator of the liver PFK. It was demonstrated that this effector also stimulated the activity of rat muscle PFK (Van Schaftingen *et al*, 1980b). Uyeda *et al* (1981), positively identified this activator as fructose-2,6-bisphosphate, and showed that the levels of fructose-2,6-bisphosphate in hepatocytes decreased after the

administration of glucagon, and increased following the addition of glucose to the medium. One of the roles of fructose-2,6-bisphosphate in hepatic tissue was the regulation of the PFK/fructose-1,6-bisphosphatase cycle (Schellenberger *et al*, 1985). The role of this metabolite in muscle tissue is not yet established but since it affects the activity of muscle PFK, it may play some role in the regulation of glycolysis.

Experiments were performed on sheep heart tissue to determine if the presence of fructose-2,6-bisphosphate in the medium would enhance the solubilisation of PFK from the membrane.

4-2 MATERIALS

Sheep hearts were obtained from Waitaki Freezing Company, Feilding. All general chemicals were reagent grade or better, and obtained from May and Baker Ltd, Dagenham, England, Serva, New York, British Drug Houses or Ajax Chemicals. ATP was the sodium salt, grade I from Sigma Chemical Company, St. Louis, U.S.A. Other biochemicals were also obtained from Sigma. All water was deionised and distilled.

4-3 METHODS

4-3.01 THE EFFECT OF CHANGING EFFECTOR CONCENTRATIONS ON THE BINDING OF PFK

Frozen sheep heart was homogenised in 2 volumes of wash buffer (10mM Tris-HCL, 2mM EDTA, pH 8.0), using an UltraTurrax homogeniser. The resulting homogenate was centrifuged at 12,000g for ten minutes and the precipitate resuspended in 2 volumes partial extraction buffer

(20mM Tris-HCL, 5mM 2-mercaptoethanol, 0.5mM EDTA, ph 8.6). Aliquots of this suspension were taken and added to solutions of extract buffer containing appropriate concentrations of the effector being tested. The final volume was adjusted with partial extract buffer so that suitable concentrations were obtained in a final volume equivalent to 4 times the weight of heart tissue taken. The adjusted samples were then homogenised with the UltraTurrax, heated to 40^{o} for five minutes, and centrifuged at 12,000g for ten minutes. Aliquots of the supernatant were taken for PFK assay to determine the amount of enzyme released.

4-4 RESULTS AND DISCUSSION

4-4.01 THE EFFECT OF CHANGING EFFECTOR CONCENTRATIONS ON THE BINDING OF PFK

The Effect of ATP Concentration

Final samples contained $5x10^{-4}$, $2x10^{-4}$, $1x10^{-4}$, $0.5x10^{-4}$, $0.2x10^{-4}$, $0.1x10^{-4}$ and zero Molar ATP. As can be seen from Figure 4.01, ATP appears to have no effect on releasing the bound PFK from the precipitate. A reasonably constant amount of PFK activity is recorded in all samples, regardless of ATP concentration.

The Effect of $MgSO_4$ and ATP

The final concentrations of the samples were as follows:

Sample 1 - No ATP or $MgSO_4$

Sample 2 - 50mM $MgSO_4$

Sample 3 - 0.5mM ATP

Sample 4 - 0.5mM ATP and 50mM $MgSO_4$

See Table 4.01. The ATP appears to have no effect at all on the association of PFK with the membrane. $MgSO_4$ alone results in the same

Figure 4.01
PFK ACTIVITY versus ATP CONCENTRATION

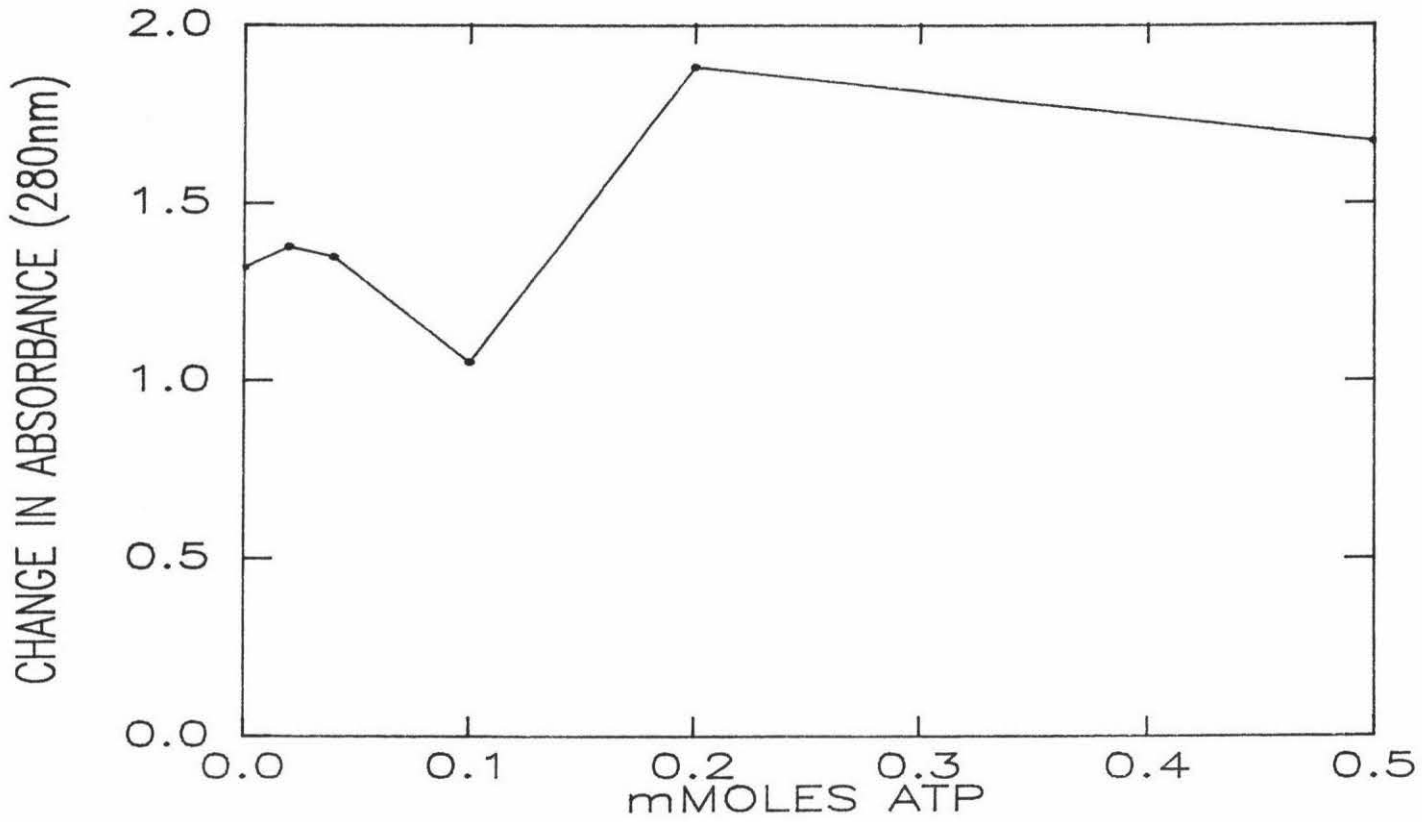


Table 4.01

THE EFFECT OF $MgSO_4$ AND ATP ON THE RELEASE OF PFK FROM THE MEMBRANE

SAMPLE CONTENTS	% PFK ACTIVITY
No ATP or $MgSO_4$	7
50mM $MgSO_4$	100
0.5mM ATP	12
50mM $MgSO_4$, 0.5mM ATP	95

degree of activity as with both $MgSO_4$ and ATP. Whereas, 5mM ATP alone results in only a small fraction of the total PFK activity.

The Effect of $MgSO_4$ and Na_2SO_4

The final concentrations of the samples were 1.0, 0.4, 0.2, 0.1, 0.04, 0.02 0.01 and zero Molar of both $MgSO_4$ and Na_4SO_4. See Figure 4.02. This graph clearly indicates the relationship between the concentrations of $MgSO_4$ and Na_2SO_4, and the degree of solubilisation of PFK from the membrane. Up to 5mM the salts have no effect, but from 5-50mM the relationship is reasonably linear.

The Specificity of the Binding Mechanism for $MgSO_4$

The samples contained 50mM Na_2SO_3, $MgSO_4$, $Na_2S_2O_3$, Na_2SO_4, NaCl, $Mg(NO_3)_2$, CH_3COONa, $MgCl_2$, $(CH_3COO)_2Mg$, $NaNO_3$, $CaCl_2$, or $MnCl_2$. According to Table 4.02, the divalent anions appear to be important in solubilising PFK from the membrane; specifically, sulphite, sulphate and thiosulphate ions. The cation seems unimportant, as can be seen from comparing the values for $MgSO_4$ and $MgCl_2$.

The Effect of Fructose-2,6-bisphosphate

The final concentrations were as follows:

Sample 1 - 1µm fructose-2,6-bisphosphate

Sample 2 - 1µm fructose-2,6-bisphosphate, 50mM $MgSO_4$

Sample 3 - 1µm fructose-2,6-bisphosphate, 0.5mM ATP

Sample 4 - 50mM $MgSO_4$

See Table 4.03. In contrast to the results by other researchers, discussed in the introduction, the fructose-2,6-bisphosphate appears to have an inhibitory effect on the activity of PFK. Without the presence of $MgSO_4$, the fructose-2,6-bisphosphate results in very little PFK activity released from the membrane. When added to the extraction buffer, along with $MgSO_4$, there is a decrease in activity as compared with $MgSO_4$ alone. This is also the case when fructose-2,6-bisphosphate is added after the PFK has been solubilised from the membrane by the $MgSO_4$. These results suggest that the fructose-2,6-

Figure 4.02
PFK ACTIVITY versus MgSO4 or Na2SO4 CONCENTRATION

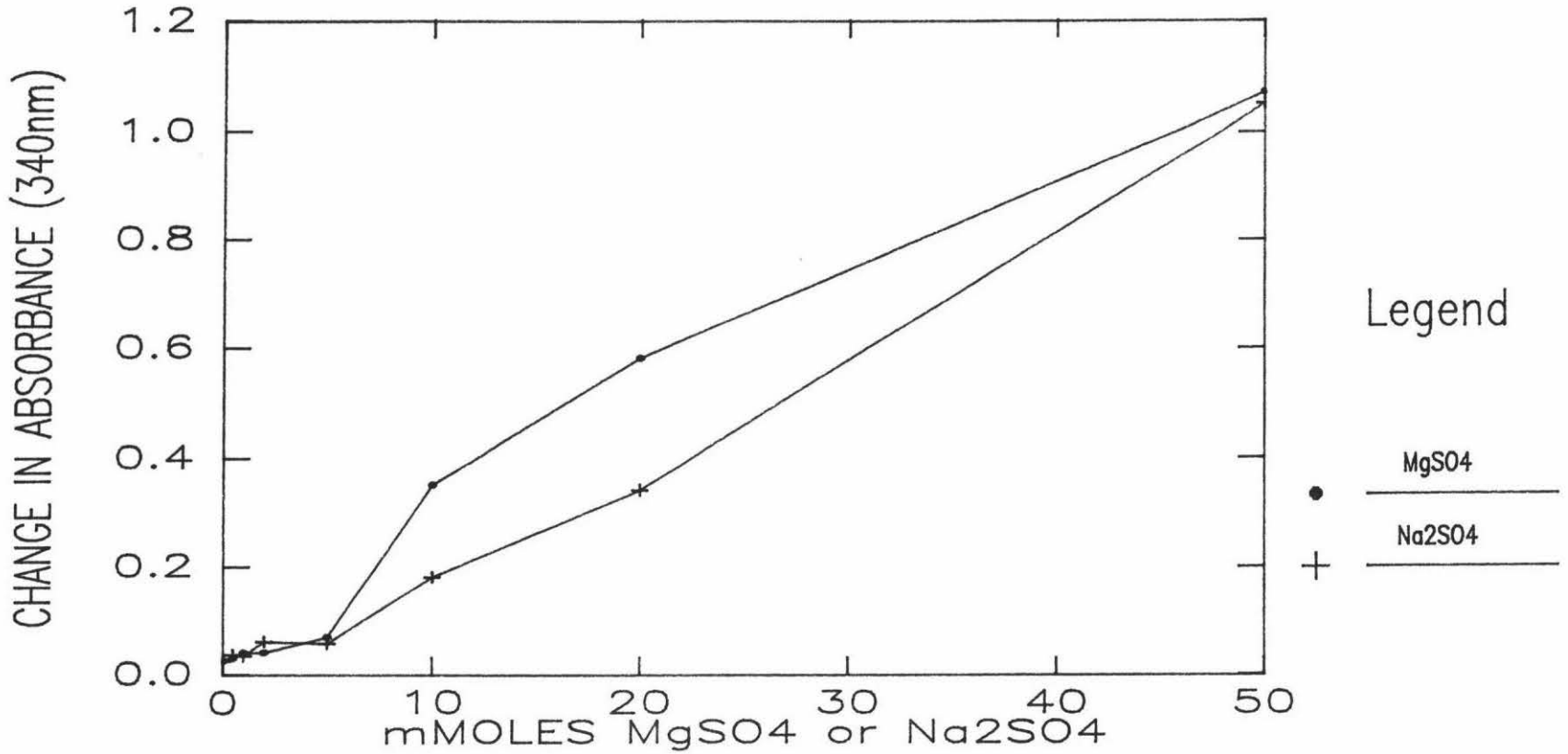


Table 4.02

THE EFFECT OF DIFFERENT SALTS ON THE ON THE RELEASE OF PFK FROM THE MEMBRANE

SALT (50mM)	% PFK ACTIVITY
$Na_2SO_3.7H_2O$	100
$MgSO_4.7H_2O$	98
$Na_2S_2O_3.7H_2O$	87
Na_2SO_4	58
NaCl	40
$Mg(NO_3)_2.6H_2O$	33
$CH_3COONa.3H_2O$	23
$MgCl_2.6H_2O$	17
-	17
$(CH_3COO)_2Mg.4H_2O$	16
$Na_2CO_3.10H_2O$	0
$NaNO_3$	0
$CaCl_2$	0
$MnCl_2$	0

Table 4.03

THE EFFECT OF FRUCTOSE-2,6-BISPHOSPHATE ON THE RELEASE OF PFK FROM THE MEMBRANE

SAMPLE CONTENTS	% PFK ACTIVITY
1uM F-2,6-P_2	2
1uM F-2,6-P_2, 50mM $MgSO_4$	71
1uM F-2,6-P_2, 0.5mM ATP	12
50mM $MgSO_4$,	100
50mM $MgSO_4$, (1)	66
50mM $MgSO_4$, (2)	13

(1) 1uM Fructose-2,6-bisphosphate was added to the solution following extraction by 50mM $MgSO_4$.

(2) 50mM $CaSO_4$ was added to the solution following extraction by 50mM $MgSO_4$.

bisphosphate has no effect on the solubilisation of PFK, but inhibits the activity of the enzyme (directly or indirectly) once it has has been extracted from the membrane.

CHAPTER 5 SPECIES AND TISSUE COMPARISONS

5-1 INTRODUCTION

This section describes the attempts to study some of the properties of PFK from sources other than sheep heart. The cross reactivity of anti sheep heart PFK antibodies with PFK from other tissues and species (especially sheep liver) was studied, with a view to possible future immunogold localisation experiments with tissues other than sheep heart.

In 1964, Lowry and Passonneau observed differences in the sensitivities to effectors of PFK activity in crude homogenates from six different rat tissues. Later, on the basis of ion exchange chromatography elution profiles and antibody precipitation tests, it was established that at least four types of PFK were present both in human tissue (Layzer and Conway, 1970) and in rat tissue (Tanaka *et al*, 1971). Kemp (1971), when comparing the properties (stability, molecular size, kinetics and electrophoretic mobility) of highly purified rat skeletal muscle and liver PFK, found them to be distinctly different. Tsai and Kemp (1973), following electrophoretic and immunochemical studies on rabbit tissue PFKs, found their data to be consistent with there being three types of PFK monomers.

Skeletal and cardiac muscle have been shown to contain an isoenzyme consisting of one type of monomer only. This isoenzyme has been labelled A. Liver and erythrocytes contain isoenzyme B, only. Lung, adipose and stomach tissue contain A/B hybrids yielding 5 isoenzymes. Tsai and Kemp (1973) found a third residual component in brain tissue, which they labelled isoenzyme C. This isoenzyme was later isolated in a pure form by Foe and Kemp (1985).

Tsai and Kemp (1973) studied the cross reactivity of rabbit liver and muscle PFK antiserum with several other tissues. Their results are outlined in Table 5.01.

In this section the cross reactivity of sheep heart PFK antiserum was studied using SDS Gel analysis of immunoprecipitates (3-3.02), and Western Blotting (3-3.10).

5-2 MATERIALS

Sprague-Dawley rats were obtained from the Small Animal Production Unit at Massey University. Sheep tissue was obtained from Waitaki Freezing Company, Feilding. Pure sheep liver PFK (6.6 units per ml) was purified and supplied by K. Markwick, Massey University. All general chemicals were reagent grade or better, and obtained from May and Baker Ltd, Dagenham, England, Serva, New York, British Drug Houses or Ajax Chemicals, Sydney. Biochemicals were obtained from Sigma Chemical Company, St.Louis, U.S.A. All water was distilled and deionised.

5-3 METHODS

5-3.01 COMPARISON BETWEEN THE SOLUBILISATION OF PFK IN SHEEP AND RAT HEARTS

Crude PFK extracts were obtained from 5g of both rat and sheep heart, according to the method described in 3-3.04. The supernatants obtained from the first centrifugation were also kept for analysis. All samples were assayed for PFK (3-3.02).

Table 5.01

PRECIPITATION OF PFK FROM CRUDE EXTRACTS BY LIVER PFK AND MUSCLE PFK ANTISERUM

TISSUE	% ACTIVITY REMOVED BY	
	LIVER PFK ANTISERUM	MUSCLE PFK ANTISERUM
Heart	2	92
Skeletal Muscle	4	92
Liver	93	25
Erythrocytes	96	32
Lung	58	58
Stomach	58	67
Adipose	64	58
Cerebrum	11	50
Thymocyte	27	21
Testes	43	52
Spleen	58	40
Uterus	62	30
Kidney Cortex	72	32

From Tsai and Kemp (1973), Table III

5-3.02 ANALYSIS OF IMMUNOPRECIPITATES FORMED BY ANTI SHEEP HEART PFK ANTIBODIES BY SDS GEL ELECTROPHORESIS

Sheep heart and sheep liver samples were prepared, electrophoresed and silver stained according to 3-3.09

5-3.03 DETERMINATION OF THE CROSS REACTIVITY OF ANTI SHEEP HEART ANTIBODIES WITH PFK FROM OTHER TISSUES AND SPECIES BY WESTERN BLOTTING

Crude rat heart, crude rat liver, crude sheep heart, crude sheep liver and crude sheep muscle extracts were prepared, Western blotted onto nitrocellulose, and stained according to 3-3.10 (Method 1). Pure sheep heart PFK and pure sheep liver PFK were also blotted and stained the same way.

5-4 RESULTS AND DISCUSSION

5-4.01 COMPARISON BETWEEN THE SOLUBILISATION OF PFK IN SHEEP AND RAT HEARTS

See Table 5.02. All the PFK (100%) in sheep heart is present on the membrane until solubilised from the membrane by the extract buffer. This is supported by results from Mansour *et al* (1966), which also show that almost 100% of the PFK activity can be found in the 'activated extract'. With rat hearts, 87% of the PFK activity can be located in the wash supernatant, indicating that at the time of death, most of the PFK was present in the cytosol.

This difference may be due to a species difference in the control of glycolysis, or due to the state of the animal at the time of death. Hormone levels ,such as insulin, glucagon and epinephrine, differ

Table 5.02

THE % OF CYTOSOLIC OR PARTICULATE PFK ACTIVITY IN SHEEP OR RAT HEARTS

SPECIES	% PARTICULATE	% CYTOSOLIC
SHEEP	100	0
RAT	13	87

Sheep or Rat hearts were homogenised with 2 volumes of wash buffer, and centrifuged at 12,000g. The PFK activity present in the supernantant was deemed 'cytosolic'. The precipitate was homogenised in 2 volumes of extract buffer and centrifuged at 12,000g. The PFK activity present in this supernatant was labelled 'particulate'.

according to the fed or starved state of the animal (Stryer, 1981; Ganong, 1975). There is evidence (Knull *et al*, 1973 and 1974) to suggest that the levels of these circulating hormones may have an effect on the proportion of soluble/particulate PFK.

5-4.02 ANALYSIS OF IMMUNOPRECIPITATES FORMED BY ANTI SHEEP HEART PFK ANTIBODIES BY SDS GEL ELECTROPHORESIS

See Figure 5.01. 25µl of immunoprecipitate, 20µl of the supernatants, and 40µl of pure PFK immunoprecipitate was applied to the gel. The samples were labelled as described in 3-4.09. The reactions of the sheep heart preparations are explained in 3-4.09. Lanes 1 and 2 (L4 and L3 respectively), show the smear of proteins expected from homogenates. Lanes 3 and 4 (L1 and L2 respectively), indicate three faint bands corresponding to PFK and the heavy and light chains of the immunoglobulin. The bands are very faint, even though silver stained, so the cross reactivity of the anti sheep heart PFK antibodies with sheep liver PFK is minimal.

5-4.03 DETERMINATION OF THE CROSS REACTIVITY OF ANTI SHEEP HEART ANTIBODIES WITH PFK FROM OTHER TISSUES AND SPECIES BY WESTERN BLOTTING

See Figure 5.02. 25µl of the crude extracts, and 20µl of the pure extracts were applied to the SDS polyacrylamide gel. The samples were applied as follows:Lane 1 - Crude rat heart

Lane 2 - Crude rat liver

Lane 3 - Pure sheep heart PFK

Lane 4 - Crude sheep liver

Lane 5 - Crude sheep muscle

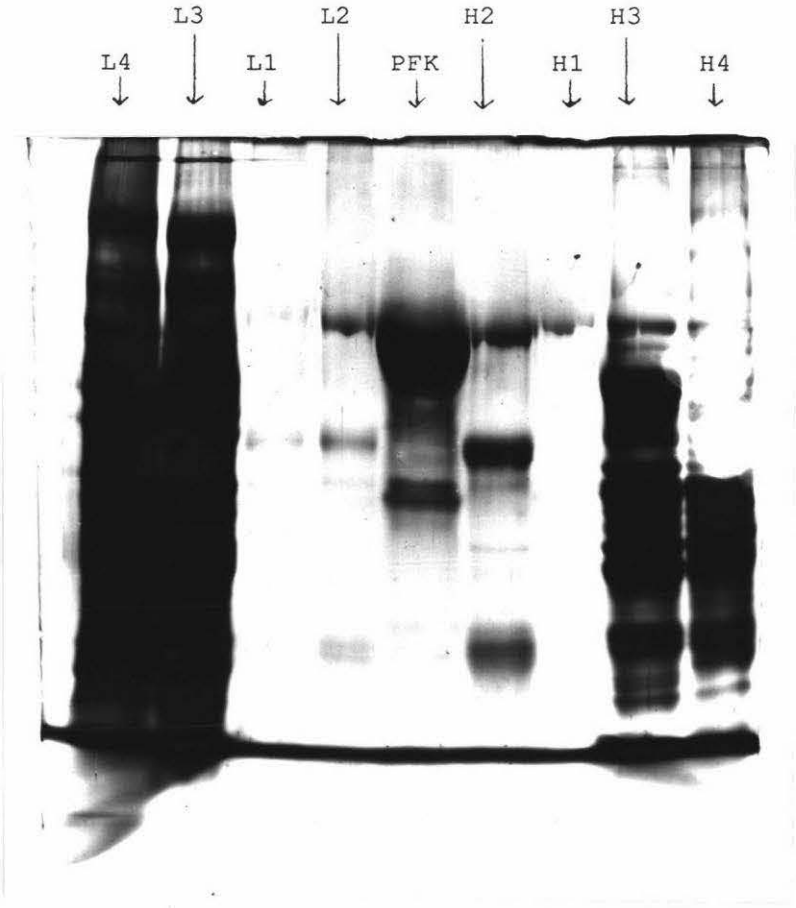
Lane 6 - Crude sheep heart

Lane 7 - Pure sheep heart PFK

Lane 8 - Pure sheep liver PFK

Figure 5.01

SDS GEL (8%) ANALYSIS OF IMMUNOPRECIPITATES OBTAINED FROM SHEEP HEART



KEY

L1 - Immunoprecipitate of Crude Sheep Liver Wash - 25µl

L2 - Immunoprecipitate of Crude Sheep Liver Extract - 25µl

L3 - Crude Sheep liver Wash - 20µl

L4 - Crude Sheep Liver Extract - 20µl

H1 - Immunoprecipitate of Crude Sheep Heart Wash - 25µl

H2 - Immunoprecipitate of Crude Sheep Heart Extract - 25µl

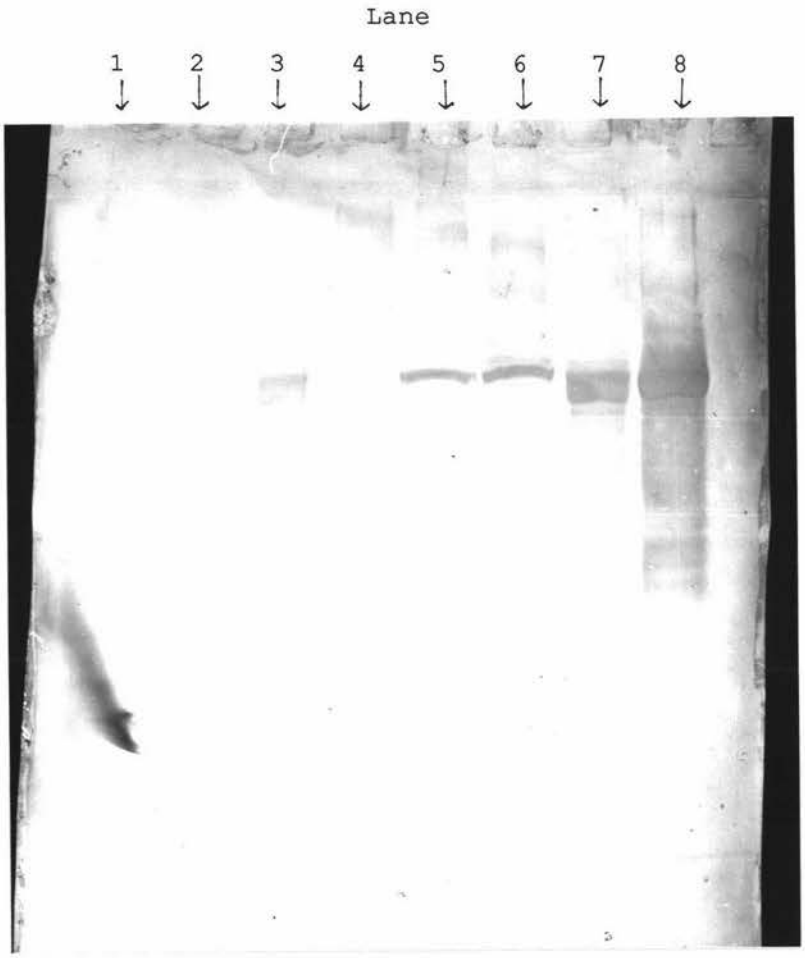
H3 - Crude Sheep Heart Wash - 20µl

H4 - Crude Sheep Heart Extract - 20µl

PFK - Pure Sheep Heart PFK - 40µl

Figure 5.02

NITROCELLULOSE BLOT OBTAINED FROM WESTERN BLOTTING OF AN SDS GEL (8%) - Method 1



KEY

Lane 1 - 25µl crude rat heart extract

Lane 2 - 25µl crude rat liver extract

Lane 3 - 20µl pure sheep heart PFK

Lane 4 - 25µl crude sheep liver extract

Lane 5 - 25µl crude sheep muscle extract

Lane 6 - 25µl crude sheep heart extract

Lane 7 - 20µl pure sheep heart PFK

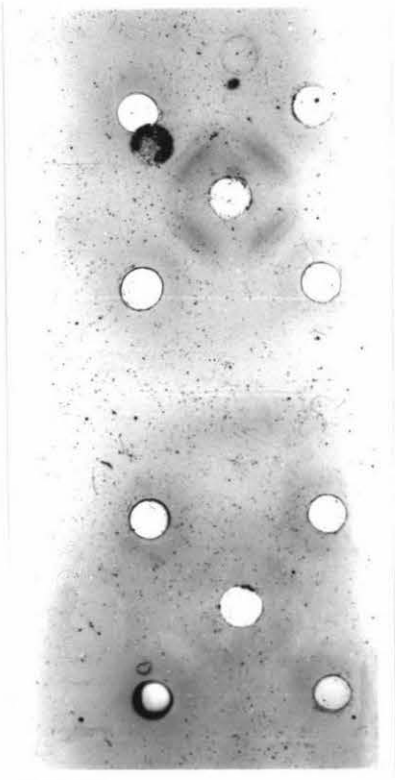
Lane 8 - 20µl pure sheep liver PFK

As can be seen from the gel, there is a distinct band, corresponding to the PFK-antibody complex, in lanes 3, 5, 6 and 7 (pure sheep heart PFK, crude sheep muscle, crude sheep heart and pure sheep heart PFK, respectively). These results are expected since heart PFK and muscle PFK are the same isoenzyme (Tsai and Kemp, 1973). Lane 1 (crude rat heart), has only a faint band in the PFK-antibody complex location, indicating that there may be a species difference in the heart/muscle isoenzyme. As expected lanes 2 and 4 (crude rat liver and crude sheep liver respectively), produced no clear band due to the difference in isoenzymes. Lane 8 produced an unexpected result since it shows a thick band corresponding to the PFK-antibody complex bands. It is not understood why this pure extract of liver PFK reacted with the antibodies, while the crude extract didn't. Possibly, there was not enough antigen present in the crude extract. The possible cross reactivity of the liver PFK with the anti sheep heart PFK antibodies was further examined with Micro-Ouchterlony precipitation plates (3-3.05) of fresh pure sheep liver PFK samples (figure 5.03). These plates showed the presence of only very faint lines of antigen-antibody precipitates. The lines may be faint due to very low levels of the enzyme in the tissue.

From these results, it can be concluded that the antibody solution prepared (3-3.03), could be used for immunogold analysis of sheep muscle and possibly rat heart. However, since not much cross-reactivity is evident with liver (rat or sheep), then immunogold experimentation would not be successful. For immunogold localisation of liver PFK, antibodies against purified liver PFK would need to be used.

Figure 5.03

OUCHTERLONY IMMUNODIFFUSION ANALYSIS OF THE CROSS REACTIVITY OF ANTI-SHEEP HEART ANTIBODIES WITH PURE SHEEP LIVER PFK



Centre Well - Pure Sheep Liver PFK
Batch 1

Outside Wells - Antiserum from
Rabbit A

Centre Well - Pure Sheep Liver PFK
Batch 2

Outside Wells - Antiserum from
Rabbit A

CHAPTER 6 CONCLUSIONS

Subcellular Fractionation.

The subcellular fractionation experiments produced ambiguous and inconclusive results. This was due primarily to the inherent difficulties in fractionating a fibrous tissue such as heart. Applying sufficient mechanical force to rupture the cardiac cells, yet maintain the intactness of the subcellular structures was difficult to achieve. However, the results did indicate that the PFK was attached to a dense organelle, since it was found to be located mainly in fractions which precipitated at 800g.

Immunogold Localisation

By determining the staining densities of the gold labelled secondary antibody on the sheep heart sections, it was shown that the myofibrils had a 4 to 4.5 times higher degree of staining compared with other organelles (mitochondria, plasmalemma, endoplasmic reticulum etc.). From these preliminary results it can be tentatively proposed that the myofibril is the probable site for PFK binding in sheep heart muscle.

Effect of Metabolites

It it apparent from the results in Chapter 4, that ATP does not play a direct role in solubilisation of PFK. In subsequent extraction experiments, the deletion of ATP from the extraction buffer, resulted in no decrease in the capacity of the buffer to release PFK. On the other hand, the concentrations of $MgSO_4$ and Na_2SO_4 in this buffer are directly related to the degree of solubilisation. Other salts, specifically sodium sulphite, and sodium thiosulphate, result in higher degrees of solubilisation. It appears from these results that divalent anions play an important role in the release of PFK from the membrane. It is unlikely that the cation is of importance since other sodium and magnesium salts, such as $MgCl_2$, Na_2CO_3, NaCl, result in

very low levels of solubilisation. The effect of fructose-2,6-bisphosphate is uncertain but it appears to inhibit PFK, once the enzyme has been released from the membrane. This is contrary to the results of Van Schaftingen *et al* (1980b), and Uyeda *et al* (1981), who state that fructose-2,6-bisphosphate stimulates the activity of rat muscle PFK. Further investigation is needed to determine the effect of fructose-2,6-bisphosphate on both PFK activity and its binding to subcellular organelles.

Cross Reactivity of Anti Sheep Heart PFK Antibodies with PFK from other Tissues and Species

The anti sheep heart PFK antibodies cross react with PFK in sheep muscle, and to a certain extent, rat heart. It should be possible therefore, to carry out similar immunogold localisation experiments on these tissues, using the anti sheep heart antibodies. No cross reactivity was evident with crude sheep or rat liver extracts, either due to lower levels of this enzyme being present in the tissue, or the heart and liver isoenzymes being sufficiently different so as not to allow cross reactivity. A band due to cross reactivity is evident in the Western blot of pure sheep liver PFK (Figure 5.02), but only faint lines of precipitation are visible with Ouchterlony immunodiffusion analysis. For successful immunogold localisation of liver PFK, antibodies against purified liver PFK would need to be used.

Future Investigations

Subcellular fractionation of tissue is proving inadequate for the investigation of ambiquitous enzymes. The following quotes from recent publications by workers in this field, indicate the potential problems with this approach.

'A number of workers over many years have sought evidence for the existence of a glycolytic complex. Most of the investigations were concerned with the cytosolic fraction of cells, however, and it is now well known that homogenisation and centrifugal fractionation are

deeply disruptive to the complex structure and organisation of the cytomatrix.' Masters, (1984).

'A thorough understanding of such binding would seem to be fundamental to any detailed understanding of the many structural and functional implications of these phenomena, but in pursuing these goals, enzymologists may have to abandon some of the ingrained conclusions derived from the *in vitro* studies of uniphasic systems. For example, many of the classical methods of extracting and fractionating enzymes and subcellular structure may not be favourable, and indeed may act to preclude the isolation of organised multienzyme systems - hence the continued references to the importance of innovative technologies in future investigations.' Masters, (1981).

'Many biochemists have adhered to the classical homogenisation-centrifugal fractionation scheme and have taken the results of such a procedure to indicate the 'localisation' of the enzyme, with the implicit assumption that such localisation was as characteristic of the enzyme as its catalytic activity. Hence such terms as 'mitochondrial matrix' enzymes, etc., have appeared in the literature. We wish to offer the suggestion that this may not be correct and that the reversible variation in 'localisation' may represent one mechanism (of several) by which regulation of catalytic activity, the *sine qua non* for metabolic regulation, may be achieved.' Nemat-Gorgani and Wilson, (1980)

Further investigation into the localisation of sheep heart PFK, and also other PFKs should centre on the refinement and further development of the immunogold technique. Although a major limitation on the localisation procedure carried out in this thesis was the disintegration of some cellular components during the embedding process, with the consequent difficulties in calculating the staining densities of specific organelles, such as mitochondria, future immunogold localisation procedures performed using the revised low

temperature embedding process, outlined in Chapter 3, should produce electron micrographs of tissue with intact subcellular structure, and thus give a more convincing location.

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