BIOCHEMICAL CHARACTERIZATION OF A NOVEL MAMMALIAN POLYPHOSPHATE DEPENDENT GLUCOKINASE

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Declaration

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgment has been made in the text.

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Abstract

Hexokinases are a family of enzymes that catalyse the phosphorylation of glucose by transferring the γ- phosphoryl group from adenosine triphosphate (ATP) to the sixth position hydroxyl group of glucose to generate Glucose 6-phosphate (G6P).

Until now, five isozymes of mammalian hexokinase (HK) have been described: types I, II, III and IV, all of them ATP dependent, and type V which is ADP-dependent. The present thesis describes a novel hexokinase, we have designated as PPGKm. The enzyme is strictly polyphosphate (pp) dependent, is correctly defined as a glucokinase (GK), by virtue of its kinetics and is present in mammalian tisues, at high activity in liver. The enzyme does not use ATP and ADP and indeed appears to be inhibited by them. As far as can be ascertained this is the first description of a mammalian enzyme using inorganic polyphosphate as a phosphoryl donor. Polyphosphate is used as phosphoryl donor in bacterial systems and is quite well characterized, although only two micro-organisms have been shown to be strictly pp-dependent like the one described here.

This novel enzyme (PPGKm) also showed unique features, differing from the others hexokinases in having a longer half-life and can be stored for several months at -20˚C without loss of activity. However, thermal stability was lower than other hexokinases studied.

The enzyme activity is concentrated in the hepatocyte nucleus has a higher molecular weight compared to Hexokinases 1, 2 and 3. The full biological significance of the enzyme is as yet unclear, and attempts to purify and sequence it have been only partly successful. Its specific role in cellular, and especailly nuclear metabolism remains unknown.

Abbreviations

- **ADP** Adenosine diphosphate
- **ANOVA** Analysis of variance
- **AP4A** Diadenosin tetraphosphate
- **AP5A** Diadenosin pentaphosphate
- **ATP** Adenosine triphosphate
- **CTP** Cyctidine triphosphate
- **DAPI** 4',6-diamidino-2-phenylindole
- **Ddppk1** Eukaryotes homolog to bacterial polyphosphate kinase1.
- **DH_2O** Distilled water
- **D_2O** Deuterium oxide
- **DTE** 1, 4-Dithioerythritol
- **DTT** Dithiothreitol
- **ESI-MS** Electrospray ionization mass-spectroscopy
- **F1P** Fructose 1 phosphate
- **F6P** Fructose 6 phosphate
- **FGF** Fibroblast growth factor
- **FK** Fructokinase
- **G1P** Glucose 1phosphate
- **G6P** Glucose 6 phosphate
- **GK** Glucokinase
- **GKRP** Glucokinase regulatory protein
- **GTP** Guanosin triphosphate
- **HK** Hexokinase
- **$HKDC_1$** Hexokinase domain containing 1
- **HMP** Hexamethaphosphate
- **HPLC** High pressure liquid chromatography
- **HSP70** Heat shock protein70
- **LC-MS/MS** Liquid chromatography mass-spectroscopy
- **MCF7** Human carcinoma cell line
- **mTOR** Mammalian target of rapamycine
- **MODY2** Maturity onset diabetes of the young type2
- **MS** Masss-pectroscopy
- **NAD** Nicotinamine adenine dinucleotide (oxidized form)
- **NADH** Nicotinamine adenine dinucleotide (reduced form)
- **NDK** Nucleotide diphosphate kinase
- **Na HEPES** Sodium salt (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
- **NMR** Nuclear magnetic resonance
- **PAP** Polyphosphate: AMP phosphotransferase
- **PCs** Human plasma cells
- **Pi** Inorganic phosphate

- **P_{700}** Polyphosphate of 700 chain length
- **PBS** Phosphate buffer saline
- **PHB** Polyhydroxybutyrate
- **Poly-Pi60** Polyphosphate has chain length of 60 phosphates
- **PPGK** Polyphosphate glucokinase
- **PPGKm** Mammalian polyphosphate glucokinase
- **Poly-Pi** Polyphosphate
- **PPK1** Polyphosphate kinase 1
- **PPX** Exoployphosphatase
- **Pvds** RNA polymerase Sigma factor gene of *Pseudomonas aeruginosa* .
- **RecA** Gene encodes DNA-recombinase
- **Rpos** Gene which encodes RNA polymerase sigma factor
- **SDS-PAGE** Sodium dodecyl sulphate- polyacrylamide gel electrophoresis
- **SOS** Global response to DNA damage
- **TOR** Target of rapamycine
- **Tri-P** Tripoly phosphate
- **Tris** (hydroxymethyl) aminomethane
- **TSP** Trimethlsilyl propanoic acid
- **UTP** Uridine triphosphate
- **VDAC** Voltage -dependent anion channel

- **Units:**

- **µg** Microgram
- **mg** Milligram
- **mM** Millimolar
- **mL** Milliliter
- **˚C** Degree celsius
- **V/V** Volume/Volume
- **W/V** Weight /Volume
- **IU** International unit
- **Ppm** Part per million

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Chapter 1

Introduction

1.1 Part One: Evolution of Hexokinases Isoenzymes and their regulatory role

Phosphorylation of glucose by hexokinases (EC 2.7.1.1) using ATP or ADP as a phosphoryl donor occurs in all eukaryotic, prokaryotic, and plant cells (Wilson, 2004). This reaction is considered as the first step in glucose utilization. The formation of glucose 6-phosphate (G6P) by hexokinase sequesters glucose inside the cell, and as a consequence, directs glucose to alternative metabolic pathways: the formation of glycogen and short-term carbohydrate storage in liver; immediate use in energy production by glycolysis; and the formation of pentose phosphates for anabolic purposes (Cárdenas., 2004).

Hexokinases were first described in yeast by Meyerhof; 1927 (Racker., 1946). There are three isoenzymes P_I, P_{II} and glucokinase (Maitra, 1970; Gancedo et al., 1977). In animal species, the activity of hexokinase isoenzymes was studied for the first time in rodents' livers (González et al., 1964). Further investigations in other animal species have shown isoenzyme activity in all animals, including humans (Cárdenas., 2004). Most invertebrates also show hexokinases activity. Four hexokinase isoenzymes are found in various vertebrates' tissues: I, II, III, and IV (Cárdenas., 2004).

In addition, hexokinases have been reported to be found in green plants – two types of glucose- and fructose-phosphorylating enzymes have been discovered in plants: hexokinases (HKs) and fructokinases (FKs) (Millerd et al, 1951; Saltman, 1953; Medina and Sols., 1956). Several HK and FK (EC 2.7.1.4) isozymes have been identified in protein extracts of various plant species. While FK activities have been found to be specific to fructose, the HKs from various species have been found to be capable of phosphorylating glucose, fructose, mannose, and glucosamine, but not galactose (Saltman., 1953), similar to fungal, mammalian, and protozoan HKs (Cárdenas et al., 1984; Fekete et al., 2004; Rui and Hahn., 2007).

Isozymes capable of phosphorylating glucose have often been identified as glucokinases. However, unlike in the fungal kingdom, no glucose-specific glucokinase has been found in plants (Dai et al., 2002b). Therefore, in plants, glucose can be phosphorylated only by HK while fructose can be phosphorylated either by HK or FK. Therefore, HK and FK are the gateways for most organic metabolism in plants. These enzymes catalyze irreversible reactions and therefore may play important roles in the regulation of plant sugar metabolism.

1.1.1 Sugar specificity and naming conventions

Sugar specificity, of hexokinases in different organisms. Glucose is the preferred substrate of hexokinases, but hexokinases can still phosphorylate other hexoses to some degree. Only a few species like bacteria are known to contain hexokinases that predominantly or solely phosphorylate glucose (Ureta et al., 1987).

This has led to a lot of confusion in the naming of these enzymes. Generally speaking microbiologists call a hexokinase, glucokinase, if it predominantly or only phosphorylates glucose. However, in man and rodent species in which metabolic studies have been conducted to better understand glucose metabolism and insulin sensitivity, the name glucokinase has been reserved for Hexokinase D (IV) because this enzyme plays a pivotal role in glucose homeostasis as the part of glucose sensor in the beta cells, and in glucose uptake in the liver.

This creates potential confusion, because the microbiological community is largely separated from the metabolic and diabetes community, and certainly with respect to hexokinases.

This has also led to the inappropriate naming of of an ADP-dependent hexokinase as a glucokinase (Ronimus & Morgan., 2004) when it clearly exhibits non-sigmoidal kinetics and has a low Km for glucose :the whole point in naming mammalian HK D (IV) glucokinase is that it has sigmoidal kinetics and a very low affinity for glucose with an $S_{0.5}$ of approximately 8mM (within the physiological range of 4.4-6.1mM).The misnamed ADP-dependent enzyme has a Km of about 0.5mM glucose , far too low to have any influence on glycemic regulation .

Clearly, this needs to be addressed at the highest level to attempt a uniform naming convention.

The discovery of a real pp-dependent glucokinase, described in this thesis, will only make appreciation of these conventions more problematic for most workers unaware of these naming conventions and conflicts.

The following table (1.1-1) summarizes different species categorized according to their hexokinases specificity. It is concluded that more specific enzymes are generally found in simpler organisms, while higher animals with more complicated metabolic systems lack enzymes with high specificity for phosphorylation of glucose, mannose, or fructose (Cárdenas et al., 1998).

Taxon	High specificity	Intermediate specificity	Low specificity
Archaea	*Pyrococcus furiosus*		
Bacteria	*Streptococcus mutans*	*Leuconostoc meserentoides*	*E.coli*
	E. coli	*Streptomyces violaceruber*	
	Bacillus stearothermophilus	*E. coli*	
	Zymomonas mobilis		
	Aerobacter aerogenes		
	Rhodospirillum rubrum		
	Pseudomonas saccharophila		
	Breĺibacterium fuscum		
Lower eukaryotes	*Euglena gracilis*	*Saccharomyces cerevisiae*	*Sac. cerevisiae*
	Dictyostelium discoideum	*Torulopsis holmii*	*Neurospora cross*
	Candida sp	*Saprolegnia litoralis*	*Entamoeba histolytica*
	Sap. litoralis		*Trypanosoma equiperdum*
Green plants	*Pisum sativum* (peas)		*Triticum* (wheat)

Table 1.1–1 Different species categorized according to their hexokinase sugar specificity.

Note: Reprinted from "Evolution and Regulatory Role of the Hexokinases," by M. L. Cárdenas et al., 1998, *Biochimica et Biophysica Acta*, *1401*, 242–264.

1.1.2 Molecular mass of hexokinases in different phyla

It has been suggested that the evolution of vertebrates' hexokinases resulted from the duplication and fusion of the 50 kDa ancestral hexokinase, which is similar to the recent yeast and mammalian hexokinase IV size.

Taxon	Organism	Isoenzyme	Molecular mass kDa (native)
Archaea	*P. furiosus*	Glucokinase	93
Bacteria	*B. stearothermophilus*	Glucokinase	67
	S. mutans	Glucokinase	41
	E. coli	Glucokinase	
Fungi	*Sac. cerevisiae*	$P_I P_{II}$	102
	Sac. cerevisiae	Glucokinase	Aggregates
Protozoa	*Try. brucei*	Hexokinase	295
Plants	Wheat germ	L_I, L_{II}	50
	Wheat germ	H_I, H_{II}	100
Nematodes	*Hymenolepsis diminuta*	Hexokinase	98
Insects	*Drosophila*	Hexokinase A	47
Echinoderms	Starfish	Hexokinase	50
Chordates	Lamprey	Hexokinase	90
	Rat	Hexokinase A	98

Table 1.1–2 showing molecular mass of different hexokinases belongs to different organisms

In figure 1.1 a phlogenetic relationship of different hexokinase was illustrated showing the family of enzymes present in a variety of living organisms including plants, bacteria, yeast and vertebrates. The mutations / duplication or fusion, that happened along in the course of evolution of these organisms were indicated.

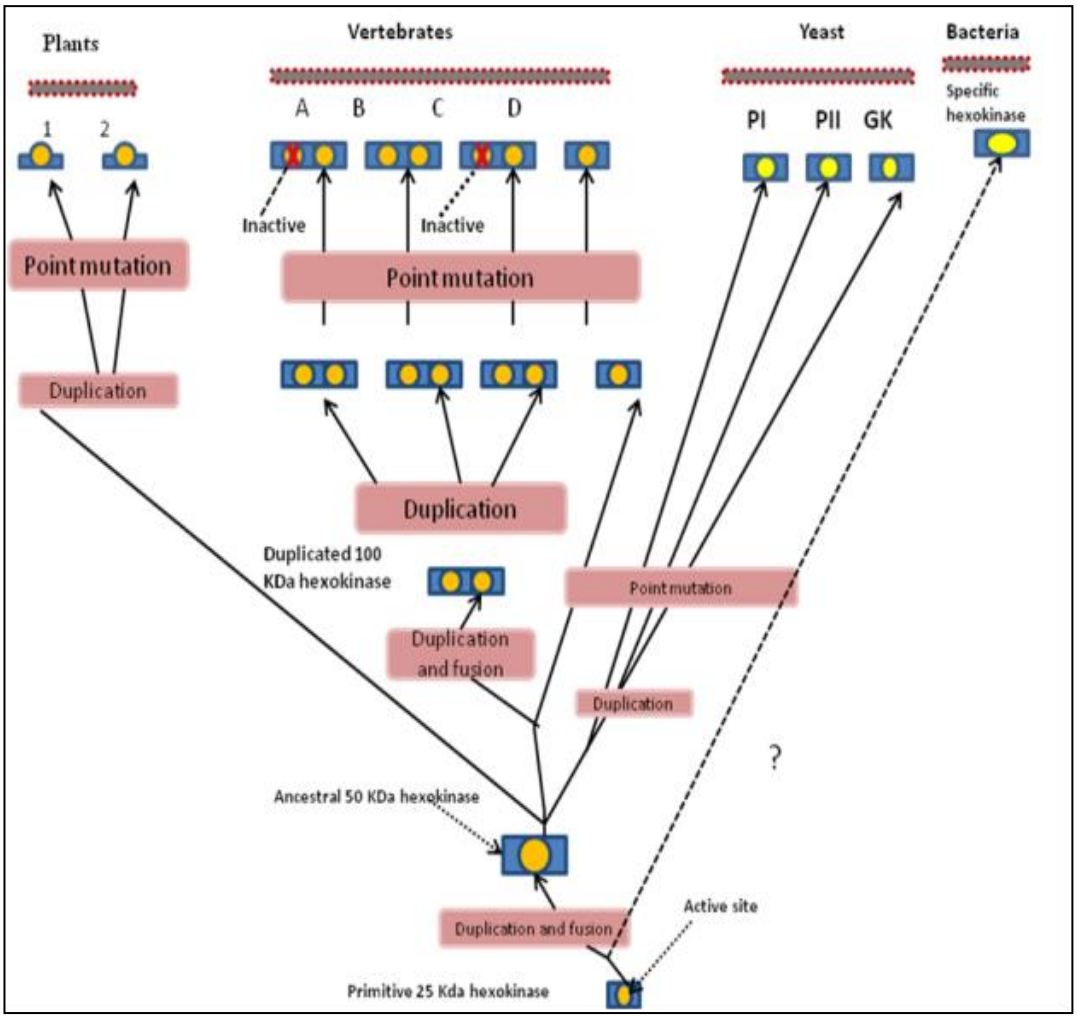


Figure 1.1–1 Structural similarities of hexokinases and phylogenetic relationships between hexokinases

Note: Reprinted from "Evolution and Regulatory Role of the Hexokinases," by M. L. Cárdenas et al., 1998, Biochimica et Biophysica Acta, 1401, 242–264.

1.1.3 Tissue distribution of hexokinase isoenzymes in mammalian systems

a) Types I, II, III, and IV (Kawai et al., 2005) are expressed in different organs of the body.

b) The liver contains all four types of hexokinases, while the kidney and intestine contain three (I–III).

c) Types I and II are found in epididymal fat pad, skeletal muscle, the brain, and the heart.

However, Type I is predominantly present in brain and kidney and Type II is predominant in skeletal muscle and epididymal fat pad (Iynedjian., 2009).

According to Katzen and Schimke, (Katzen et al., 1965) the properties of different types of hexokinase do not vary from tissue to tissue. Instead, they are present in variable amounts depending on age, stability of the enzyme, and nutritional status.

Different metabolic pathways can be linked to tissue specificity in the body, and therefore, these differences may be correlated to the differences between the structures; affinity for substrates and inhibitors; and subcellular localization of the isozymes of hexokinase, for example, HK_I and HK_{II} have a hydrophobic leader sequence that is important in binding hexokinase to mitochondria. On the other hand, HK_{III} and HK_{IV} do not contain this leader sequence and are nottherefore, bound to mitochondria. This suggests that these isozymes may be involved in metabolic pathways other than glycolysis as illustrated in Figure 1.1.2.

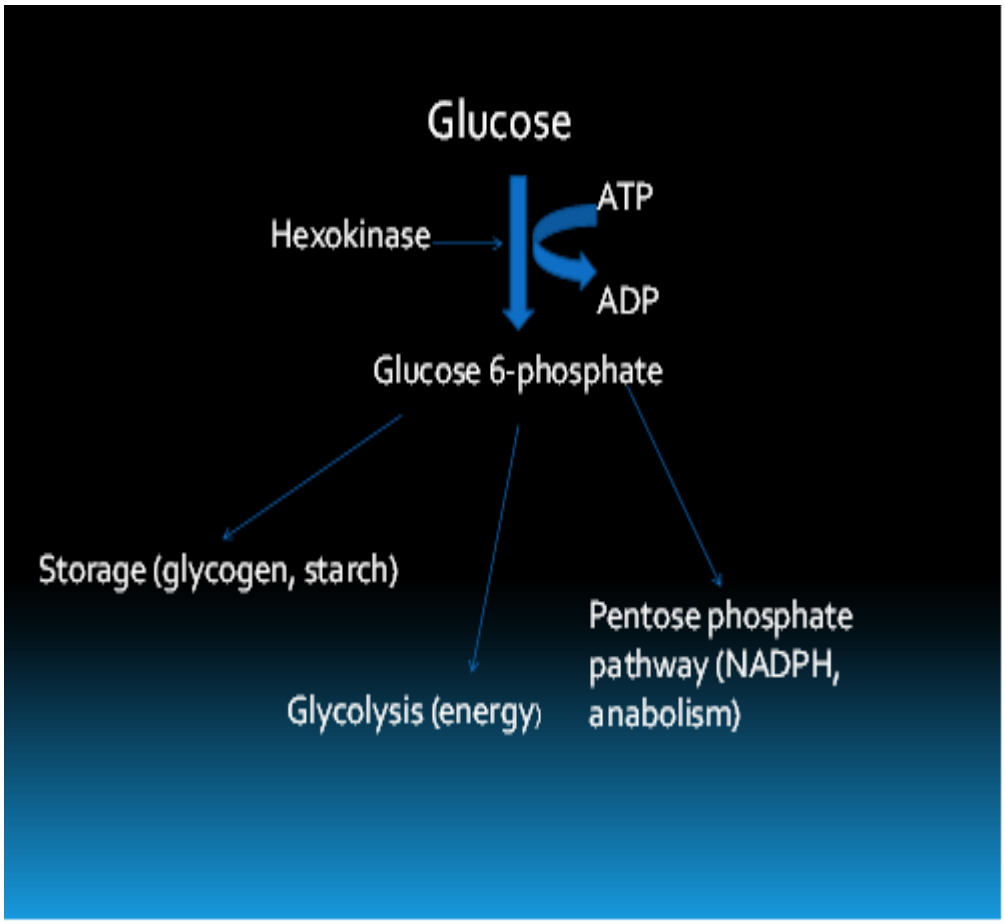


Figure 1.1–2 Phosphorylation, catalysed by hexokinase, is the initial step in common pathways of glucose metabolism.

1.1.4 Subcellular Distribution of Isoenzymes

Type I–III have a molecular mass of 100 kDa while Type IV, also known as glucokinase has a molecular mass of 50 kDa. HK Types I–III are thought to have evolved by the duplication and fusion of an ancestral gene that encodes a 50 kDa hexokinase (Kamata et al, 2004), the result of which is N- and C-terminal halves of similar sequence (Kogure et al., 1993).

In spite of the sequence similarity, the N- and C-terminal halves have different functions for the isozyme Types I–III. For isozyme II, both halves have a catalytic function, whereas for isozymes I and III, the C-terminal half has catalytic functions and the N-terminal has a regulatory function (Tsai and Wilson, 1996). All three isozymes are inhibited by the product Glucose-6-phospate (G6P). It has been hypothesized that inhibition by G6P is the regulatory function of the N-terminal half of HK_I and HK_{III}.

Other differences between the three isozymes are substrate affinity, the role of inorganic phosphate (Pi) in regulation, and sub-cellular location. Of the four isozymes, HK_{III} has the highest affinity for glucose and lowest affinity for ATP. G6P inhibits isozyme IIII, but Pi antagonizes inhibition by G6P in the Type-I isozyme only; further, it is an inhibitor of isozymes II and III. Isozymes I and II have a conserved hydrophobic leader sequence at the N-terminus. Isozyme III lacks the hydrophobic leader sequence.

Properties	**HK_I**	**HK_{II}**	**HK_{III}**	**HK_{IV}**
MW (KD)	100	100	100	50
Tissues	Brain, kidney, RBCs	Skeletal muscle, fat	Liver, intestine, kidney	Liver, pancreas
Km Glc (mM)	0.03	0.3	0.003	6-8
Km ATP (mM)	0.5	0.7	1	0.6
Function, N-terminal half	Regulatory	Catalytic	Regulatory	-
Function, C-terminal half	Catalytic	Catalytic	Catalytic	-
Inhibition by Glc-6-P	Yes	Yes	Yes	No
Pi relief	Yes	No	No	-
Subcellular localization	Mitochondria	Mitochondria	Nuclear periphery	Shuttles between nucleus and cytoplasm.

Table 1.1–3 Cellular and molecular properties of the human hexokinase enzymes: *Glucokinase (HKIV) does not exhibit Michaelis-Menten kinetics, and its substrate affinity is defined by its half-saturating glucose concentration (S0.5) (Wilson J.E, 2003).

Immunolocalization studies have shown the association of isozymes Types I and II with mitochondria, whereas the Type-III isozyme is co-localized with the envelope of the nucleus (Amparo et al., 2011). It is thought that the hydrophobic leader sequence for the Types I and II isozymes confer mitochondrial binding properties,

which allow the association between the outer mitochondrial membranes to occur by selective targeting (Wilson., 2004).

A voltage dependent anion channel (VDAC) – also called Porin – is located in the mitochondrial membrane. The function of this channel is to control the flux of metabolites across the membrane and interact with hexokinase I. The channel may facilitate the phosphorylation of glucose by providing a sufficient supply of ATP from oxidative phosphorylation inside mitochondria (Tsai and Wilson., 1995).

In cancer cells, HK (mainly II) is upregulated and its binding to the mitochondria through the VDAC channel plays an important role in the growth and survival of cancer cells. This happens in most aggressive tumors, such as glioma and hepatoma (Pastorino and Hoek., 2003).

These phenomena contribute to the Warburg effect (Pedersen., 2007), which is considered as the hallmark of cancer. In addition, it facilitates the evasion of apoptosis by cancer cells through increased expression of hexokinase II and antiapoptotic proteins of the BCL-2 family. This in return protects the cancerous cells against mitochondria-mediated cell death (Mathupala, Ko, and Pedersen., 2006).

Glucokinase (HK_{IV}) shuttles between the nucleus and cytoplasm of hepatcoytes according to the cellular glucose concentration. The mechanism of translocation is highly sophisticated, is under allosteric control, and is thought to be ciritcal to the regulation of blood glucose in mice, rats and man. This is considered in detail in section 1.1.4.3 below.

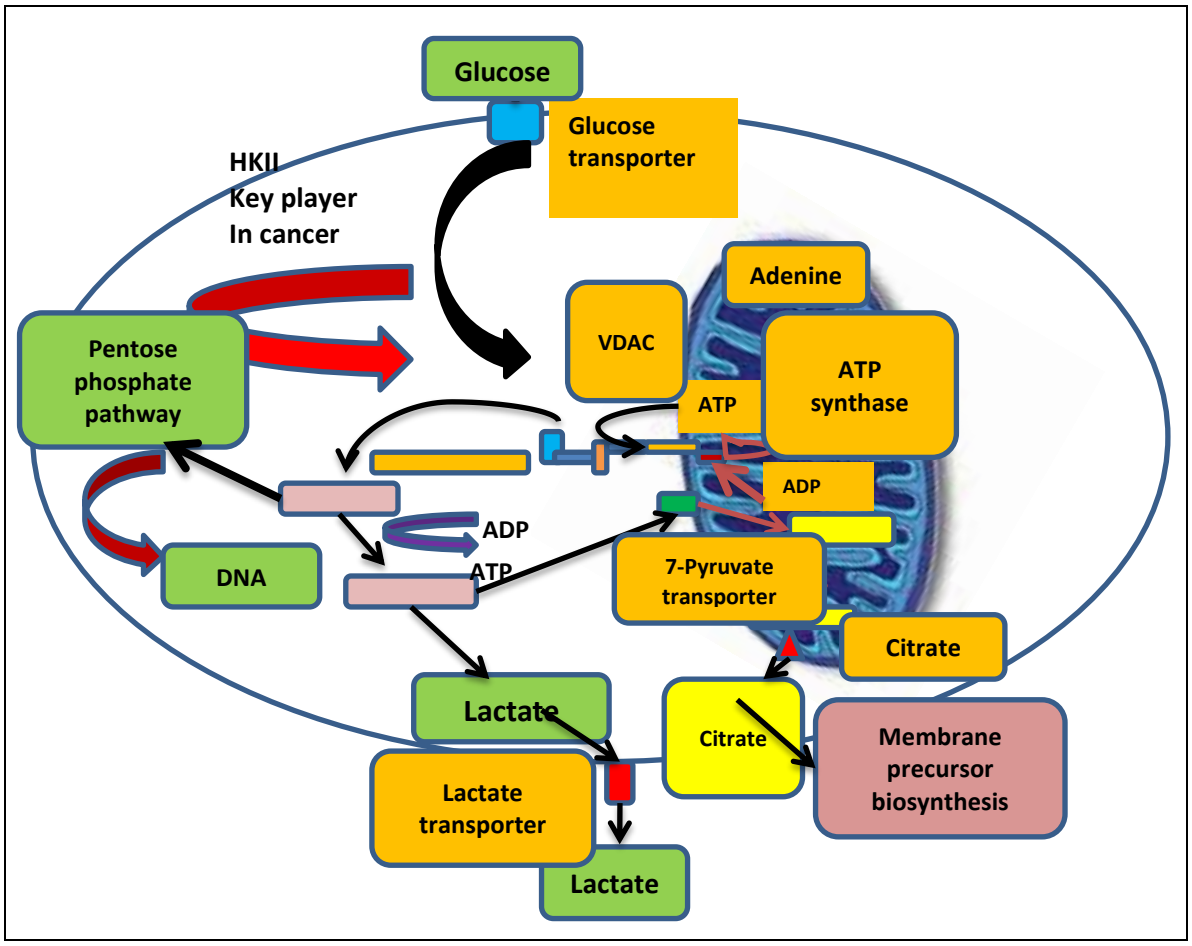


Figure 1.1–3 Interaction between hexokinase and mitochondria and their involvement in the maintenance of cancer cell survival (glucose and ATP are delivered to hexokinase II) (Galluzzi, Kepp, Tajeddine, and Kroemer, 2008).

1.1.4.1 Recent advances in hexokinase isozymes

In addition to the previously mentioned hexokinases, a hexokinase II pseudo gene has been identified (Malkki et al., 1994), which maps to chromosome Xq21. It has no introns, and this may indicate that it has been derived from cDNA by reverse transcription from mRNA, and contains internal stop codon that would prevent the synthesis of functional protein (Malkki et al., 1997; Ardehali et al., 1995).

Further, bioinformatics analysis and genomic search has been conducted (Irwin and Huanran., 2008), and the results of these searches have identified five hexokinase-like genes (HKDC1). The data obtained from these studies also revealed that HKDC1 is ubiquitously expressed and its sequence has been conserved among different vertebrate species and retains amino acid residues responsible for hexokinase activity. The phylogenetic analysis showed a very close relationship

between HKDC1 and hexokinase I, which indicates that HKDC1 is more likely to be a hexokinase family member.

1.1.4.2 ADP ribokinases and polyphosphate kinase

A new family of kinases with glucose phosphorylation activity was established initially from bioinformatics searches: these are ADP-dependent kinases (the first incorrectly coined ‘glucokinase') and also phosphofructokinase; these two enzymes are homologous and little sequence similarity to the known ATP- dependent enzymes described above (Guixe and Merino., 2009).

Three-dimensional structural studies of ADP-dependent enzymes allowed them to be classified as a member of the ribokinase family (Guixe and Merino., 2009).

Further structural studies have revealed that the ribokinase superfamily also contains enzymes that can transfer γ-phosphate of ATP to some vitamins involved in B6 synthesis, such as pyridoxal kinase (Li et al., 2002).

This superfamily can be classified into three major groups: ATP-dependant sugar kinase, ATP-dependent vitamin kinases and the ADP-dependent sugar kinase (Zhang et al., 2004).

Some homologous kinases have been identified in the genome of higher eukaryotics. The gene present in the genome of *Mus musculus* was cloned and expressed in *E. coli* – characterisation of the enzyme revealed that it is a monomer and quite specific for glucose, classifying it as an ‘ADP-dependent glucokinase’, (It is a hexokinase with a Km of 0.5mM and is not involved in glycemia regulation), whose physiological function is suggested to be effective during ischemia/hypoxia (Ronimus and Morgan., 2004).

These genes are expressed in different mammalian tissues, including man, suggesting that they may be housekeeping genes. Furthermore, ADP-dependent glucokinase has been found to be highly expressed in prostate cancer (Ronimus and Morgan., 2004).

1.1.4.3 Glucokinase (EC 2.7.1.2)

Glucokinase (GK) is a hexokinase isozyme related homologously to the other three hexokinases (Kawai et al., 2005). Human glucokinase is a monomeric protein of 465 amino acids with a molecular weight of about 50 kD. There are at least two clefts: one for the active site binding glucose and Mg^{2+}- ATP, and the other for a putative allosteric activatorthat has not yet been identified (Kamata et al., 2004).

This hexokinase is half the size of the other mammalian hexokinases, which preserve a degree of dimeric structure, a number of sequences, and the three-dimensional structure of the key active sites. The binding domain for ATP is shared with hexokinases, bacterial glucokinases, actin and Hsc70, the common structure they share is the actin fold (Holmes et al., 1993).

All of the hexokinases can catalyse the phosphorylation of glucose to glucose 6-phosphate, which is the first step of both glycogen synthesis and glycolysis pathways. However, glucokinase is encoded by a different gene, and its unique kinetic properties allow it to perform a completely different function. Glucokinase has a lower affinity for glucose than the other hexokinases do, and its activity is confined to a few cell types, such as those of the liver, pancreas, gut, and brain of humans and most other vertebrates. In each of these organs, it has an important role in the regulation of carbohydrate metabolism by acting as a glucose sensor, creating shifts in metabolism/ cell function as a result of glucose concentrations changes (Kawai et al., 2005).

Under specific conditions, glucokinase, like other hexokinases, can induce phosphorylation of other hexoses (six carbon sugars) and similar molecules (Cárdenas., 2004). Therefore, the general glucokinase reaction is more preciesly described as:

$$\text{Hexose} + \text{MgATP}^{2-} \rightarrow \text{Hexose-PO}_3^{2-} + \text{Mg ADP}^{-} + \text{H}^{+}$$

Hexose substrates phosphorylated by glucokinase are mannose, fructose, and glucosamine, but the affinity of glucokinase for these needs concentrations not usually found in cells for significant activity (Magnuson and Matschinsky, 2004).

1.1.4.4 Glucokinase genetics

Human glucokinase is coded for by the GCK gene on chromosome 7. This single autosomal gene has 10 exons (Matsutani et al., 1992; Stoffel et al., 1992). Genes for glucokinase in other animals are homologous to human GCK (Wilson, 2004; Bell Cuesta-Munoz, & Matschinsky., 2002).

A distinctive feature of the gene is that it begins with two promoter regions (Iynedjian, Jotterand, Nouspikel, Asfari, and Pilot., 1989). The first exon from the 5'-end contains two tissue-specific promoter regions. Transcription can take place at both promoters (depending on the tissue type) so that the same gene can provide a slightly different molecule in the liver and in other tissues. The two isoforms of glucokinase vary only by 13–15 amino acids at the N-terminal end of the molecule, which results in only a minimal variation in structure. The two isoforms have a very similar kinetic and functional characteristic (Iynedjian., 2009).

The first promoter from the 5'-end, referred to as the "upstream" or neuroendocrine promoter, is active in pancreatic islet cells, neural tissue, and enterocytes (small intestine cells) to yeild the "neuroendocrine isoform" of glucokinase (Iynedjian et al., 1989). The second promoter, the "downstream" or liver promoter, is active in hepatocytes and directs production of the "liver isoform" (Iynedjian et al., 1989). The two promoters have little or no sequence homology and are separated by a 30 kbp sequence which has not yet been shown to sustain any functional discrimination between isoforms (Iynedjian., 2009). The two promoters are functionally exclusive and controlled by distinct sets of regulatory factors, so that glucokinase expression can be regulated separately in different tissue types (Iynedjian., 2009). The two promoters correspond to two large categories of glucokinase function: In the liver, glucokinase play a role in the "bulk processing" of available glucose, while in the neuroendocrine cells, it acts as a glucose sensor. (Cárdenas et al., 1998). The glucose sensing mechanism initiated as the level of glucose increases, this in return triggers a number of cellular responses: glucose enters the cell via glucose transporter 2 (GLUT 2), then it is phosphorylated by glucokinase. As a consequence the cytosolic ATP/ADP ratio elevates leading to the closure of K^{+}_{ATP} channels, neuronal depolarization and entry of Ca^{2+} ions through Ca^{2+} channels causing the release of neurotransmitter. (Backer et al., 2016).

1.1.4.5 Clinical significance

About 200 mutations of the human glucokinase gene GCK have been identified, which can affect and alter the effectivness of glucose binding and phosphorylation, the sensitivity of beta cells to increase or decrease insulin secretion in response to glucose, and resulting in clinically significant hyperglycaemia or hypoglycemia (Arden et al., 2007).

1.1.4.5.1 Diabetes

Around 190, of these mutations affect the functional effectivness of the glucokinase molecule. Heterozygotes for the alleles with low enzyme activity have a higher threshold level for insulin secretion and persistent, mild hyperglycemia. This condition is known as maturity onset diabetes of the young, type 2 (MODY2).

Homozygosity for GCK alleles with lowered function can result in severe congenital insulin deficiency, the outcome of this termed as persistent neonatal diabetes (Matschinsky and Magnuson., 2004).

Chronic hyperglycemia will result in a number of complications if it remains untreated. The main consequences of hyperglycemia are nephropathy, neuropathy (damage to the peripheral nerves), cardiovascular disease, retinopathy (damage to the blood vessels in the eye, which may cause blindness), and in the long term, stroke and comma (Matschinsky and Magnuson, 2004).

1.1.4.5.2 Hyperinsulinemic hypoglycemia

As of 2004, five mutations have been identified which stimulate insulin secretion. Heterozygous for gain of function mutations reduces the threshold level of glucose that enhances insulin release. This produces hypoglycemia of varying patterns, including transient or persistent congenital hyperinsulinisim, or fasting or reactive hypoglycemia appearing in old age.

1.1.4.6 Hepatic glucokinase

Most of the glucokinase in mammals is present in the liver, although its presence in pancreatic beta cells, the small intestine, and the brain play a crucial integrative role in glycemic regulation.

Glucokinase can be rapidly stimulated and inhibited in hepatocytes by a regulatory protein (glucokinase regulatory protein or GKRP), which functions to maintain an inactive reserve of GK that can be made quickly available in response to increasing levels of portal vein glucose (Cárdenas and Maria., 1995).

GKRP shuttles between the nucleus and cytoplasm of the hepatocytes and may be attached to the microfilament cytoskeleton (Payne et al., 2007 and Shin et al., 2007). It forms reversible 1:1 complexes with GK and can transport it from the cytoplasm into the nucleus. It acts as a competitive inhibitor with glucose, therfore the enzyme activity is declined to near-zero when it is bound. GK–GKRP complexes are sequestered in the nucleus while glucose and fructose levels are low. Nuclear retention of GK may act as a protective mechanism from degradation by cytoplasmic proteases (Cardenas & Luz., 1995). GK can be released very fast from GKRP as a result of increasing levels of glucose. Contrary GK in beta cells, GK in hepatocytes is not associated with mitochondria (Zhang et al., 2006). Fructose in micromolar concentration (after phosphorylation by fructokinase EC 2.7.1.4, to fructose 1-phosphate (F1P) EC 4.1.2.13 dissociates GK from GKRP. This sensitivity to the occurence of small amounts of fructose enables GKRP, GK, and ketohexokinase EC 2.7.1.3 to act as a "fructose sensing system", which indicates that, a mixed carbohydrate meal is being digested and enhances the utilization of glucose. However, fructose 6-phosphate (F6P) stimulates the binding of GK by GKRP. F6P decreases phosphorylation of glucose by GK when glycogenolysis or gluconeogenesis is taking place. F1P and F6P both bind to the same site on GKRP. It is suggested that they produce two different conformations of GKRP: one able to bind GK and the other not (Futamura et al., 2006).

Regulation takes place at several levels and speeds and is affected by many factors that influence mainly two general mechanisms:

1. Glucokinase activity can be increased or decreased in minutes by the influence of the glucokinase regulatory protein. The roles of this protein are influenced by small molecules such as glucose and fructose.

2. The amount of glucokinase can be increased by the nuclear sequestration system far beyond that which could be produced through the period of post-prandial transcriptional activiation by insulin (Murphy et al., 2003, Iynedjian, 2009).

3. The perivenous concentration of glucokinase also enables the liver to respond quickly when changing from the fasting to early post-prandial state: glucose uptake can occur in the perivenous cells even whilst the periportal cells are still producing glucose via gluconeogenesis (Burns et al., 1996, Burns et al., 1997 and Murphy et al., 2003). The overall weight of these two processes, periportal glucose output from gluconeogenesis, and perivenous glucose uptake determining net glucose uptake or output, according the kinetics of glucokinase and the concentration of the enzyme stored in the perivenous hepatocytes nucleus (Burns et al., 1997, Burns et al., 2001). The architecture of the liver lobule provides a system for fetal programming of the weighting of these two groups of cells and generates an epigenetic glucostat in the liver architecture (Burns et al., 1997, Burns et al., 2000b).

1.1.4.7 Pancreatic glucokinase

Although most of the glucokinase in the body is in the liver, smaller amounts are present in beta and alpha cells of the pancreas, certain hypothalamic neurons, and specific cells (enterocytes) of the gut, which play a significant role in regulation of carbohydrate metabolism. Regarding glucokinase function, these cell types are referred to as neuroendocrine tissues, and they share some aspects of glucokinase regulation and function, especially the common neuroendocrine promoter. Of the neuroendocrine cells, the beta cells of the pancreatic islets are the most studied and best understood. It is likely that many of the regulatory relationships discovered in the beta cells will also exist in the other neuroendocrine tissues with glucokinase.

In islets beta cells, glucokinase activity serves as the principal control for the secretion of insulin in response to rising levels of blood glucose. As G6P is consumed, increasing amounts of ATP initiate a series of processes that result in release of insulin. One of the immediate consequences of increased cellular respiration is a rise in the NADH and NADPH concentrations (collectively referred to as NAD (P) H). This shift in the redox status of the beta cells results in increasing

intracellular calcium levels, closing of the KATP channels, depolarization of the cell membrane, merging of the insulin secretory granules with the membrane, and release of insulin into the blood (Moukil et al., 2001).

1.1.4.8 Hypothalamic glucokinase

Glucokinase has been found in the brain mostly in the same areas that contain glucose-sensing neurons, including both of the hypothalamic nuclei. Inhibition of glucokinase negatively affects the ventromedial nucleus response to a meal. However, brain glucose levels are lower than plasma levels – typically 0.5–3.5 mmol/L. Although this range matches the sensitivity of the glucose-sensing neurons, it is below the optimal inflection sensitivity for glucokinase (Roncero et al., 2000). These workers speculated that neuronal glucokinase is somehow exposed to plasma glucose levels even in the neurons, since hypothalamic glucose-sensing neurons involve subgroups of cells in the lateral, arcuate and ventromedial parts of hypothalamus, these neurons are inhibited or excited according to the changes in extracellular glucose (Wang et al., 2004).

1.1.4.9 Glucokinase distribution in species belong to different nutritional categories

Liver glucokinase occurs widely but not universally throughout vertebrate species. The gene structure and amino acid sequence are highly conserved among most mammals (e.g., rat and human glucokinase is more than 80% homologous). However, there are some unusual exceptions: For example, it has not been discovered in cats, ruminants and bats (Ballard., 1965, Irwin et al., 2013), though some reptiles, birds, amphibians, and fish have it.

Whether glucokinase occurs similarly in the pancreas and other organs has not yet been determined. It has been postulated that the presence of glucokinase in liver reflects the ease with which carbohydrates can be included in the animals' diets (Irwin et al., 2013).

A comparative study has been done by Ballard in 1965, between different mammalian animals belonging to different dietary categories (omnivorous, carnivorous and herbivorous) to evaluate their glucokinase activity. The data obtained from this study revealed that, omnivorous animals like rat and pig showed

high glucokinase activity and an increased rate of glycogenesis, whereas carnivorous animals such as dogs and cats, showed either high glucokinase activity in dogs or lower activity in cats (Ballard., 1965). In ruminants, which belong to the herbivorous category, the data obtained showed no glucokinase activity, the lowest hexokinase activity and low rates of glycogenesis.

1.1.5 Glucose metabolism in ruminants

Gluconeogenesis is a continual and critical process in ruminants to maintain a steady blood glucose level, because almost all dietary carbohydrates are fermented to volatile fatty acids in the rumen by ruminal micro flora (Aschenbachet et al., 2011).

Therefore ruminants are only able to absorb about 10% of dietary glucose from the gut (Emmanuel., 1981), as a consequence this may explain the absence of any glucokinase activity in ruminant liver (Ballard., 1965; Ureta., 1982).

Many surveys examined hexokinases activity from livers of numerous species, and their chromatographic profiles indicated that some mammals including ruminants (cow, ovine) bats and cats have little if any glucokinase activity (Vandercammen & Van Schaftingen., 1993; Ureta., 1982). Furthermore, the same studies also reported that the other hexokinases (I, II, III) are expressed normally in these species, suggesting that the absence of glucokinase activity was not compensated by up regulation of other hexokinases.

Bioinformatics studies showed that mutations that inactivate glucokinase function seems to be unlikely, on the other hand mutations in the GKRP have been reported (Irwin et al., 2013). As ruminants have not been reported to show any symptoms of diabetes or impaired glucose metabolism, it is more likely that they handle glucose metabolism differently. Glucose is not normally present in their blood at high concentrations, and therefore ruminants are less dependent on GK to remove excess glucose. On the other hand glucose required by other tissues is produced in the liver and its production is regulated by the hormones insulin and glucagon.

This also suggests that the control of glucose steady state in the circulation of these species may be more geared to glucose production (gluconeogenesis) rather than glucose uptake (absorption from the gut) (Wang et al., 2013).

Studying glucose metabolism in ruminants, specifically ovine is challenging since it has been reported previously that they are lacking glucokinase activity in their liver extracts, furthermore studies have shown that the glucokinase regulatory protein is mutated in this species (Irwin et al., 2014).

The previous studies on ovine GK misinterpreted the very high blank background absorbance at high glucose concentration, subtracting it from GK activity and concluding this enzyme was absent. However, the high 'blank' absornabnce has been shown to the result of an ATP-indepdendent hexokinase enzyme which has been proven to phosphoryale glucose using 13C labelled glucose using NMR (Bursn et al., unpublished data). The current thesis takes this observation and discovers two key new pieces of information: that the phosphorylation substrate is polyphosphate, and that the enzyme is inhibited by Adenosine phospahtes such as ATP and ADP. This data has allowed the enzyme to be measured in a variety of mammalian tissues including rat liver, which show that existing assay methods must now take account fo this enzyme and remove it from total phosphorylating capacity to obtain true ATP- and ADP-depdendent hexokinases and glucokinases in particular in muscle and liver tissue. Successful purification of the enzyme and sequencing will shed the light on the relevance of this novel enzyme, and any other similar enzymes in mammals and therefore help to elucidate their physiological function and probably their implications in the pathophysiology of certain metabolic disorders such as diabetes.

1.2 Part Two: Introduction to polyphosphate

Inorganic polyphosphate (Poly-Pi), a linear polymer of tens to hundreds of phosphate residues is linked together by 'high-energy' phosphoanhydride bonds. The molecular structure of P_i is shown in Figure 1.2-1 below:

Inorganic phosphate can be found in the cytoplasm like many others inorganic ions but in living organisms it is predominantly found as P_i or bound to organic molecules.

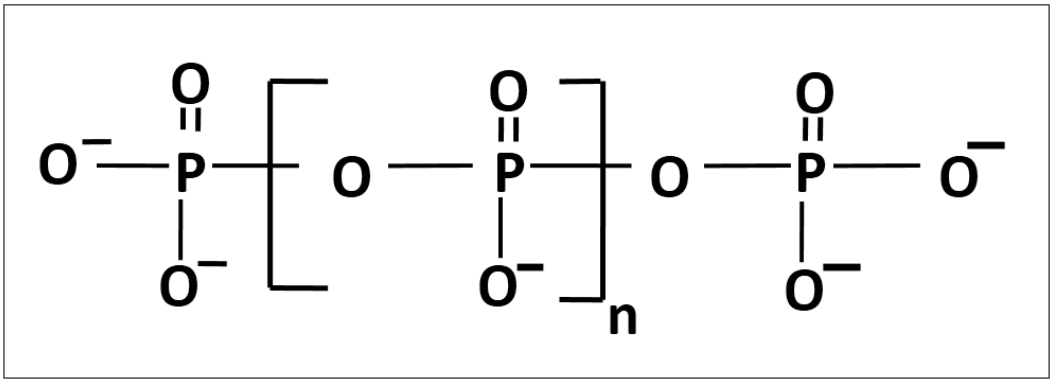


Figure 1.2–1 shows the linear molecular structure of inorganic polyphosphate, "n" is the number of [PO3−], residues vary from tens to thousands, (n=10-1,000).

Poly-Pi etc. can be formed from (ortho)-phosphate salts by dehydration at high temperature, which occurs under some special geological conditions like volcanoes and deep-oceanic steam vents. Hence, many scientists have concluded that Poly-Pi may have been involved in prebiotic evolution; it resembles a source of stored energy in a pre-ATP world (Jones & Lipmann., 1960; Schwartz., 2006).

In 1904 "volutin", a type of 'metachromatic granule", or intracellular particle that could be stained pink by basic blue dyes, was first observed in yeast and deemed to be nucleic acid (Meyer., 1904).

Many years later, with analytical techniques such as electron microscopy, these granules were identified to be Poly-Pi (Wiame., 1947).

Yet due to a lack of sensitive and accurate analytic methods to determine its functions, Poly-Pi was regarded as a "molecular fossil" for a long time. During the past couple of decades, a variety of techniques have been developed that have

enabled researchers to explore the various forms and functions of Poly-Pi in cells, the results show that it is present in every cell in nature: archaea, bacteria, fungi, plants and animals(Kulaev & Vagabov., 1983; Kumble & Kornberg 1995; Zhang & Kornberg., 2002).

1.2.1 The main functions of polyphosphate in cells of living organisms

Polyphosphates are involved in many biological functions associated to their chemical structure and properties, being found acting as chelator, buffer or energy source and reservoir, explained in more detail below. Although more work is required, especially in mammals, the importance of polyphosphate in some biological processes has been demonstrated, including Ca^{2+} channels, basic metabolism, biofilm formation, cell capsule, SOS response, sporulation and motility, osmotic regulation, ATP substitute and energy source, buffer against alkali, apoptosis, bone formation, blood coagulation, and regulation of mTOR.

1.2.1.1 Substitute and energy source

The amounts of Poly-Pi in cells are much higher than those of ATP. Under normal conditions, the cellular ATP levels are around 5-10mM. However, the vacuoles of yeast cells may contain more than 200mM of Poly –Pi (200mM PO_3^- units joined as polyphosphate chains of variable length), and in *E. coli*, Poly-Pi levels may increase to 50mM in a response to stress (Ault-Riche et al., 1998).

It has been shown that Poly-Pi may serve as a substitute for ATP, acting as a phosphate-containing substrate for several enzymatic reactions including Poly-Pi/ATP dependent NAD kinase and Poly-Pi/ATP dependent glucokinase (Kornberg, 1995). Most remarkably, in combination with polyphosphate kinase 1 (PPK1), Poly P: AMP phosphotransferase may regenerate ATP from AMP. Considering the key role of ATP and the high level of Poly-Pi in cells, Poly-Pi plays an important role as a substitute for ATP as a phosphate donor and energy source (Van Veen et al., 1994).

1.2.1.2 Reservoir for phosphate

A constant amount of phosphate is crucial for metabolism and cell growth. Poly-Pi may act as a reservoir for phosphate, which can be hydrolyzed to phosphate by the actions of phosphatase enzymes (e.g. exopolyphosphatase, Ppx) to maintain a stable level of phosphate within the cell. As long chain Poly-Pi can bind and form complexes with many multivalent counter-ions simultaneously, it has little effect on cellular osmotic pressure while at the same time act to preserve a constant level of crucial metabolite such as free P_i and ATP. (Harold., 1966).

1.2.1.3 Metal Ions Chelator

The long-chain phosphate residues allow Poly-Pi to powerfully chelate metal ions inside the cells. The Poly-Pi-metal complexes have been shown to play multiple roles in the cell. In *Lactobacillus plantarum*, the Mn^{2+}-Poly-Pi complexes act as an inorganic superoxide dismutase (Archibald & Fridovich., 1982). Within the vacuoles of yeast, the chelation of Ca^{2+} by Poly –Pi act as Ca^{2+} sink (Dunn, Gable & Beeler., 1994). In the cell walls of gram-positive bacteria, the complexes of Poly-Pi, Ca^{2+} and Mg^{2+} play an antibacterial role of Poly-Pi (Lee et al., 1994).

1.2.1.4 Buffer system against alkali

The ability of Poly-Pi to act as a buffer against alkali has been demonstrated in one study involving a species of algae (Weiss, Bental & Pick., 1991). Similar to yeasts, Poly-Pi is accumulated in vacuoles within algal cells. In *Dunaliella salina*, a type of halophilic green algae, the levels of Poly-Pi can be as high as 1M. In an alkaline environment, Poly-Pi is hydrolyzed to tri-Poly-Pi by specific endopolyphosphatase to neutralize amines within the vacuoles. Thus, Poly-Pi can act as a high-capacity buffer to maintain the pH stability of the intracellular environment.

1.2.1.5 DNA entry Channels

Chemical transformation facilitates the entry of exogenous DNA into cells. Its mechanism has been shown to involve PHB (polyhydroxybutyrate)/calcium/Poly –P complexes. During the process of competent cell preparation, the addition of Ca^{2+} leads to the formation of PHB/calcium/Poly-Pi complexes in the membranes of the

competent cell, which cause profound physical changes and facilitate DNA entry. However, many details of the transport process are still unclear (Reusch & Sadoff., 1988).

1.2.1.6 Metabolism

Poly-Pi plays an important role in the regulation of metabolism. Kusano and Ishihama showed that Poly-Pi could tightly bind to the RNA polymerase of *E. coli* in the stationary growth phase and selectively reduce the transcription of the genes that were dominant in the exponential growth phase (Kusano & Ishihama 1997). Poly-Pi interacts with ribosomes, mainly through its binding to specific ribosomal proteins, which plays an important role in maintaining the fidelity of translation (McInerney, Mizutani & Shiba 2006).

Another significant discovery is that Poly-Pi can form complexes with the Lon protease to stimulate the degradation of free ribosomal proteins when the cell undergoes amino acid starvation (Kuroda et al., 2001).

This process is illustrated in Figure 1.2—2 below

Poly-Pi formed in large amounts in response to amino acid starvation ‘capture’ the Lon protein from DNA, forming a Poly-PiLon complex. The binding of Poly P specifically activates the hydrolytic (proteolytic) activity of Lon towards free ribosomal proteins, resulting in them being degraded to amino acids. These amino acids can then be used for the synthesis of many housekeeping proteins in the cell. This pathway is of great importance for cell survival during periods of amino acid starvation, as it promotes the synthesis of key proteins/enzymes.

Further studies have revealed that in addition to its effects on the protease activities of Lon, Poly -Pi also affects the DNA-binding abilities of Lon. The major reason is that the affinity of Lon to Poly -Pi is higher than its affinity to DNA. This means Poly -Pi effectively competes with DNA for Lon protein binding, thereby inhibiting the formation of DNA-Lon complexes (Nomura et al. 2004). In addition, the Poly -Pi-Lon complex has been shown to be involved in the degradation of nucleoid proteins under certain conditions (Kuroda et al., 2006).

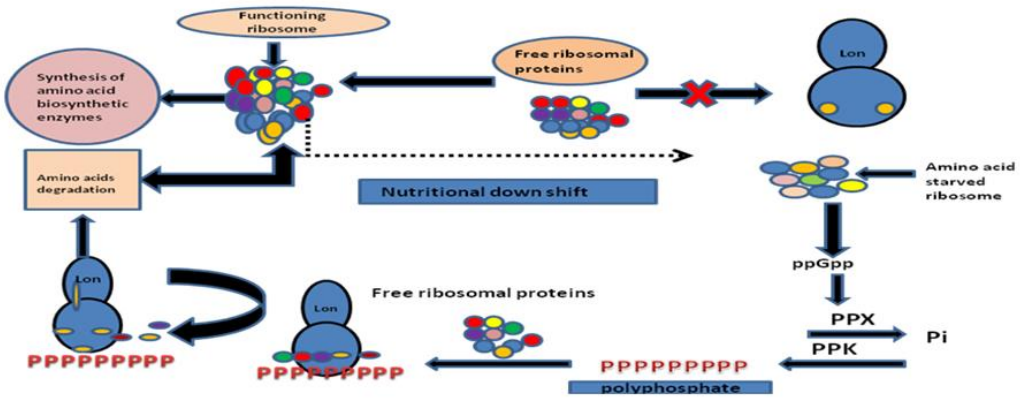


Figure 1.2–2 The proteolysis of ribosomal proteins activiated by the Poly Pi-Lon complex in response to amino acid starvation.

This figure is taken from *Rao, NN, et al. Annu Rev Biochem, 2009*. (Rao, Gomez-Garcia & Kornberg 2009)

1.2.1.7 Bio- film formation

Bio-films are surface-attached, structured communities containing single or multiple species of micro- organisms. They are commonly present in a wide range of ecosystems, and play a vital role in the (synergistic) growth and survival of most bacteria (Hall-Stoodley, Costerton & Stoodley., 2004) , such as protecting them against predation by protozoa or white cells (Van Oss., 1978), reduce UV penetration (Elasri & Miller., 1999) and increase their resistance to antibiotics and biocides, etc., Studies in *Pseudomonas aeruginosa*, *Porphyromonas gingivalis* and *Bacillus cereus* have revealed that the Poly-Pi null cells (achieved by knock-out mutation of the *PPK1* gene) are deficient in their ability to form biofilms (Rashid et al., 2000; Chen, Palmer & Kuramitsu 2002; Shi, Rao & Kornberg, 2004). This suggests that Poly-Pi plays an important role in the process of biofilm formation, and thereby influences the virulence and the resistance of bacteria.

1.2.1.8 Cell capsule

In several *Neisseria* species, including two important human pathogens, *Neisseria meningitidis* and *Neisseria gonorrhoeae*, about half of Poly-Pi is found loosely attached to the exterior of the cell, and forms part of the capsule (Tinsley, Manjula & Gotschlich., 1993).

The Poly-Pi capsule is speculated to contribute to the pathogenesis of infections by providing an antiphagocytic capsule, by chelating metals needed in complement fixation or by complex essential nutrients as iron.

1.2.1.9 DNA Damage and SOS response

During the SOS response, which is induced in response to the detection of DNA damage, various sets of DNA repair proteins mend the breaks in the chromosome, and facilitate the progression of the DNA replication machinery.

However, in cells with low levels of Poly P (generated by the over-expression of the exoployphosphatase Ppx) the expression of *rpoS* (which encodes the RNA polymerase sigma factor, RpoS) and *recA* (which encodes a DNA-recombinase) are reduced (Shiba et al., 1997; Tsutsumi, Munekata & Shiba., 2000). These results indicate that Poly -P is involved in the expression of SOS genes.

1.2.1.10 Osmotic stress

Dehydration (water loss) leads to an increase in osmotic pressure, and this usually leads to cell death. Many studies have found that osmotic stress can induce cells to accumulate large amounts of Poly-Pi, and have speculated that Poly-Pi could enhance the ability of cells to survive changes in osmotic pressure (Ault-Riche et al, 1998). Recent studies show that cells with low concentrations of Poly-Pi (resulting from the deletion of the *PPK1* gene) showed lower survival rates towards desiccation compared to the wild cells, indicating that Poly-Pi may play an important role in resistance to osmotic pressure (Fraley et al., 2007).

1.2.1.11 Bacterial Motility and Sporulation

The spore is an important cell form that resists environmental stresses. In *Bacillus cereus*, the *ppk*, *ppx* and *pap* genes were individually ‘knocked-out’ (mutationally-inactivated) from the genome, and changes in cell properties were evaluated. The results showed that only in the *ppx* mutant, the sporulation efficiency was reduced (Shi, Rao & Kornberg., 2004). In the delta-proteobacterium *Myxococcus xanthus*, inactivating the *PPK1* gene resulted in delayed fruiting-body formation, fewer spores and delayed germination (Zhang, Rao, Shiba, Brown & Kornberg., 2005).

Similarly, in the soil-dwelling amoeba *Dictyostelium discoideum*, the *PPK1* mutant had smaller fruiting bodies and few spores (Zhang, Gomez-Garcia, and Brown & Kornberg., 2005). In addition, the motility of all the above mutants was significantly reduced. Thus, Poly -P is regarded as a regulator or mediator of sporulation and motility in micro-organisms, but its molecular mechanisms remain to be explored.

1.2.1.12 Regulation of mTOR

Though Poly-Pi widely exists in tissues and subcellular compartments (Kumble & Kornberg., 1995), little information is available about the functions of Poly-Pi in animal cells.

The few known functions mainly include regulation of mTOR (mammalian target of rapamycin), modulation of the mitogenic activity of fibroblast growth factors; its participation in apoptosis, bone formation and blood coagulation.

TOR (target of rapamycin), a serine-threonine protein kinase, plays central roles in signalling pathways relating to cell growth and proliferation (Schmelzle & Hall., 2000), and is highly conserved in all eukaryotes analyzed so far (Jacinto & Hall., 2003).

mTOR is composed of two proteins: mTORC1 and mTORC2.*in vivo*, mTOR can respond to the signals from amino acid supply, cellular energy state, various hormones (such as insulin) and growth factors.

Activated by autophosphorylation, mTOR can initiate cascade reactions and promote cell growth, and proliferation (Scott et al., 1998).

However, in *in vitro* studies, signals such as insulin or amino acids failed to stimulate mTOR as they do *in vivo*, and surprisingly Poly-Pi was found to be capable of strongly activating mTOR (Wang et al., 2003).

The same result was also seen *in vivo* with MCF-7, a human carcinoma cell line. When the level of Poly-Pi in MCF-7 cells was reduced by supplying the Ppx exopolyphosphatase, cells showed low response to insulin and amino acids, and the cell growth rate significantly decreased. Based on the above results, Poly-Pi is

regarded to be a regulatory factor of mTOR, controlling the growth and proliferation of animal cells.

1.2.1.13 Modulation of mitogenic activity of fibroblast growth factors

Poly -P plays a role in the proliferation of normal human fibroblast cells. Cell proliferation could be enhanced by adding Poly -P into the culture medium.

The mechanism might relate to acidic fibroblast growth factor (FGF-1) and basic fibroblast growth factor (FGF-2), the study revealed that (i) their mitogenic activities could also be enhanced by Poly-Pi; (ii) a physical interaction was found between Poly-Pi and FGF-2; (iii) FGF-2 could be stabilized by Poly -P; (iv) the binding of FGF-2 to its cell surface receptors was facilitated by Poly-Pi (Shiba et al., 2003).

1.2.1.14 Apoptosis

Apoptosis plays a vital role in various cell activities, e.g. normal cell turnover, cell development and chemical-induced cell death. A recent study discovered that Poly Pi was related to apoptosis (Hernandez-Ruiz et al., 2006). When adding Poly-Pi to human plasma cells (PCs), the secretion of immunoglobulin was inhibited and apoptosis was stimulated. Moreover, the Poly-Pi stimulated apoptosis was specific for PCs, myeloma (malignant PC) cell lines, primary myeloma cells, and B lymphoid cell lines. For normal B cells, T cells, total blood mononuclear cells, and non-lymphoid cell lines, no apoptotic effects were detected. The relevance of Poly-Pi to the humoral immune response makes it an attractive, novel target for the therapy of myeloma.

1.2.1.15 Bone Tissue mineralization and formation

Poly-Pi has been suggested to be involved in the control of vertebrate skeletal mineralization (Omelon & Grynpas 2008; Omelon et al., 2009) based on the following observations: (i) vertebrate bones contain crystals of apatite, a calcium- and phosphate based mineral; (ii) amorphous, electron-dense granules containing calcium and phosphate have been identified in skeletal tissue and mitochondria (proposed to participate in apatite biomineralization) (Halstead., 1969); (iii) Poly-

Pi is identified in areas of resorbing bone and calcifying cartilage; (iv) tissue nonspecific alkaline phosphatase is able to cleave orthophosphate from polyphosphate. The roles of Poly-Pi in the bio-mineralization processes are speculated to be in circumstances where the apatite formation is not required (such as cartilage tissue or during bone resorption), calcium-Poly-Pi complexes will be formed to reduce the concentrations of free orthophosphate and free calcium; whereas when the mineralization is required, orthophosphate will be released from Poly-Pi catalyzed by tissue-nonspecific alkaline phosphatase, concurrently accompanied with the release of calcium previously sequestered by Poly-Pi.

1.2.1.16 Poly-Pi role in blood coagulation cascade and Platelets activiation

Poly -P has been shown to be present in the dense granules of human platelets by DAPI staining, NMR and gel electrophoresis (Ruiz et al., 2004). Studies showed that Poly-Pi could be released from platelets to accelerate clot formation when platelets were activated. While the decrease of Poly-Pi resulting from hydrolysis by (the exopolylphosphatase) Ppx led to the inhibition of clotting and the activation of fibrinolysis during wound healing (Smith et al., 2006).

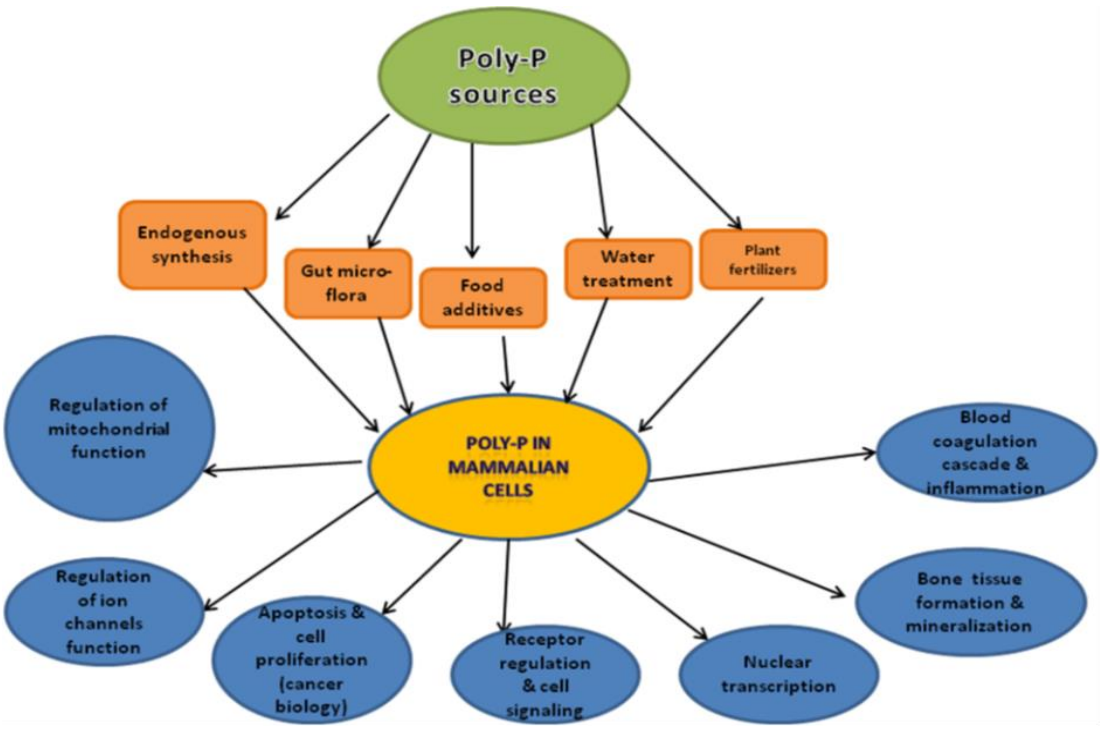


Figure 1.2–3 A schematic illustration summarizes polyphosphate sources and pivotal functions in mammalian cells.

1.2.2 Intracellular polyphosphate levels and subcellular distribution

Studies show that polyphosphates are widely distributed and have been found in different cellular compartments in prokaryotes, fungi, plants and eukaryotes.

1.2.2.1 Prokaryotes

There is difference in the abundance and intracellular distribution of polyphosphate in bacteria, as reviewed in (Kornberg, Rao & Ault-Riche, 1999). Some bacteria, such as *Acinetobacter johnsonii* (Van Groenestijn et al., 1989) and *Microlunatus phosphovorus* (Van Groenestijn et al., 1995) isolated from activated sludge, can accumulate high levels of Poly-Pi. In *A. johnsonii*, Poly –P (200mM) accounts for up to 30% of the dry biomass (Deinema, Van loosdrecht & Scholten 1985), whereas in many other bacteria, Poly-Pi levels are far lower under normal growth conditions. For example, *E. coli* in stationary phase contains about 0.1-50mM of Poly-Pi, however it is able to accumulate several hundred-fold more Poly-Pi in response to certain nutritional deficiencies and environmental stressed (Kornberg, Rao & Ault-Riche., 1999).

Poly-Pi may be found in all compartments of the bacterial cell: cytoplasm, cell surface, periplasm and plasma membrane. Poly-Pi appears in the cytoplasm of various prokaryotes (Kulaev & Vagabov 1983; Kulaev., 1979) in the form of granules, whose size and number depends on the concentration of phosphate in the culture medium. Poly-Pi granules are even used as a diagnostic feature of some major human pathogens, such as *Corynebacterium diphtheriae*. But this application is not entirely reliable, as Poly-Pi granules have also been identified in some strains of non-pathogenic *C. glutamicum* (Pallerla et al., 2005). *Vibrio cholerae* (01, biotype El Tor) accumulates high level of Poly -P stored as granules under normal growth conditions (Ogawa et al., 2000). *Pseudomonas aeruginosa* is also able to accumulate a large amount of Poly-Pi during the stationary phase (Kim, 1998) and in response to phosphate and amino acid starvation (Ault-Riche et al., 1998).

Poly-Pi is also present in the periplasm (Rao & Torriani 1988; Nesmeyanova, Dmitriev & Kulaev, 1974), in the vicinity of the bacterial nucleoid (Kulaev & Vagabov, 1983) and on the cell surface (Tinsley, Manjula & Gotschlich 1993; Tinsley & Gotschlich., 1995).

In the plasma membranes of bacteria, Poly-Pi possesses the capacity to form complexes with Ca^{2+} and poly-β-hydroxybutyrate (PHB). These adopt a double helix structure, with an outer chain of PHB and an inner chain of Poly-Pi linked together by Ca^{2+} ions (Reusch & Sadoff 1988; Reusch., 1992). The number of PHB/calcium/Poly -P complexes is elevated in cells to make it competent for transformation (Huang & Reusch., 1995). Also, the high concentration of PHB/calcium/Poly-Pi complexes in the membranes can form rigid domains which disrupt the liquid bilayer conformation (Castuma et al., 1995). Consequently, it has been speculated that these complexes are involved in the process of DNA transport into the cell, forming voltage-activated calcium channels (Huang & Reusch., 1996). This conserved PHB complex has also been identified in the membranes of a variety of fungi, plant tissues, and animal cells (Reusch., 1992).

1.2.2.2 Fungi

In simple eukaryotes, Poly-Pi is found in many different cell compartments, including cytosol and cell envelope (Kulaev 1979; Kulaev, 1994), vacuoles (Urech et al., 1978; Lichko, Okorokov & Kulaev, 1982), nuclei (Kulaev, 1979), mitochondria (Beauvoit et al., 1989) and plasma membrane (Pilatus, Mayer & Hitdebrandt, 1989).

The content of Poly-Pi in each compartment is best studied in *Saccharomyces cerevisiae*. The levels of Poly-Pi in the cytosol largely depends on the cell growth phase and cultivation conditions; for example, in the cytosol of *Saccharomyces cerevisiae*, the amount of Poly-Pi may vary from 10% (Okorokov, Lichko & Kulaev 1980) to 70% (Kulaev, Vagabov & Kulakovskaya, 1999) of total intracellular Phosphate during different growth phases.

In yeast cells, a fraction of the cytosolic Poly-Pi (about 14% of total intracellular phosphate) exists in 'volutin granules' which act as P_i reserve and cation sequestration (Jacobson, Helman & Yariv 1982).Some studies have suggested that

the main function of Poly-Pi in the cytosol is phosphate storage (Kulaev 1979; Kulaev, Vagabov & Kulakovskaya., 2004).In*S. cerevisiae* cells, vacuoles may contain the major part of Poly-Pi when it is grown in a medium containing arginine as the only nitrogen source (Urech et al., 1978).

Furthermore, the amount of Poly -Pi in the vacuoles increases seven-fold than in the cytosol when the cells are cultured with phosphate, glucose and K+, and is ten-fold in the presence of Mn^{2+} ions (Lichko, Okorokov & Kulaev., 1982).

However, under other conditions, the proportion of Poly-Pi in vacuoles is significantly lower; less than 15% (of total Poly-Pi) when *S. cerevisiae* is cultured with Rider medium (a phosphate-limited medium) (Reader., 1927).All of these studies implicate Poly-Pi in regulatory metabolic processes, either arising as a result of alternative metabolic processes during nutrient depletion or, perhaps even driving glycolysis under cell stress.

For example, in the fungus *Candida utilis*, vacuoles contain 30-50% of the total Poly-Pi depending on the growth rate and the nitrogen source in the medium (Nunez & Callier., 1989).When other *Candida*spp. Were cultured in phosphate-deficient medium, no Poly-Pi could be detected in the vacuoles (Bourne., 1991).

Poly-Pi vacuoles are also found in *Neurospora crassa (*Cramer & Davis., 1984) and *Dunaliella salina* (Pick & Weiss., 1991). It has been suggested that the main function of Poly-Pi in vacuoles is to chelate the cations accumulated inside the organelles (Lichko, Okorokov & Kulaev., 1982), but alternative metabolic explanations might exist.

In addition, the levels of Poly-Pi in the cytosol and vacuoles of yeast cells are strongly correlated to the phosphate concentration of the growth medium. In a Pi-deficient medium, the levels of Poly-Pi both in complete cells and in vacuoles are sharply decreased. After 7h of Pi starvation, the Poly-Pi level in vacuoles decreases by 85%. When cells are transferred from Pi-deficient medium back to Pi-containing medium, the intracellular Poly-Pi levels increase considerably (over six-fold), compared to those in the initial culture.

This phenomenon is referred to as *phosphate overcompensation*. It is observed that the levels of Poly-Pi in the vacuoles does not respond to the re-supply of phosphate in the growth medium (Kulaev, Vagabov & Kulakovskaya 1999).

Poly-Pi is often localized within the cell envelope. This Poly -P fraction can be effectively extracted under weakly alkaline conditions (e.g. at pH 9.0) (Kulaev & Vagabov., 1983).

Short chain Poly-Pi Ca^{2+}· 14 phosphate residues) has also been identified in the mitochondria of yeast cells by 31P-NMR, which accounted for ca. 10% of the total Poly-Pi. This fraction of intracellular Poly-Pi is speculated to participate in bio energetic processes (Beauvoit et al., 1989). Several studies have indicated that Poly -Pi can form complexes with nucleic acids present in the nuclei of yeast cells (Kulaev & Vagabov 1983; Kulaev., 1979), whose function is thought to be probably related to the regulation of gene expression (Kulaev 1979; Kulaev, Vagabov & Kulakovskaya., 2004), though much of this remains speculation.

1.2.2.3 Higher eukaryotes

Poly-Pi is also present in all higher eukaryotic organisms investigated, but in smaller amounts compared to those found in microorganisms (Gabel and Thomas, 1972). Kumble *et al.* reported that micromolar levels of long chain Poly-Pi (consisting of 50 to 800 phosphate residues) were present within certain tissues/organs in rodents (e.g., brain, heart, kidneys, liver, and lungs). This was localized within various subcellular compartments such as nuclei, mitochondria, plasma membranes, and microsomes (Kumble & Kornberg., 1995). However, there is limited understanding of the distributions and functions of Poly-Pi in higher organisms including *H.sapien.* At the time of submission no enzymes of Poly-Pi utilization in mammalian organisms have been identified, and the current work is therefore the first demonstration of a Poly-Pi dependent enzyme in mammals.

1.2.3 Main enzymes of polyphosphate metabolism

As we have seen, polyphosphates are involved in different vital processes and, therefore, many enzymes are expected to be involved in their metabolism.

Main enzymes of polyphosphate synthesis and degradation	Process
Polyphosphate kinase 1 (PPK1)	nATP → Poly -Pn + nADP →NDP + Poly-Pn → NTP + Poly -Pn-1→(N, nucleotide: A, G, C or U)→GDP + Poly -Pn → ppppG + Poly-Pn-2
Polyphosphate kinase 2 (Ppk2) (1-domain Ppk2 protein)	Poly-Pn + GDP → Poly-Pn-1 + GTP→Poly -Pn + ADP → Poly-Pn-1 + ATP→nATP → Poly-Pn + nADP→nGTP → Poly-Pn + nGDP
Polyphosphate kinase 2 (Ppk2) (2-domain Ppk2 protein)	Poly -Pn + GMP → Poly -Pn-1 + GDP→Poly -Pn + AMP → Poly-Pn-1 + ADP
Poly-P:AMP phosphotransferase (PAP)	Poly-Pn + NMP → Poly-Pn-1 + NDP→(N, nucleotide: A, G, C or U)→nADP → Poly-Pn + nAMP→nGDP → Poly -Pn + nGMP
Poly-P/ATP dependent NAD kinase	Poly-Pn + NAD → Poly -Pn-1 + NADP→ATP + NAD → ADP + NADP
Poly-P/ATP dependent glucokinase Exopolyphosphatase (Ppx)	Poly-Pn + Glucose → Poly-Pn−1 + glucose 6-P→ATP + Glucose → ADP + glucose 6-P Poly -Pn → Poly-Pn-1 + Pi → nPi
Guanosine pentaphosphate phosphohydrolase (Gppa)	pppGpp → ppGpp + Pi→Poly-Pn → Poly -Pn-1 + Pi → nPi
Endopolyphosphatase (Ppn)	Poly-Pn → Poly-Px + Poly-Py→(x + y = n)
Eukaryotic poly P synthesis: VTC (Vacuolar Transporter Chaperone)	nATP → Poly -Pn + nADP
dDPpk2	Arp (G) + nATP ↔ Arp (F) + Poly-Pn + nADP→G: small→ligomer; F: filament

Table 1.2–1 Main metabolic enzymes involved in polyphosphate synthesis and degradation

1.2.3.1 Polyphosphate kinase1 (EC 2.7.4.1)

Polyphosphate kinase 1 (PPK1), also referred to as polyphosphate: ADP phosphotransferase, is highly conserved in prokaryotes and is considered to be the main synthesizer of Poly-Pi in a majority of bacterial species. Poly-Pi accumulation can be understood by in-depth study of all the enzymes involved in it and their regulation. Polyphosphate kinase- PPK, polyphosphate adenosine monophosphate phosphotransferase, polyphosphate glucose-6-transferase, exopolyphosphatase and adenylate kinase are the main enzymes involved in the metabolism of prokaryotic polyphosphate. The first step of Poly-Pi synthesis by PPK1 includes autophosphorylation of its histidine residues, and accordingly PPK1 is a histidine

kinase (Zhang et al., 2007).Among the growing list of enzymes that make and hydrolyze Poly-Pi, polyphosphate kinase 1 (PPK1) is the most widely conserved. Poly-Pi PPK1 homologs have been found in more than 100 prokaryotic species, including 20 major pathogens, and to date, only in a few eukaryotes.

The process of polymerization is highly active with no need for oligo- or polyphosphate primers, and there are no detectable intermediates (Ahn & Kornberg., 1990). The PPK1 protein from *E. coli* has been crystallized (Zhu et al, 2005), which provides valuable information about the active site, spatial structure and catalytic mechanism of PPK1.

In addition to its Poly-Pi synthesis activity, PPK1 also exhibits other enzymatic activities including nucleotide diphosphate kinase (NDK) activity and guanosine 5'-tetraphosphate (ppppG) synthesis activity (Kuroda & Kornberg, 1997). The reactions catalysed by PPK1 are shown in Table (1.2-1).

The Poly-Pi and ATP synthesis reactions can be considered as two results of one reaction. In general, PPK1 catalysing the forward reaction, that is the Poly-Pi synthesis reaction. However, when ADP is present in excess, the reverse reaction (ATP synthesis) can be driven to completion. This reaction could be of importance in cells that are highly energy depleted, or which undergo sudden increased demand for ATP resynthesis. In mammalian muscle cells this reaction is undertaken by the transfer of a phosphate from creatine phosphate to ATP during muscle exhaustion. The use of Poly-Pi in this circumstance would be highly favourable.

Among all the enzymes involved in the metabolism of Poly-Pi, PPK1 is the most highly conserved enzyme in bacteria and archaea. PPK1 gene homologues are present in the genomes of most bacteria sequenced to date, including numerous pathogens responsible for major infectious diseases. PPK1 plays an important role in the metabolism of Poly-Pi and enables cells to accumulate large amounts of Poly-Pi in response to unfavourable growth conditions or other environmental stress. Studies have shown that PPK1 can serve as a useful gene target for the regulation of Poly–P content in cells. Usually, 'knocking-out' (i.e. mutationally-inactivating) the PPK1 gene leads to a dramatic decrease in the concentration of intracellular

Poly-Pi. Therefore, PPK1 has been used for functional studies of Poly-Pi in a variety of micro-organisms.

PPK1-null mutants have been shown to be defective in many notable aspects; such as cell motility, quorum sensing, biofilm formation, virulence and predation (Rashid et al., 2000). PPK1 genes are present in several species of archaea, including *Methanosarcina* and *Haloarcula*, but none have been functionally characterized to date. Very few PPK1 homologs have been identified within eukaryotes.

Only the DdPPK1 from *Dictyostelium discoideum* (Zhang et al., 2005), and EcPPK1-like enzyme from *Candida humicola* (McGrath et al., 2005) have been characterized. Similar to EcPPK1, DdPPK1 also possesses the conserved residues for ATP binding and autophosphorylation, but at the N terminal it has a unique extension of 370 amino acids that is necessary for enzyme activity, cellular localization and physiological functions. The *D. discoideum* PPK1-null mutant has been shown to have slower rates of cytokinesis and cell division, and is partially defective in various aspects of development, sporulation and predation (Zhang et al., 2007).

1.2.3.2 Polyphosphate kinase2 (EC: 2.7.4.1)

The discovery of Polyphosphate kinase 2 (Ppk2) in *Pseudomonas aeruginosa* PAO1(wild type) was inspired by the finding that the *PPK1*-null mutant still possesses about 20% of the wild-type levels of Poly-Pi (Rashid et al., 2000). Although the PPk2 protein from *P. aeruginosa* also possesses the activity of Poly-Pi synthesis, it is different from PPK1 in many ways: (i) Whilst PPK1 is mainly involved in the synthesis of Poly-Pi, PPk2 favors the synthesis of nucleotide triphosphates (GTP/ATP) from nucleotide diphosphates (GDP/ADP), using Poly-Pi as the phosphate donor. This reaction is 75-fold faster than the synthesis of Poly-Pi from ATP/GTP. (ii) Ppk2 shows similar selectivity for adenosine and guanosine nucleotides; both in Poly-Pi synthesis and in the synthesis of nucleotide triphosphates.

This is quite different to the PPK1 protein, where the phosphate donor is strictly limited to ATP for Poly-Pi synthesis; it also has an 80-fold preference for ADP over

GDP in the synthesis of nucleotide triphosphates. (iii) PPk2 prefers Mn^{+2} over Mg^{2+}, whereas PPK1 shows the reverse selectivity for Poly-Pi synthesis. (iv) The Poly-Pi synthesis activities of PPk2 are stimulated in the presence of Poly-Pi, while for PPK1, no stimulatory effect is detected (Zhang et al., 2002).

The *ppk2* gene is present in many bacterial species. Some species, e.g. *P. aeruginosa* contain multiple *ppk2* gene homologues. Some species contain neither *PPK1* nor *ppk2* gene homologues (e.g. *S. aureus*, *T. denticola*). *Corynebacterium diphtheria* and several other *Corynebacteria* species are quite distinct in containing two *ppk2* homologues, but no identifiable *PPK1* gene.

The *ppk2* gene shares significant amino-acid sequence homology to the Fur controlled genes, RNA polymerase sigma factor 70 (PvdS), which is involved in the biosynthesis of the fluorescent siderophore molecule pyoverdine (Ishige et al., 2002).

The PPk2 proteins can be sub-divided into two groups based on the number of domains contained in the proteins. By using Poly-Pi as phosphate donor, proteins with a single Ppk2 domain are able to catalyse the phosphorylation of ADP to ATP (or GDP to GTP), whereas proteins with two fused Ppk2 domains also possess the ability to catalyse the phosphorylation of AMP to ADP (or GMP to GDP) (Nocek et al, 2008). Consequently, the combination of these two groups of Ppk2 proteins allows Poly-Pi to be used as intracellular energy reserve for the regeneration of the essential 'energy' molecule ATP (and to a lesser extent, GTP).

The 1-domain Ppk2 protein (SMc02148) from *Sinorhizobium meliloti* and the 2-domain Ppk2 protein (PA3455) from *Pseudomonas aeruginosa*, have been bio-physically and bio-chemically characterized, and their crystal structures have been solved. The single-domain SMc02148 protein, and both the N- and C-terminal domains of the PA3455 monomers, are all folded into similar structures: 3-layer α/β/α sandwiches with an α-helical lid. In solution, SMc02148 is a tetramer and PA3455 is a dimer, suggesting a similar overall quaternary structure (Geourjon et al., 2001).

Alanine replacement mutagenesis has indicated that 9 conserved residues are required for the activities of Ppk2 proteins, which are located in Walker A and

Walker B motifs [two major conserved motifs required for ATP binding and hydrolysis (Geourjon et al., 2001) and in the lid module.

Poly-Pi is synthesized in *D. discoideum* using two enzymes namely DdPPK1 and DdPPK2. Homology to the bacterial polyphosphate kinase (PPK) sequence identified DdPPK1. Biochemical purification of polyphosphate kinase activity was performed and then the second protein DdPPK2 was recognized. It was found to comprise one actin-related protein and two actin-associated proteins (Zhang et al., 2005).

With reference to the functions of Ppk2 in *P. aeruginosa*, it is thought to play a role in the synthesis of alginate, an exopolysaccharide that envelops the bacteria and is an important virulence factor. Supporting this hypothesis, the expression of Ppk2 is elevated hundreds of times during the stationary growth phase of *P. aeruginosa* which should substantially increase the intracellular concentration of GTP. This closely matches the increased demand for GTP for alginate synthesis (Ishige et al., 2002). Up to now, PPK1 and/or Ppk2 homologues have been found in more than 500 bacterial species including many major human pathogens.

Table 2 displays the compositions of PPK1 and Ppk2 homologues in selected bacterial species.

Although there are no identifiable PPK1 or Ppk2 homologues in *S. aureus* and *T. denticola*, *ppnk* gene homologues (putatively) encoding Poly-Pi/ATP dependent NAD kinases, have been found in the genomes of all these bacteria. It remains to be seen whether there are additional unknown enzymes involved in the metabolism of Poly-Pi in the bacteria without identifiable PPK1 or Ppk2 homologues.

1.2.3.3 Polyphosphate: AMP phosphotransferase (EC 2.7.4.B2)

Polyphosphate:AMP phosphotransferase (PAP) enables Poly-Pi to be used as phosphate donor in the phosphorylation of AMP to ADP, which can be further catalysed to ATP by PPK1 also using Poly-Pi as phosphate donor.This cyclic reaction offers an additional pathway for ATP regeneration using Poly-Pi as energy source (Kameda et al., 2001).

It was first found in *Acinetobacter* Strain 210A (Bonting et al., 1991) and exhibits its highest activities with Poly-Pi with chain lengths ranging from 18 to 44 phosphate residues.

The degradation of Poly-Pi is processive. Pyro-, tri-, and tetraphosphate cannot be used by PAP, but serve as competitors to inhibit its activities. PAP has been identified in various bacteria, such as *P. aeruginosa* (Ishige & Noguchi, 2001), *Myxococcus xanthus* (Zhang et al., 2005) and *Bacillus cereus* (Shi et al., 2004).

Further studies have shown that in addition to its Poly-Pi-dependent AMP kinase activities, PAP protein from *A. johnsonii* 210A also has the ability to phosphorylate other NMPs and dNMPs (N: nucleotide, G, U, or C). However, compared to the reaction for AMP, PAP protein for GMP reacts at a rate 10 times lower, and for the pyrimidine nucleotides (UMP and CMP) it is around 1,000 times lower. It can also phosphorylate dNMPs, but the reaction rates are ca. 10-fold slower than those of the corresponding NMPs (Shiba et al., 2005).

PAP protein has also been shown to possess Poly-Pi synthesis activity using ADP or GDP as phosphate donors, with ADP is preferred over GDP (Itoh & Shiba., 2004). The activities of the PAP protein from *A. johnsonii* 210A described above are quite similar to those of the 2-domain Ppk2 proteins characterized to data, except for the ADP (GDP)-dependent Poly-Pi kinase activity.

Beyond that, the amino acid sequence of PAP protein cloned from *A. johnsonii* 210A is shown to be highly homologous to that of Ppk2 from *P. aeruginosa* PAO1 (Itoh & Shiba., 2004). Based on the above two studies, it's highly possible that PAP proteins are actually 2-domain Ppk2 proteins. However, this speculation needs to be further verified through the biochemical and biophysical evaluation of additional protein homologues.

1.2.3.4 Exopolyphosphatase (Polyphosphate phosphohydrolase, EC 3.6.1.11)

Poly-Pi are synthesized by polyphosphate kinase, and hydrolysed by exopolyphosphatase and endopolyphosphatases. Exopolyphosphatase (Ppx) catalyses the processive hydrolysis of Poly-Pi, progressively releasing phosphate monomers (Pi, orthophosphate) from the terminals of the Poly-Pi chain. Based on

their protein primary structure (i.e. sequence), Ppx and Gppa/ppx present in E. coli and other bacteria and archaea belong to the sugar kinases/actin/hsp70 protein superfamily ScPpx and Ppx from fungi and protozoa belong to the superfamily of DHH phosphoesterases (Reizer et al., 1993).

1.2.3.5 Guanosine pentaphosphate phosphohydrolase (EC 3.6.1.40)

The guanosine pentaphosphate phosphohydrolase protein (denoted by Gppa or Gppa/ppx) is similar to Ppx in its structure and functionality. The Gppa/ppx protein from Aquifex aeolicus has been characterized by it structure but not by its functionality.

The E. Coli Gppa/ppx protein has been shown to possess exopolyphosphatase activity, but its main function within the cell appears to be as a guanosine pentaphosphate phosphohydrolase: catalysing the hydrolysis of the 5'-γ-phosphate of guanosine pentaphosphate (pppGpp) to form guanosine tetraphosphate (ppGpp). The crystal structure of the Gppa/ppx protein from Aquifex aeolicus reveals a monomeric protein folding into two domains, with the active site located in the interdomain cleft (Kristensen et al., 2004).

1.2.3.6 Endopolyphosphatase (EC 3.6.1.10)

Endopolyphosphatase (Ppn) has been discovered in cells from archaea to mammals (Kumble & Kornberg., 1996).

The functional study of Ppn protein from *S. cerevisiae* reveals that this enzyme is able to cleave internal phosphoanhydride bonds within long chains of Poly-Pi to produce shorter chains: limited digestion predominantly produces chains containing 60 and 3 phosphate residues (Sethuraman et al., 2001). The mature Ppn protein from *S. cerevisiae* is a homotetramer composed of 35-kDa subunits that are generated by the proteolytic digestion of a 78-kDa pre-Ppn protein (Shi & Kornberg., 2005).

1.2.3.7 Poly-Pi/ ATP dependent NAD kinase (EC 2.7.1.23)

Nicotinamide adenine dinucleotide (NAD) and it phosphorlyated form NADP are the two factors essential for the proteins that are involved in a variety of cellular

metabolic and biosynthetic pathways. NAD kinase is responsible for catalysing the phosphorylation of NAD (at the 2'-position) to form NADP.In some bacteria, such as *M. tuberculosis*, *Micrococcus flavus*, and *Bacillus subtilis*; NAD kinase can utilize both Poly-Pi and ATP as the phosphate donor ((Kawai et al., 2000), and thus is denominated as Poly-Pi/ATP dependent NAD kinase.

However, in other organisms, such as *E. coli, S. cerevisiae* and the plant *Arabidopsis*, only ATP (and not Poly-Pi) can be used as the phosphate donor for this enzyme, which is correspondingly named NAD kinase.

1.2.3.8 Poly-Pi dependent glucokinase (EC 2.7.1.63)

Poly-Pi dependent glucokinase catalyses the transfer of the terminal phosphate residue of Poly-Pi to glucose, forming glucose 6-phosphate. The Poly-Pi dependent glucokinases from *M. tuberculosis* (Hsieh et al., 1996), *C. glutamicum*, *Propionibacterium shermanii* (Kowalczyk et al., 1996) and *Arthrobacter* sp. (Mukai et al., 2003) have been functionally characterized. All these enzymes optimally use Poly-Pi as the phosphate donor, although ATP can also be utilized. However, the glucokinase from *Microlunatus phosphovorus*, can only use Poly-Pi as a substrate (Tanaka et al., 2003).

Bork and colleagues, established that the three-dimensional structures of actin, hexokinase, and Hsp70 protein families included a common ATP binding motifs which are the "Phosphate-1" and "Phosphate-2" motifs joining with β- and Y-phosphates of ATP and the "Connect-1" and "Connect-2" motifs at the interface between the subdomains (Bork et al, 1992, Bork et al., 1993). Residues present in these motifs which play a role in the interaction of ATP, are highly conserved in many glucokinases; these residues are Asp and Gly (in Phosphate-1), Asp (in Connect-1), Gly and Thr (in Phosphate-2), and Gly (in Connect-2). Analysis of the deduced amino acid sequences of the *ppgk* gene demonestrated that this enzyme involves regions that are homologous to Phosphate-1 and Phosphate-2 regions of yeast glucokinase. Moreover, sequence alignment studies on other prokaryotic glucokinases sequences also revealed the exsistance of phosphate binding motifs. These phosphate binding sites are conserved from eukaryotic hexokinases to prokaryotic glucokinases.

The recognition of sequences participating in polyphosphate binding is not clear yet. Previously, Bork and co-workers showed that tryptophans in the peptide, KNDWTYPKWAKQ, of Poly -P-glucokinase from H_{37} Ra strain were found to be particularly oxidized by *N*-bromosuccinimide with associated loss of enzymatic activity (Hsieh et al, 1993). Tetrapolyphosphate or long chain polyphosphate substrate sustains protection toward this oxidation and the loss of activity. Residues 177-216 of the Poly-Pi-glucokinase sequence from H_{37} Rv encode a closely related sequence, RKDWSYARWSEE. Therfore this region might be a binding site for polyphosphate, in addition to Phosphate 1 and 2, which specifically enables the Poly-Pi-glucokinase to utilize polyphosphates. Several charged groups around this region as Lys_{188}, Glu_{189}, Lys_{190}, Asp_{192}, Lys_{197}, and Lys_{200} may play a crucial role in Poly-Pi binding mechanism (Bork et al., 1993).

$Poly\text{-}Pi_n$+D-glucose→$Poly\text{-}Pi_{n-1}$+D-glucose 6-phosphate.

This enzymatic activity was first observed in *Mycobacterium phlei* (Szymona, 1957), and then in numerous bacteria (Szymona et al., 1962; Szymona and Ostrowsky., 1964), including other *Mycobacteria* (Szymona and Szymona, 1978), *Corynebacterium diphtheriae* (Szymona and Szymona., 1961) and *Nocardia minima* (Szymona and Szymona., 1979).

The screening for polyphosphate glucokinase activities in different organisms showed its presence in the phylogenetically ancient bacteria belonging to the Actinomycetales (Szymona et al, 1967, 1969, 1977; Kulaev and Vagabov., 1983). The enzyme activity was not found in eukaryotes. The discovery of this enzyme was of the greatest significance to understand the role of Poly-Pi: it provided the first evidence of the possible function of Poly-Pis as a phosphate and energy donor without the nucleoside phosphate system.

There have been numerous reports on the occurrence of various isoenzymes of polyphosphate glucokinase in different microorganisms and on differences in the molecular weights of the enzyme in the same organism (Szymona et al., 1977; Kowalska et al., 1979; Pastuszak and Szymona., 1980). Szymona et al, 1977 found that the molecular mass of native enzyme from *M. tuberculosis* when using Sephacryl-200 size exclusion gel, was 118 kDa, while Pastuszak and Szymona

(1980) found a larger form of the enzyme. The enzyme from M. phlei was found to be a protein of 113 kDa (Szymona and Ostrowski., 1964) or 275–280 kDa (Girbal et al., 1989). The native enzyme from P. shermanii was reported to have a molecular mass of 31 kDa (Clark., 1990). The purified enzymes from *P. shermanii* (Phillips et al., 1993) and *M. tuberculosis* (Hsieh et al., 1993b) also showed multiple proteins by HPLC gel filtration, native PAGE and isoelectric focusing (lEF)–PAGE, although a single band was observed by SDS–PAGE.

One explanation for the existence of varying molecular masses of these glucokinase, is variable chain lengths of strongly bound Poly-Pis (Phillips et al., 1999), showed the enzymes from *P. shermanii*, *M. tuberculosis* and *Propionibacterium arabinosum* were all homodimers of 30 kDa sub units, variability in molecular mass being ascribed to binding of Poly-Pi of varying chain length: this remains speculative but plausible.

Poly-Pi glucokinase have been shown to contain a polyphosphate glucokinase activity and an ATP- dependent activity. Stable co-purification of these activities suggested that both of them can be catalysed by a single enzyme (Szymona et al, 1977; Pepin and Wood., 1986).

A detailed characterization of enzyme preparations unequivocally revealed that a single enzyme from these sources catalyses both Poly-Pi and ATP-dependent glucokinase activities (Hsieh et al., 1993a, Phillips et al., 1993, Kowalczyk et al., Phillips et al., 1999).

The most convincing evidence was cloning the gene from M. tuberculosis (Hsieh et al, 1996a). It was shown that the recombinant protein, expressed and purified from E. coli, contained both activities. The ability to utilize both inorganic (Poly-Pi) and organic (ATP) phosphoryl donors in glucose phosphorylation shows that the fundamental differences in the structures of the two phosphate donors brings differences in the residues involved in their binding also :Horn et al., (1991), Phillips et al, (1993a), and Hsieh et al., (1993a) provided evidence of separate binding sites for the substrates.

Despite the lack of similarities in sequence between eukaryotic hexokinases and prokaryotic glucokinases in the putative adenosine site, Hsieh (1996) found some

structural similarities between the adenosine site in polyphosphate glucokinase and the proposed adenosine site in yeast hexokinase.

Comparison of the kinetic features of Poly-Pi and ATP-dependent reactions for the enzymes from different sources supports the hypothesis that glucokinase in the earliest organisms may have predominantly been dependent on Poly-Pi rather than ATP (Phillips et al., 1999). There is a progressive decrease in the efficiency of Poly-Pi utilization by glucokinases, from older to newer organisms. The polyphosphate glucokinase from *Microlunatus phosphovorus* was closely related to the polyphosphate/ATP–glucokinase of *Mycobacterium tuberculosis*, but it could not phosphorylate glucose with ATP (Tanaka et al., 2003).

The catalytic sites are shared for Poly-Pi-dependent phosphorylation and ATP-dependent phosphorylation of the enzyme, and the Poly-Pi-utilizing mechanism of the enzyme was shown to be non-processive (Mukai et al., 2003).

The deduced amino acid sequence of the polypeptide exhibited homology to the amino acid sequences of the Poly-Pi/ATP–glucokinase of *M. tuberculosis* (level of homology, 45 %), ATP-dependent glucokinases of *Corynebacterium glutamicum (*45 %), *Renibacterium salmoninarum* (45 %) and *Bacillus subtilis* (35 %) (Mukai et al., 2003).

All of these observations show that Poly-Pi was a precursor of ATP in bioenergetics processes at the earliest stage of evolution (Kulaev, 1971, 1974). There might have been a gradual transition from Poly-Pi to ATP as the phosphoryl donor in glucose phosphorylation (Phillips et al., 1999).

1.2.4 Applications of polyphosphate

1.2.4.1 Removal of phosphate in the environment

Traditionally, metal salts (such as lime, alum or ferric chloride) are used to precipitate and remove phosphate (Pi) from waste water. This stimulates the ‘algal blooms’ that are unfavourable to the river water and estuarine ecosystems.

This is especially important for water contaminated by the agricultural waste containing fertilizers, or various discharges from industrial sources. However, this

method has limited application because of its high cost and the need for extra operations to remove the toxic metal from water.

These disadvantages can be eliminated by the use of enhanced biological phosphate removal processes, which utilize a group of polyphosphate accumulating organisms (PAOs) to accumulate a large amount of phosphate in the form of Poly-Pi in cells. Subsequently, the accumulated Poly-Pi can be efficiently removed along with the bacteria as sludge (McGrath & Quinn., 2003).

To further develop this process, genetically engineered bacteria may be used for their increased ability in taking up Pi and converting it to Poly-Pi.

Currently, there are numerous studies aimed at modifying microbial populations in 'activated sludge' to make the Poly-Pi accumulation process more efficient.

1.2.4.2 Chelation of mercury, Copper and Uranium

When the synthesis of Poly-Pi is induced or up-regulated in bacteria, they have enhanced abilities to take-up and chelate heavy metals from the growth medium.

The high chelating efficiencies of Poly-Pi towards mercury, copper and uranium have been reported in several studies (Pan-Hou et al., 2002-Renninge et al., 2004).

This approach may be used to remove these toxic elements from the environment.

1.2.4.3 Antibacterial actions and use as a food additive

Short-chain Poly-Pi (oligomers containing 3-10 phosphate units) is used as a food additive because of the following properties that are desired: (i) it is a safe, biodegradable polymer; (ii) it can inhibit microbial growth, especially for grampositive bacteria (Loessner et al, 1997, Maier et al, 1999); (iii) it is able to enhance the water binding, emulsification and colour retention of food, as well as retard oxidative rancidity.

The above effects are thought to be mainly due to the metal chelating activities of Poly-Pi (Jen &Shelef 1986, Denny et al., 2003). The characterization of two D. discoideum enzymes DdPPK1 and DdPPK2 in synthesizing Poly-Pi in vitro

suggested that both enzymes might contribute to the synthesis of Poly-Pi in vivo. The ability of Poly-Pi to chelate is considered to be responsible for the inhibitory effects. It is observed that certain Poly-Pi macromolecules bind to the cell wall, remain bound, and chelate structurally essential metals, which then destabilize the cell wall.

1.2.4.4 Regeneration of ATP and other nucleotide triphosphates

Generally, acetylphosphate, creatine phosphate and phosphoenolpyruvate are used as substrates for the enzymatic formation or regeneration of ATP.However, this method is exorbitantly expensive to be performed on an industrial scale. Poly-Pi is an inexpensive chemical that can be easily synthesized by heating NaH2PO4 at 700°C. Several studies have showed that Poly-Pi and its associated enzymes (such as Ppk and PAP) can be employed to regenerate ATP, which should be significantly cheaper than existing methods if scale-up is feasible (Kameda et al., 2001, Butler 1977-Haeusler et al., 1992). Moreover, when combined with the NDK (nucleotide diphosphate kinase) activities of PPK1, Poly-Pi can also be used as a substitute for phosphoenolpyruvate in the regeneration of uridine triphosphate, for the production of oligosaccharides (Noguchi & Shiba., 1998).

1.2.4.5 Insulating fibres

Poly-Pi can also be used to synthesize calcium polyphosphate fibres that have properties similar to asbestos. These fibres can be used to form insulating materials for possible use in a variety of household and industrial applications (Griffith, 1992). However, products containing asbestos release tiny fibres in to the air which cause a lot of health issues. This application is not currently being pursued because of these health related issues.

1.2.4.6 Medical applications of polyphosphate

As discussed above, Poly-Pi plays a number of roles essential for bacterial growth during the stationary-phase and during times of environmental stress.

Cells with low levels of Poly-Pi exhibit a wide range of deficiencies in their resistance to various challenges from an infected host organisms or harmful agents,

e.g. antibodies, lysozyme, antibiotics, etc. The intimate relationship between Poly-Pi levels and cell survival offers new clues and possible targets for future antimicrobial therapies.

In microbial cells, low Poly-Pi levels can be induced and maintained by inactivating the *PPK1* (ppk) gene, which encodes the main protein responsible for Poly-Pi synthesis. This gene is present in the majority of bacterial species sequenced to date, including many important pathogens, e.g. *Mycobacteriumtuberculosis*, *B. anthracis*, *Clostridium difficile*, *Neisseria meningitidis* and *P. aeruginosa* (Zhang et al., 2002).

Results of a number of studies conducted in this field shows that the deletion of the *PPK1* gene results in a variety of deleterious effects on bacterial metabolism, and remarkably decreases their persistence and virulence (Chen et al., 2002, Chouayekh & Virolle., 2002 and Sureka et al., 2007).

PPK1 gene homologues appear to be absent in all higher eukaryotes, which suggests that this protein, or polyphosphate metabolism in general, may be a possible target for future antibiotic therapies.

1.2.5 Various forms of Polyphosphate in cells

Poly-Pis may be present in living cells both in the free and bound states. Modern methods like ^{31}P NMR spectroscopy, give some evidence of this theory. Since the classic work of MacFarlane (1936), it has been considered that acid-soluble low-molecular-mass Poly-Pis are present in cells in the free -state. However, the question of the state of polymeric Poly-Pis within a cell cannot yet be considered as having been finally resolved.

1.2.5.1 Polyphosphate–Cation Complexes

Being polyanions, Poly-Pis can form complexes with different cations including biologically important Ca^{2+} and Mg^{2+} (Van Wazer and Campanella., 1950; Van Wazer, 1958; Corbridge, 1980; Bonting et al., 1993a; Cini et al., 2000). The dissociation constants for Mg^{2+}– and Ca^{2+}–polyphosphate complexes were 9.3 × 10−2 Mand 1.5 × 10−2 M, respectively (Bonting et al., 1993a).

Analysis with electron microscopy and energy disperse X-ray showed that Poly-Pi granules of *cyanobacteria* contain Mg^{2+}, Ca^{2+}, Mn^{+2} and other cations (Baxter and Jensen, 1980a, b; Jensen et al, 1982). Large Poly-Pi granules of *Acinetobacter* contain Mg^{2+}, Ca^{2+} and K+ in a ratio which depends on the extracellular concentrations of the above cations (Bonting *et al.*, 1993a). In bacteria, Poly-Pi complexes with heavy metals were observed: with Sr^{2+} and Ba^{+2} in *Plectonema boryanum* (Baxter and Jensen., 1980 a,b), with Ni^{2+} in *Staphylococcus aureus* (Gonzales and Jensen., 1998), and with Cd^{+2} in *Anacystis nidulans* (Keyhani et al, 1996) and *Escherichia coli* (Keasling and Hupf, 1996; Keasling, 1997; Keasling et al., 2000).

Poly-Pis can form complexes with arginine, spermidine, lysine, Mg^{2+}, Ca^{2+} and Mn^{2+} in vacuoles of yeast (Wiemken andD¨urr, 1974; D¨urr *et al.*, 1979; Okorokov et al, 1980; Lichko et al., 1982; Westenberg et al, 1989; D¨unn et al., 1994) and *Neurospora crassa* (Cramer and Davis, 1984). The cells of protozoa (Docampo and Moreno., 2001; Rodrigues et al., 2002a,b) and some algae (Ruiz et al., 2001b) possess an acidic organelle, acidocalcisome which contain pyrophosphate and Poly-Pis bound with Ca^{2+}, Mg^{2+}, Zn^{2+} and other cations. In these organelles, low-molecular-weight Poly-Pis, including the alkali soluble Poly-Pi3, is present in the bound state.

1.2.5.2 Polyphosphate–Ca2+–Polyhydroxybutyrate Complexes

Specific complexes containing polyhydroxybutyrate (PHB) and Poly-Pis have been found in, membranes of many organisms (Reusch and Sadoff., 1988; Reusch, 1992, 1999a, 2000). When such components of *E. coli* membranes were isolated and analysed, Ca^{2+} was found to be the predominant neutralizing cation (Reusch and Sadoff, 1988). Non-aqueous size-exclusion. Chromatography showed that the polymer length was 130–150 residues for PHB (~ 12 kD), (Seebach et al, 1994a), and 55–70 residues for Poly-Pi (~ 5 kD), as determined by SDS-PAGE electrophoresis (Castuma et al., 1995). Non-aqueous size-exclusion chromatography results estimated the molecular weight of the complex as 17 (± 4) kDa (Reusch et al., 1995).

1.2.5.3 Complexes of Polyphosphates with Nucleic Acids

Poly-Pi–ribonucleic acid complexes have been extracted from different organisms (Belozersky, 1955, 1958, 1959a; Chayen et al, 1955; Chaloupka and Babicky, 1957, 1958). The central cylinder represents the Poly-Pi helix with binding sites for Ca^{2+}, with the Ca^{2+}–PolyP complex being surrounded by the PHB helix (Reusch and Sadoff, 1988; Das et al., 1997).

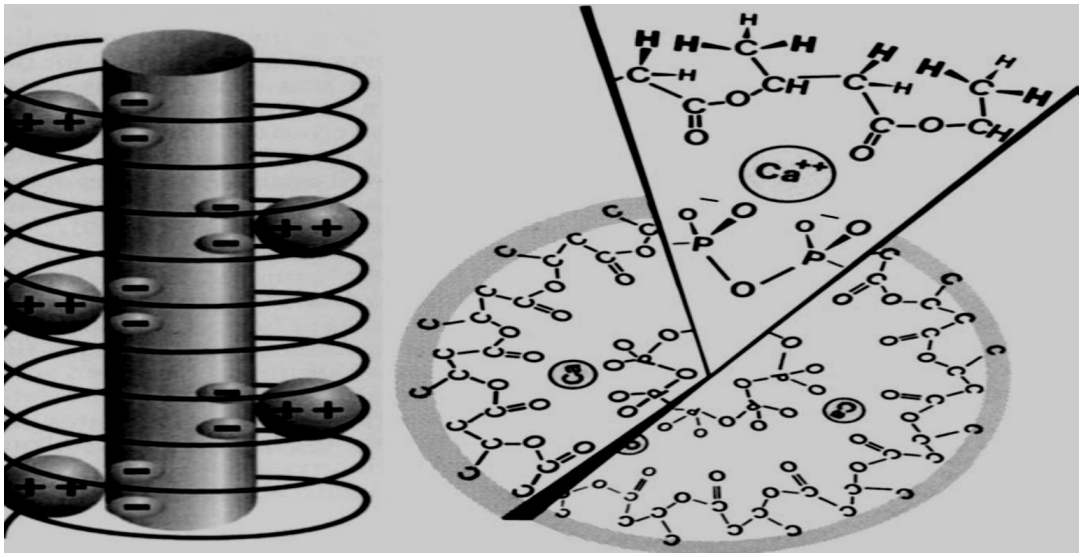


Figure 1.2–4 Model of the PolyP–PHB channel structure as proposed by Reusch and co-workers (from Reusch, 1999a).

Kulaev and Belozersky., 1957, 1958; Ebel et al., 1958c, 1962, 1963; Langen and Liss., 1958; Stahl and Ebel., 1963; Wang and Manchini., 1966).

These studies did not address the question if RNA was combined with the Poly-Pi or it was co-precipitated during extraction and separation from the cells because of the chemical and physio-chemical properties that are similar to them: It was found impossible to separate these compounds completely by precipitation and re-precipitation in the presence of Ba^{2+}, Mg^{2+}, Ca^{2+} and other metal ions. The use of different conditions for RNA separation (by sodium dodecyl sulphate (SDS) or phenol, or by a combination of the two) from yeast cells, which contained large amounts of Poly-Pis, failed to yield RNA fractions free from Poly-Pis. It proved especially difficult to separate Poly-Pis from such RNA fractions when they

contained relatively small amounts of Poly-Pis. For example, the RNA–PolyP complex obtained from *Aspergillus niger* could not be separated by electrophoresis in a Tiselius apparatus (Kulaev and Belozersky., 1958). Similar results were observed when paper chromatography was used in an attempt to separate the PolyP–RNA complex from yeast, in which RNA predominated (Chayen et al., 1955).

However, it was found possible to separate the latter from the RNA either by electrophoresis (Chayen et al., 1955; Ebel et al., 1962; Dirheimer et al., 1963) or by paper chromatography (Ebel et al., 1962; Dirheimer and Ebel., 1964a).

Belozersky and Kulaev (1970) and Stahl and Ebel (1963) showed that Ca^{2+} and Mg^{+2} ions were responsible for the formation of very stable and 'difficult-to-separate' PolyP/RNA complexes. Investigations into the possible existence of covalent or hydrogen bonds in these complexes have shown that both forms of bonding are absent, while electrostatic interactions mediated by Ca^{2+}, Mg^{2+} and other metal ions are possibly present (Ebel et al., 1962; Belozersky and Kulaev., 1964, 1970).

There might be some similarity between RNA–Poly-Pi and PHB–Poly-Pi, at least, participation of divalent cations in the linkage of the two polymers. It is probable that the complexing with Poly-Pi enhances the RNA stability. Some evidence has been obtained that in *E. coli*, Poly-Pis inhibits RNA degradation by degradosome (Blum et al., 1997).

The possibility of Poly-Pi interaction with DNA is now confirmed by evidences of data showing its participation in gene activity control (Kornberg., 1999; Kornberg et al., 1999). Earlier, Poly-Pi60 was found in DNA preparations from filamentous fungal species of *Colleotrichum* (Rodriguez., 1993).

1.2.5.4 Binding of Polyphosphates with Proteins

Liss and Langen (1960 a,b) showed that the most highly polymerized yeast Poly-Pi fraction, extractable only with strong alkali (0.05 M) or when incubated for a long period with diluted $CaCl_2$ solution, is apparently firmly bound to some cell components other than RNA. The removal of RNA by RNAase had no effect on the

extraction rate of this Poly-Pi fraction. It was considered that in this case Poly-Pi was bound to protein.

Numerous PolyP-binding proteins were detected in crude cell extracts from different organisms, including yeast and animals, using a filter-binding technique or affinity chromatography on PolyP–zirconia (Lorenz et al., 1994a).

Poly-Pi complexes with proteins play an important role in cell regulatory processes. It is observed that RNA polymerase isolated from the stationary-phase cells of *E. coli* was closely bound with Poly-Pi (Kusano and Ishihama., 1997). Poly-Pi competes with the DNA for the DNA binding sites at histones while Poly-Pis can interact with non-histone proteins in the nucleus. It is observed that Poly-Pis and PHB (polyhydroxybutyrate) are related to the ion-conducting proteins such as the human erythrocyte Ca^{2+}- ATPase pump.

Cellular Poly-Pihosphate has the ability to bind with other compounds, including polysaccharides, such as polyhexamines and chitin. Poly-Pis was shown to form complexes with polysaccharides of the cell wall of *N. crassa, in vitro* (Harold and Miller., 1961). The complex-forming reaction depended on both pH and the Poly-Pi chain length.The complexing ability of Poly-Pi is one of the major properties of this negatively charged biopolymer, it determines its regulatory function in living cells to a significant extent.

Such metabolically active molecules as Poly-Pis do not exist in cells in the free form in large amounts (Kulaev et al., 1999). They are compartmentalized and combined in the cell, either permanently or temporarily, with other compounds through the chelate bridges. As described above, Poly-Pis can form complexes with such biologically active cations as Ca^{2+}, Mg^{2+}, K^{+}, etc., with polyhydroxybutyrate and it is of great importance with nucleic acids and proteins. Probably, the interactions of Poly-Pis with other biopolymers are mediated in some cases by Ca^{2+}.

However, this has only been established with certainty for PolyP–polyhydroxybutyrate–Ca^{2+} complexes. The ability of Poly-Pis to form complexes with different components of living cells allows them to perform many specific functions in such cells.

1.2.5.5 ATP-dependent protease

Lon formed a complex with Poly-Pis under degradation of ribosomal proteins at amino acids starvation (Kuroda et al., 2001). Poly-Pi is able to compete with DNA for the DNA binding sites at histones (Schr¨oder et al., 1999), while Poly-Pis can interact with non-histone proteins in the nucleus (Offenbacher and Kline., 1984).

Poly-Pis and PHB have been found to be associated with ion-conducting proteins such as the human erythrocyte Ca^{2+}–ATPase pump (Reush et al, 1997) and the *Streptomyces lividans* potassium channel (Reusch., 1999b). Some enzymes of Poly-Pi metabolism, such as polyphosphate glucokinase (Phillips et al., 1999) and yeast high-molecular-weight exopolyphosphatase can contain tightly bound Poly-Pi (Andreeva et al., 2001, 2004).

1.3 Aims of research

The aim of this thesis is the biochemical characterization and study of PPGKm, a new discovered enzyme in ovine liver, its possible physiological functions and distribution in other tissues.

1.4 Specific objectives

1 - Kinetics studies and biochemical characterization of the novel enzyme.

2 - Purification, sequencing and recombinant expression of the enzyme.

3 - Investigate the cellular and subcellular distribution in different tissues using cell fractionation.

4 - Aim to establish possible physiological function(s) of the enzyme using a variety of biochemical strategies and bioinformatics where possible.

Chapter 2

Materials and Methods

All chemicals and reagents used for enzyme assays, buffers and NMR reagents were purchased from Sigma Aldrich, UK, Dorset, unless stated otherwise. All ovine tissues (liver, kidney, heart, skeletal muscle, bone marrow, lung and lymph node) and Porcine liver tissues were obtained from a local market.

2.1 Preparation of buffers

1.4.1 Homogenization buffer

HEPES buffers were made up in a concentrated form (4 x concentrations) and frozen. When diluted ready for the assay procedure the concentrations of the components were as follows:

7.5 mM $MgCl_2$, 2.5 mM DTE (1, 4-Dithioerythritol) or DTT (Dithiothreitol), 100 mM KCl 50mM Na HEPES (4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid sodium salt, N-(2-Hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid) sodium salt.

Appropriate amounts were weighed and dissolved in deionized water and frozen or refrigerated daily until required. Final assay buffers were completed by the addition of fresh substrates and linking enzymes and adjusted to the correct pH with NaOH/HCl as described below.

1.4.2 Assay buffer

Assay buffer (A) was prepared by the addition NAD, ATP or ADP to achieve 0.5 mM NAD and 5mM ATP or ADP in the final homogenization buffer, hexamathaphosphate (glass sodium salts), sodium tripolyphosphate salts, diadenosine tetra or penta phosphates, UTP, CTP and GTP were used as phosphoryl donor substrates at a concentration of 5mM, and then the pH was adjusted to 7.6 at 22 °C (pH = 7.4 at 38°C (see Appendix III for the optimum assay temperature). Blank assay buffer (buffer B) was prepared in the same way without adding any phosphoryl donor.

Approximately 200 IUof G6PDH was added per 50ml of assay buffer from a stock solution containing 958 IU per mg protein (40k IU per ml) to each of the buffer solutions.(One unit will oxidize 1.0 mmole of D-glucose 6-phosphate to 6-phospho-D-gluconate per minute in the presence of NAD at pH 7.4 at 30 °C).

2.2 Homogenization procedure

For the preparation of the homogenate, 1 gram of liver (fresh liver samples or frozen liver samples at -80ºC) were weighed and homogenized in 9ml of ice-cold homogenization buffer (producing a 10% homogenate) using a pestle and mortar for 10 minutes. All tissues (kidney, heart, muscle, lymph node, adipose tissue, Lung and bone marrow) were homogenised on ice. The homogenates were centrifuged using a Beckman coulter ultra-centrifuge for 60 minutes at 40,000rpm (100,000 g) at (4˚C). The 50,2Ti Rotor was used. The supernatant was collected and kept on ice to be used the same day or frozen for use on subsequent days.

2.3 Spectrophotometric determination of a novel PPGK activity in Ovine liver

Spectrophotometric coupling assays were conducted using a micro-plate reader spectrophotometer-Nano (BioTek) model number S/N 601-0029, at 38˚C Absorption readings were taken every minute at 340nm on each well over a period of 30 minutes following addition of homogenate to start the reaction. The assay being equivalent to the continuous method used in previous studies (Davidson &Arion 1986).

All assays were carried out in duplicate or triplicate (see relevant section for details) using a 96 well plate .The homogenate was assayed using ATP, ADP, AMP, UTP, UDP, CTP, AP4A, AP5A, long chain inorganic polyphosphate (P_{700}), sodium tripolyphosphate salts, Hexamethaphosphate (glass sodium salts), and a blank buffer (which contains the HEPES buffer with NAD and G6PDH) i.e. none of the phosphoryl donors were added to the blank buffer. The principle of this reaction is to measure the activity of hexokinases in an NAD-coupled reaction system, by measuring the accumulation of NADH causing an increase in absorbance at 340nm; this requires that G6PDH is in excess.

The appearance of NADH is then directly linked to the rate of formation of G6P, which gives the activity of the hexokinase enzymes.

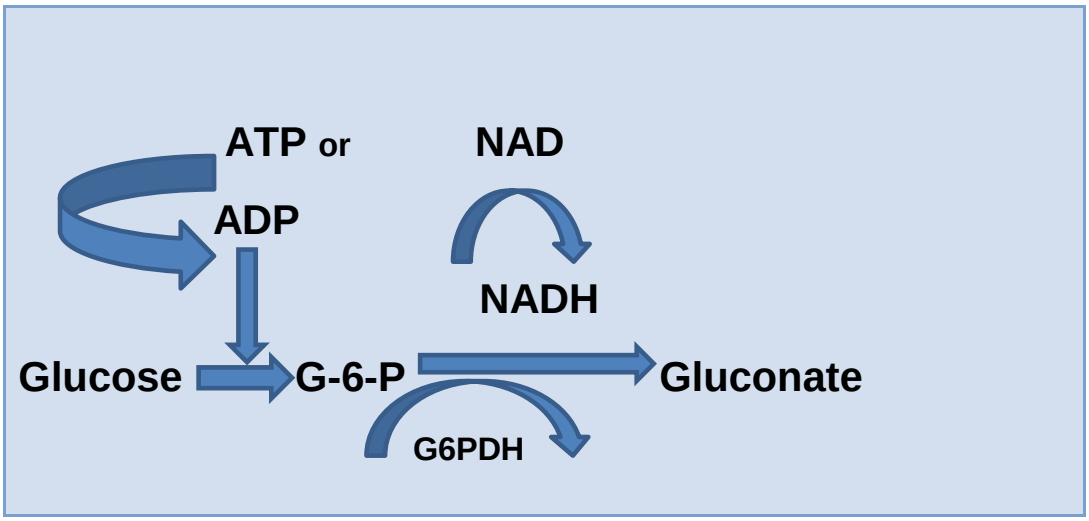


Figure 2.3–1 Glucose phosphorylation in the coupled enzyme reaction

Using high and low concentrations of glucose (0.5 or 100 mM) allows for the discrimination between low Km hexokinases and the glucokinases. This is because of the specific kinetics of the hexokinase isoenzymes in mammalian systems, which have either very high affinity for glucose (low Km=0.05-0.2 mM glucose) or very low affinity for glucose (Km or $S_{0.5}$ =8-20 mM glucose).

The final volume in each well was 300µl of which:

- 20µl homogenate or enzyme.
- 250µl reaction buffer (containing substrates or blanks).
- 30µl (1M or 5mM Glucose stock solution) which is diluted 10- fold in the well to 100mM and 0.5mM respectively.

2.4 Optimization of enzyme assay temperature

The hexokinase enzyme assay was conducted at a range of temperature from 25°C to39°C in the spectrophotometer. Experiments were made in triplicate, and the results recorded in Appendix III .The optimum temperature for the PPGK was found to be 38.5°C, and all subsequent enzyme assays conducted at this temperature. The optimum temperature for the other HKs was similar (Appendix III).

2.5 Thermostability of the novel enzyme

Ovine liver homogenate prepared as described in (section 2.2 above) was incubated at a range of temperature in pre-heated water bath at the following temperatures (40°C, 45°C, 50°C, 55°C, and 60°C) in 1ml aliquots for10 and 30 mins at each temperature, and then returned to ice for 20 mins until assayed, prior to assay samples were micro-fuged for 5 minutes at 1000g using microcentrifuge to remove any precipitated protein.The clear supernatant assayed for hexokinase and novel kinase activity using ATP and hexamethaphosphate as phosphoryl donors.

2.6 Calculations of PPGKm Activity

To calculate HK activity, the blank values (at0.5mM glucose) were subtracted from the results in response to 0.5mM glucose concentration with 5mM ATP, as previously described (Arion and Davidson).

GK activity was calculated by the subtraction of 'blank' values (activity in response to5mM ATP without glucose).HK activity (activity with 0.5mM glucose and 5mM ATP) was then substracted to give the high Km PPGK activity (in the presence of 2mM hexamethaphosphate ,HMP) free from the non-specific reduction of the sample blank and any low Km hexokinases

Present. This works well as PPGK has a very low affinity for glucose and is all, but inactive at 0.5mM glucose (see results section 4).

The enzymatic activity (IU) was calculated using Microsoft Excel. One IU is equivalent to 1µmole G6P formed per min per g liver (or mg of enzyme).

The calculation takes into account the change in absorbance per minute in the linear (continuous assay) range (see below) and converts this to µmoles NADH formed per min by accounting for the well volume and then conversion to IU by taking account of the amount of homogenate or enzyme added to the well.The assay has stoichiometric equivalence for NADH formation and G6P production ,because G6PDH is in excess (see above):this was assessed by adding more G6PDH to the wells with highest activity and checking that the rate does not increase.

2.7 Determination of kinetic parameters (Km, Vmax, Kcat)

A range of glucose concentration was prepared from a stock solution of 1M glucose (9 grams glucose in 50 ml dH_2O). Different glucose concentrations prepared: 0.5mM, 10mM, 20mM, 30mM, 40mM, 50mM, 60mM, and 100mM. Then they were assayed with ovine liver homogenate to investigate the Km of the novel enzyme using fixed hexamethaphosphate (HMP) concentrations (1mM or 2mM).

2.8 PPGKm activity dependence on HMP concentration

Eight Ovine livers were screened for the activity of the novel kinase with and without phosphoryl donors. The liver sample with lowest activity of the novel kinase (PPGK) in the absence of any phosphoryl donor was assayed with increasing concentrations of hexamethaphosphate (HMP) to examine the response of the enzyme toward the substrate.

2.9 Assessment of PPGKm inherent substrate

1.4.3 Screening for the novel enzyme activity and Hexokinase in different Ovine tissues & in different species

A variety of Ovine tissues collected, homogenized and ultra-centrifuged were then assayed for the presence of hexokinase and PPGKm activities using different phosphoryl donors (ATP, ADP, AMP, and Hexamethaphosphate).

Ovine tissues used for this purpose were: Kidney, skeletal muscle, cardiac muscle, liver, lymph node, bone marrow, lung and adipose tissue. Also liver samples from rat, pig and cow have been assayed for hexokinase and novel kinase activities using a variety of phosphoryl donors.

2.10 Temporal stability and Longevity of the enzyme

This experiment has been done to assess the stability and longevity of the novel enzyme compared to hexokinase .The ovine liver homogenate prepared and aliquots, some of the aliquots were left at room temperature (about 20°C) , others were left at (4°C and -20°C) all samples were checked daily for the enzymes activity , the samples left at room temperature were checked for 5 days then the activity

disappeared while the other samples at (4°C and -20°C) were checked for two weeks .

Hexokinase and PPGKm enzyme activity in ovine liver homogenates was monitored at room temperature and at 4^{o}C at set points for 5 days. This experiment was designed to assess the stability and longevity of the novel enzyme compared to Hexokinase.

2.11 Substrate specificity of PPGKm

The efficacy of a variety of phosphoryl donors on the novel kinase was studied, by assaying the homogenate using different phosphoryl donors, such as (ATP, ADP, AMP, Diadenosine tertaphosphate (AP4A), Diadenosine pentaphosphate (AP5A), hexamethaphosphate (HMP), sodium trimethaphosphate (Tri-P), UTP, CTP, GTP, and long chain inorganic polyphosphate (P_{700}) then PPGK activity was calculated as previously described.

2.12 Metal ions dependency assay

Sheep liver homogenate was assayed using 3 metal ions Zn^{2+}, Cu^{2+}, and Mn^{2+} at 2.5 and 7.5 mM, to check the activity of the sheep PPGK. This was carried out by replacing the Mg^{2+} in the assay buffers. In a different assay a range of Mg^{2+} concentrations (2mM, 4mM, 6mM, 7.5mM, 15mM, 20mM, 30mM, 40mM, and 50mM) were used to check the dependency of PPGK on Mg^{2+}.

2.13 Partial separation (purification) of Glucokinase, Hexokinase and Novel Enzyme Using Gel Filtration (sephacryl S-200 Gel)

Ovine, rat and porcine liver tissue were homogenized and centrifuged as described in section 2.1.3.The gel was prepared by mixing 3 volumes of sephacryl S-200 to 1volume of homogenization buffer. The supernatant of sheep, rat and pig liver homogenates were collected and transferred into centricon(Spin-X® UF concentrator MW cut off 50,000kDa) then centrifuged to be concentrated using a bench top centrifuge (BeckMan AllEGRA,X15-R, Refrigerated bench top centrifuge SX4750 rotor) at 5000 RPM for 30 minutes for Ovine liver sample and 50min for Porcine liver sample. Then 1 ml of each sample loaded on the top of the

gel column, the buffer was added and 1 mL fractions were collected, and kept on ice.

The fractions were assayed for enzymatic activity using the coupling enzyme assay previously described with high and low glucose as well as with ATP and Hexamethaphosphate.

2.14 Sub cellular fractionation and enzyme markers assay for the localization of the novel kinase enzyme

Ovine liver (50g) was weighed then washed 3 times for 5 minutes by immersing in cold PBS (15 ml for each wash). To minimize the rupture of cell organelles all homogenizers and solutions were kept on ice during the whole procedure of subcellular fractionation.

1.4.4 Homogenisation Buffers

- **Buffer I (0.25M sucrose):** 0.25M Sucrose .5mM Tris HCL.

5mM $Mgcl_2$, 1mM DTT all components were dissolved in 250 mL of distilled water. The pH of the buffers adjusted to 7.4.

- **Buffer II (2M sucrose):** 2 M sucrose, 5mM Tris HCL.

5mM $Mgcl_2$, 1mM DTT all components were dissolved in 250 mL of distilled water, with stirring on hot plate to dissolve the sucrose. The pH of the buffers adjusted to 7.4.

- **Buffer III 7.5:** mM Mgcl2. 2.5 mM DTT. 100 mM Kcl. 50mM Na HEPES

1.4.5 Methods

Ovine liver (10g) was homogenized by adding 50ml of homogenisation buffer (I) using Pestle & Mortar for 10 minutes on ice. The suspension was filtered through two layers of muslin cloth in order to remove any tissue debris, and then 3 x 1ml eppendorfs (original homogenate) were stored at -80, the remaining homogenate transferred to a 50ml centrifuge tube, and spun at 600g (2760 rpm) for 10 minutes.The supernatant retained (cytoplasmic fraction) and the pellet resuspended (nuclear fraction) in 6ml of homogenisation buffer II. To increase the yield of

nuclear fraction, the cytoplasm fraction was spun for another 10 minutes at 600g (2760 rpm), then the pellet (nuclear) resuspended in 6 ml buffer II.

The supernatant (cytoplasmic) was retained on ice, and the nuclear fraction centrifuged at 800g (Beckman coulter Avanti J-26XPI centrifuge S/N JXT08C02) for 15 minutes. The supernatant was discarded and the nuclear pellet resuspended in buffer I, and kept on ice for further steps. The supernatant from the first spin spun in the high speed centrifuge at 8000g (10,000 rpm) using (Beckman coulter Avanti J-26XPI centrifuge S/N JXT08C02), for 10 mins, the supernatant was saved and the pellet resuspended (mitochondrial fraction) in 3ml of homogenisation buffer I. To improve the mitochondrial fraction yield the previous step repeated. The remaining supernatant was then centrifuged at 15,000g (13,800rpm) (Beckman coulter Avanti J-26XPI centrifuge S/N JXT08C02) for 10 min, the pellet (lysosomal fraction) was resuspended in 6ml of homogenisation buffer III, and 1ml aliquots kept at -80°C, the final supernatant and the nuclear pellet were centrifuged at 100,000g (40,000 rpm) for 1hour using (Beckman coulter optima L-look ultra-centrifuge 52, Ti rotor S/N colo8E23).

After this last spin, 3 x 1ml aliquots of particle free supernatant were collected carefully avoiding the upper fatty layer and stored at-80°C and the pellet was resuspended (microsomal fraction) in 3ml of homogenisation buffer III, then 3 x 1ml aliquots. The nuclear pellet resuspended in 6ml of buffer III. (Cox&Emili 2006).

The nuclear and the mitochondrial fractions were sonicated on ice for 20 seconds, at the highest speed with 5 sec interval to rupture the nuclear and mitochondrial membranes and release the soluble proteins. Then, both fractions were centrifuged for 30 minutes at 9,000 g (Beckman coulter Avanti J-26XPI centrifuge S/N JXT08C02) to get rid of membranes debris. The supernatants were collected and aliquot in 1 ml eppendorfs, and stored at -80 the pellets were discarded.

All aliquots were examined for the PPGKm and hexokinase activity, using ATP, HMP, and without any phosphoryl donor.

All subcellular fractions were assayed for enzyme markers to check their purity as previously described (Lee & Chung., 2010).

The marker enzymes were used to identify the specific subcellular fractions as follows:

Mitochondria: Glutamate dehydrogenase.

Lysosomes: Acid phosphatase.

Microsomes: Glucose-6-phosphatase.

Particle free supernatant: Lactate dehydrogenase

Errors can arise where the fractions are cross-contaminated, and also where organelles are damaged due to improper homogenization and handling. This particularly applies to the acid phosphatase of lysosomes, which are released into the supernatant if the particles are ruptured by adverse physical or chemical conditions. The marker enzymes therefore provide an assessment of separation technique and relative success.

1.4.6 Determination of protein concentration (Lowry method)

A standard curve was constructed using the following concentrations of BSA: 20, 40, 80,120&160µg. The kit used was the modified Lowry protein assay kit from Thermo scientific the protocol was followed as breifly described below:

Preparation: 1ml of modified Lowry reagent reagent was added to each tube of standard and unknown protein sample, vortexed briefly, and then allowed to stand at room temperature for 10 minutes to allow any precipitated protein to re-dissolve.

A 100µl of 1X Folin Ciocalteu reagent added to each tube of standards and unknown protein, then incubated at room temperature for 30 minutes to allow the colour to develop.

To each tube, 2ml of distilled water was added and the absorbance acquired at 750nm against the control blank containing no protein.

The calibration curve was plotted, and the protein concentration determined in each fraction (including the original homogenate) the values were expressed in mg protein/ml of each sub-cellular fraction.

1.4.7 Enzyme marker assays

1. Glucose-6-Phosphatase assay

Principal: Glucose-6-phosphatase catalyses the hydrolysis of glucose-6-phosphate to glucose and phosphate. The Phosphate is measured following a simple inorganic reaction with ammonium molybdate producing a coloured product which can be monitored using a spectrophotometer.

Preparation:

Buffers and solutions were prepared as showen below:

A. Citrate Buffer, Citric acid (2.101g/100ml water), Adjust to pH 6.5 with NaOH.

B. Glucose-6-phosphate, 80mM Glucose-6-phosphate (243.3mg/10ml water). (Pre warmed to 37 °C prior to analysis) 10% TCA

C. 0.5% Ammonium Molybdate, Ammonium Molybdate 2.5g/500ml water, Add 14ml conc H_2SO_4 to 200ml water. Diluted acid poured into molybdate solution and adjusted up to 1litre.

D. Reducing agent: 5.7g metabisulphite & 0.2g sodium sulphite (Na_2SO_4) were dissolved in 50ml water, then 0.1g of 1-Aminonapthol-4-sulphonic acid (4 Amino-3-hydoxynapthalene-1-sulphonic acid) dissolved in the above mixture and diluted to 100ml with distilled water.(stored in dark bottle and used within 10 days .)

E. Phosphate standard: Pottasium dihydrogen phosphate 438mg/100ml (stored at 4 °C).

Methods

A. First step: For each fraction a 100µl aliquot was added into a test tube (a negative control tube containing 100µl of citrate buffer pH 6.5 was prepared). All samples Incubated in a preheated water bath at 37°C for 5 minutes.
100µl of pre-warmed (80mM) glucose-6-phosphate was added to each sample. (The exact time of addition for each tube addition was the same). All tubes were incubated the exactly15 minutes at 37°C, and then 2ml of 10% TCA (w/v)

added to stop the reaction. Each tube centrifuged for 5 minutes at 2300 rpm, and the clear supernatant saved for the later stages of the assay.

B. Second step: For each fraction1ml of the previous supernatant pipette into a test tube together with 5ml of 0.5% Ammonium molybdate. (In addition, a standard tube was prepared containing 100µg of phosphorous i.e. 100µl of standard into 1ml of distilled water.) When all the tubes were prepared, 1ml of reducing agent was added and the time noted. The absorbance measured at 660nm after exactly the same time for each fraction. The timing is not critical in itself but (15min – 60 min) time should be the same for all samples. The calculation was made to estimate the amount in µg of phosphate produced i.e. Absorbance sample / Absorbance standard x 100 x 2.2.

Given that 1µg of Phosphate = 0.032µmoles of glucose-6-phosphate: the enzyme activity calculated as µmoles of glucose-6-phosphate used/min/mg protein for each subcellular fraction and of the original homogenate.

2. Acid Phosphatase assay

Principle: Both Alkaline and acid phosphatase will catalyse general phosphatase reactions. The standard method of assaying is by incubating the phosphatase enzyme with a colourless substrate (p-nitro phenyl phosphate). Following catalysis, the products are p-nitro phenol and phosphate.

Conveniently, for assaying alkaline phosphatase, the reaction can be monitored continuously as the p-nitro phenol is coloured under alkaline conditions. Under acidic conditions however, p-nitro phenol is colourless, therefore assaying acid phosphatase is a little more difficult. As the reaction proceeds, no obvious changes can be seen, however, after a given period, the reaction is terminated by the addition of an alkaline buffer. The p-nitrophenol becomes coloured and can be determined spectrophotometrically

Preparation:

A. Acetate Buffer pH 4.5, 0.2M Sodium acetate trihydrate, 0.2M Acetic acid (11.5ml/l glacial acetic acid).

B. P-Nitro phenyl phosphate , P-Nitrophenyl phosphate (di-Tris salt) (0.369g/l)

C. Alkali-Tris buffer pH9, 1M Tris HCl, adjusted to pH7 containing 1M Na_2CO_3 and 0.4M K_2HPO_4. (Addition of Na_2CO_3 & K_2HPO_4 will raise pH to required 9).

D. P-Nitro phenol standard , 5mM p-Nitro phenol (0.06956g/l/100ml Alkali Tris), (final concentration was50µM), 10% Triton x-100

Method:

Each sub cellular fraction diluted (10 fold in 0.2M acetate buffer pH 4.5) and 200µl of each fraction was added to a separate test tubes, together with 1.2ml of acetate buffer and 100µl of Triton X-100 (10% v/v) a negative controls were included in which the fractions were replaced with buffer.

0.5ml of substrate (8mM p-nitro phenyl phosphate) was added, the contents of each tube mixed thoroughly, and incubated for 10 min at room temperature.

The reaction was stopped by adding 2ml of alkaline tris buffer (pH9.0). The precipitate was spun down in the bench centrifuge for one minute at top speed and the absorbance determined at 405nm for each sample.

As with the Lowry determination, a calibration curve will be necessary to determine the enzyme activity. Therefore a standard curve of p-nitro phenol was constructed.

3. Glutamate Dehydrogenase assay

Principle: Glutamate dehydrogenase catalyses the reversible oxidative deamination of glutamate to α-ketoglutarate. NADH is required as a coenzyme so the reaction can be readily measured following the change in absorbance at 340nm. EDTA is present in the reaction mixture to remove heavy metal ions which might otherwise inactivate the enzyme. As indicated earlier, glutamate dehydrogenase is a mitochondrial enzyme; therefore, in order to measure the enzyme activity, the mitochondria must be disrupted. This is achieved by addition of the detergent Triton X-100.

Preparation:

A. 0.1M potassium phosphate buffer pH7.4 was prepared.

B. 0.15M Sodium-2-Oxoglutarate, Prepared by adding 0.5475g α-Ketoglutaric acid, in 25ml phosphate buffer PH7.4.

C. 0.75M Ammonium acetate, 1.44g Ammonium Acetate, in 25ml phosphate buffer.

D. 30mM EDTA, 0.2192g EDTA, in25ml phosphate buffer.

E. NADH, 2.5mg NADH /ml in phosphate buffer.

F. 10% Triton

Method:

Each sub cellular fraction diluted (10 fold in 0.1M phosphate buffer pH 7.4) and 200µl of each fraction was added to a separate test tube, together with 2.1ml of phosphate buffer, 0.1ml of NADH (2.5mg/ml), 0.2ml of Ammonium acetate (750mM), 0.2ml of 30mM EDTA and 0.1ml of Triton X-100 (10%w/v) then mixed thoroughly.

The reaction was terminated by addition of 100µl of α-ketoglutarate (0.15M), the tubes were mixed, and the contents transferred into a cuvette, the absorbance monitored at 340nm for each fraction separately.

Provided that the Molar Absorptivity Coefficient of NADH at 340nm is 6220, the enzyme activity calculated as µmoles of NADH oxidised /min/mg protein for each sub-cellular fraction and as well as the original homogenate.

4. Lactate Dehydrogenase assay

Principle: The principle of this assay is that the coenzyme NADH is required for the enzymatic catalysis of pyruvate to lactate. As the reaction proceeds, the NADH will be oxidised to NAD and this form of the coenzyme no longer absorbs light at 340nm. Thus, the rate of change of OD at 340nm is a measure of the activity of the lactate dehydrogenase.

Preparation:

A. 0.1M Potassium phosphate Buffer PH7.4.

B. 2.5mM NADH

C. 50mM Na-Pyruvate

All above components were added to a 1.5ml cuvette the assay for each fraction was measured one at a time. Each sub cellular fraction diluted (10 fold in 100mM phosphate buffer pH 7.4) and 50µl was added to a 1.5ml cuvette, together with 600µl of 100mM phosphate buffer (pH 7.4), 230µl of distilled water and 60µl of 2.5mMNADH.

Contents of the cuvette were mixed thoroughly, and then 60µl of Na-Pyruvate (50mM) was added, mixed again and immediately the cuvette placed in a spectrophotometer and the absorbance measured at 340nm.

Readings were taken every 10 seconds and the reaction monitored until a linear rate of NADH oxidisation was observed.

Given that NADH has a molar absorptive coefficient of 6220, the absorbance/min and enzyme activity as µmoles NADH oxidised per minute/mg protein were calculated for each sub cellular fraction and of the original homogenate.

5. Protein Identification by LC-MS/MS

Analysis was performed by LC-MS/MS post in-gel digestion with trypsin. Peptides were eluted over a 20 min gradient using a Waters nanoAcquity UPLC interfaced to a Bruker maXis HD mass spectrometer. Peptide identification was achieved by searching tandem mass spectra against the expected protein database using the Mascot search program. Matches were filtered to accept only peptides with expect scores of 0.005 or better. Protein inferences required a minimum of two unique peptide sequences.

A. In-gel Digestion: In-gel digestion with trypsin performed after reduction with DTE and *S*-carbamidomethylation with iodoacetamide. Gel pieces were washed two times with 50% (v/v) aqueous acetonitrile containing aqueous 25 mM ammonium bicarbonate, then once with acetonitrile before drying in a vacuum concentrator for 20 min. A 0.2 g aliquot of sequencing grade modified porcine

trypsin (Promega) was added in 10 L aqueous 25 mM ammonium bicarbonate prior to incubation overnight at 37ºC.

B. LC-MS/MS: Peptide mixtures were loaded onto a nanoAcquity UPLC system (Waters) equipped with a nanoAcquity Symmetry C_{18}, 5 µm trap (180 µm x 20 mm Waters) and a nanoAcquity HSS T3 1.8 µm C_{18} capillary column (75 µm x 250 mm, Waters). The trap wash solvent was 0.1% (v/v) aqueous formic acid and the trapping flow rate was 10 µl/min. The trap was washed for 5 min before switching flow to the capillary column. Separation used a gradient elution of two solvents (solvent A: aqueous 0.1% (v/v) formic acid; solvent B: acetonitrile containing 0.1% (v/v) formic acid). The capillary column flow rate was 350 nl/min and the column temperature was 60°C. The gradient profile was linear 2-35% B over 20 mins. All runs then proceeded to wash with 95% solvent B for 2.5 min. The column was returned to initial conditions and re-equilibrated for 25 min before subsequent injections. The nanoLC system was interfaced with a maXis HD LC-MS/MS system (Bruker Daltonics) with CaptiveSpray ionisation source (Bruker Daltonics). Positive ESI-MS and MS/MS spectra were acquired using AutoMSMS mode. Instrument control, data acquisition and processing were performed using Compass 1.7 software (microTOF control, Hystar and DataAnalysis, Bruker Daltonics). Instrument settings were: ion spray voltage: 1,450 V, dry gas: 3 L/min, dry gas temperature 150°C, ion acquisition range: *m/z* 150-2,000, MS spectra rate: 2 Hz, MS/MS spectra rate: 1 Hz at 2,500 cts to 10 Hz at 250,000 cts, cycle time: 3 s, quadrupole low mass: 300 *m/z*, collision RF: 1,400 Vpp, transfer time 120 ms. The collision energy and isolation width settings were automatically calculated using the AutoMSMS fragmentation table, absolute threshold 200 counts, preferred charge states: 2 – 4, singly charged ions excluded. A single MS/MS spectrum was acquired for each precursor and former target ions were excluded for 0.8 min unless the precursor intensity increased fourfold.

C. Database Searching: Tandem mass spectra were searched against the *Ovis* subset of the NCBI database (147,277 sequences; 77,737,218 residues) using a locally-running copy of the Mascot program (Matrix Science Ltd., version 2.5.1), through the Bruker ProteinScape interface (version 2.1). Search criteria

specified: Enzyme, trypsin; Fixed modifications, Carbamidomethyl (C); Variable modifications, Oxidation (M), Deamidation (N, Q); Peptide tolerance, 10 ppm; MS/MS tolerance, 0.1 Da; Instrument, ESI-QUAD-TOF. Results were filtered to accept only peptides with an expect score of 0.005 or lower. Protein inferences required a minimum of two unique peptide sequences.

6. NMR Assay

Standard solutions of the following were prepared:

1ml of each component ATP, ADP, AMP, NAD, NADH was prepared at 10mM concentration. Dissolved in homogenization buffer, pH adjusted to 7.4 +10% D_2O.

A. NMR standards preparation

A number of standards for different metabolites (G6P, F6P, G1P, Hexamethaphosphate, 6 phosphogluconic acid barium salts, ATP, ADP, NAD and NADH) were prepared.The mixture for each standard was made as following:

300µl of 20% D_2O, 210µl of 100mM TSP (in D_2O), 264µl of 1M MDP, 50mM of each metabolite. The total volume was 3ml; the final volume was made up by adding homogenisation buffer (described in section 2.1.3). The pH of each sample adjusted to 7.4 at 22 °C. All standards were freshly prepared the same day of the experiment.

For each standard 600µl was added to the NMR tube (Aldrich color -spec NMR tubes, tube diameter L5mmx7in, thrift color coded yellow, limit 400MHZ frequency). ^{31}P,^{13} C, and ^{1}H NMR spectra were acquired for each standard at 500MHz. Spectra were acquired using 30^0 pulses and long relaxation times (at least 3 times the T_1 so that spectra were fully relaxed and could be used for quantitation. ^{1}H NMR spectra were obtained with H_2O saturation using a standard Bruker presaturation protocol (Bruker PRESAT). 32K spectra were collected with a 20ppm sweep width. ^{31}P NR spectra were obtained with ^{1}H-decoupling with a 50ppm sweep width. ^{13}C NMR spectra were obtained ^{1}H –decoupled using a 400ppm sweep width. All spectra were recorded on a Bruker AMX500 NMR sepctrometer.

NMR assay for Ovine liver PGK

Ovine liver homogenate freshly prepared and concentrated using the MWC 50kDa (section 2.1.4).

Three samples were prepared for NMR experiment as following:

Sample (1)	Sample (2)	Sample (3)
Contains 70µl of 100mM TSP/D_2O. 300µl Ovine liver homogenate. 300 µl homogenisation buffer containing 50mM NAD. 70µl of 1M ^{13}C glucose. 100µl 20%D_2O. 10µl G6PDH. 50µl of hexamethaphosphate (64mg /200µl DH_2O	Contained all the previous components except G6PDH was replaced by 10µl of DH_2O. NB: no MDP was added to the reaction mixture, because it was observed that the addition of MDP caused turbidity of the mixture.	Contained no G6PDH and no hexamethaphosphate they were replaced by DH_2O.

Table 2.14–1 Samples for NMR experiment

Components of each sample were mixed and pH adjusted to 7.4 at 22°C. 600 µl of each sample was pipette into the NMR tubes (Aldrich color -spec NMR tubes, tube diameter L5mmx7in, thrift, color coded yellow, limit 400MHZ frequency), then phosphorus, carbon and proton spectra were acquired at 25˚C.

The reactions were monitored over couple of hours (see results section). In other experiments the reaction carried out and monitored for 24hours. Spectra were recorded with a variety of acquisition parameters according to the requirements of each experimental condition (see above for more details on NMR parameters. Briefly, spectra were accumulated with 90+% relaxation, using a recycling time of at least 3^xRT, so that spectra could be used quantitatively. Sweep wifth was not altered to achieve this but an additional delay following the acquisition time was added where required.

7. SDS gel electrophoresis and silver staining of partially purified PPGK

A. Samples preparation: A few fractions obtained from different chromatography procedures were analyzed using denaturing SDS gel, and then stained using silver staining. In this experiment, total Ovine liver homogenate prepared as described in (section 1.4), then the homogenate ran through size exclusion gel filtration using sephacryl 200-gel (as described in section 2.1.15) the fractions contained the PPGKm activity were pulled together , also the nuclear fraction obtained from sub-cellular fractionation experiment (as described in section 2.1.16) used in this experiment , some of the nuclear fraction ran through the size exclusion gel filtration, and the fractions that showed PPGKm activity were used in this experiment.

Additional fractions were used from an experiment applying different chromatography methods including size exclusion, ion exchange and overnight depletion assay, in which a mixture of Ovine liver homogenate, glucose, NAD and G6PDH were incubated for 24 hrs at 37 ˚C to exhaust the inherent substrate, and then the mixture ran through the size exclusion gel filtration.The fractions that showed activity with HMP and was also used as a partially purified PPGKm.

- 16% precise- mini Tris-Glycine gel (10x8cm) was purchased from Thermo scientific, all samples described above were used in this experiment.
- 2µl of each sample was mixed with2.5µl of4X LDS sample buffer (from Thermo scientific), and 5.5µl ultrapure water (total volume 10µl).
- Samples heated for 2 minutes at 85 C̊ to be denatured.
- The running buffer prepared by adding 50mL of 10X Tris-Glycine-SDS running buffer to 450mL ultrapure water.
- The gel rinsed with ultrapure water, then placed in gel electrophoresis unit (Xcell sureLock mini-cell invitrogen).
- 200mL of running buffer was added to the inner (cathode) chamber and 300mL of running buffer to the outer (anode) chamber.

- 5 µl of the molecular weight marker and protein samples were loaded on the gel, the gel ran at 185V for 40 minutes.

B. Silver staining: Silver Xpress staining kit was purchased from invitrogen.

- **Fixing:** Using 90mL ultrapure water, 100mL Methanol and 20mL (200mL total volume) the gel was immersed for 10 minutes.
- **Sensitizing:** By adding 105 mL ultrapure water, 100mL Methanol and 5mL of the sensitizer (total volume 200mL). The gel immersed in 100mL for 30 minutes, then poured and the other 100mL added and the gel incubated for 30minutes.
- **Washing:** The gel washed twice using 200mL ultrapure water for 5 minutes each.
- **Staining:** 5mL from each of stainer A and stainer B (from the kit) were mixed with 90mL ultrapure water, and then the gel incubated in it for 15 minutes at room temperature.
- **Washing:** The gel washed twice with 200mL ultrapure water 5 minutes each.
- **Developing:** 5mL of the developer mixed with 95mL ultrapure water, the gel placed in it and incubated at room temperature for 10 minutes.
- **Stopping:** 5mL of the stopper was added directly to the developing solution.
- **Washing:** The gel washed 3 times with 200mL ultrapure water for 10 minutes each.

Gel was photographed and the desired bands were cut, and placed in clean eppendorfs. Samples were sent to be sequenced to York University (proteomic facility) Biology department, the method used was mass-spectrometry, and liquid chromatography mass-spectrometry (LC-MS/MS).

2.15 Statistical analysis of results

All experiments were conducted in triplicate as a minimum standard and ‘n’ for each experiment is shown in the legends to individual figures.

Significance of mean differences was analysed using ANOVA (one-way ANOVA), and P-values reported where significant differences were found, and are detailed in the legend to figures, accordingly. The results are shown plotted as the mean ± sem, and the full data for each plot is shown in the relevant section of Appendix IV. Values with $P < 0.05$ were considered statistically significant. The statistics were performed using Minitab statistical software (Minitab17).

Chapter 3

PPGKm phosphoryl donor substrate specificity for glucose phosphorylation in Ovine liver 3.1 Introduction

Glucose metabolism in Ovine species differs in a number of aspects from that in humans and other non-ruminants species. Apart from the observations that small amounts of glucose is actually absorbed from the gut (Lindsay., 1978), and also ovine are less sensitive to insulin compared to non-ruminants species (Brockman, 1983), one of the most significant differences from a metabolic point of view is that Ovine do not have a specific GK enzyme, according to Ballard (Ballard., 1965) or, multiple factors in the assay can significantly affect underestimation of glucokinase activity in liver homogenate (Davidson & Arion., 1986).

The observations of Davidson & Arion highlighted the fact that glucokinase activity may be inhibited in vivo by specific binding with other cellular proteins.

Thus in ovine liver we observed certain phosphorylation activity of the glucose at high concentrations in the absence of any common known phosphate donor, the first of our aims was to study PPGKm specificity for possible phosphate donors, including nucleoside phosphate as well as inorganic phosphate.

In order to determine the Km for the phosphate donor, we will study the PPGKm activity with inherent substrate and, the concentration that show the lowest PPGKm activity (low inherent substrate) will be used to further investigate the response of PPGKm to the increased concentrations of HMP, and to estimate the Km for phosphate donor.

3.1 Results and discussion

1.4.8 Phosphate donor specificity of PPGKm using different substrates.

To determine the specificity of our enzyme, PPGKm, for the phosphate donor ovine liver homogenate was prepared as described in methodology section 2.2, and assayed with different phosphate donors.

Figure 3.1-1 below shows the specificity for possible -and most common- phosphate donors in Nature, inorganic and nucleosides phosphates, although none

of them showed increase in the activity of PPGKm when compared to the activity with inherent substrate (0.44 IU). As it is seen in the graph, the activity of the enzyme was even decreased when trinucleoside phosphates, were used in the assay buffer.

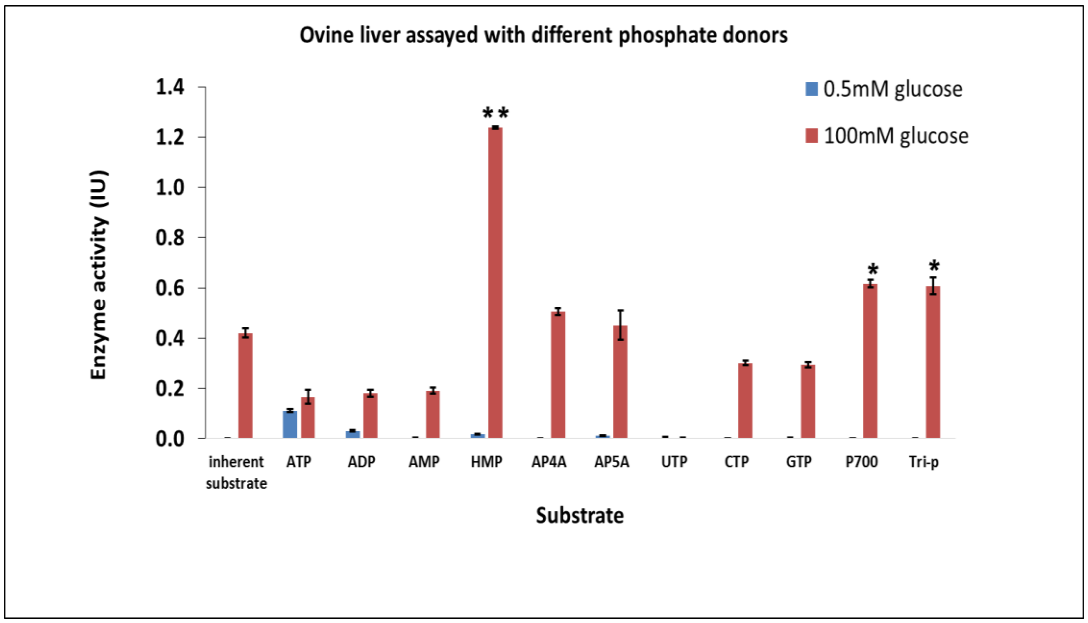


Figure 3.1–1 Glucose phosphorylating activity of PPGKm using different phosphate donors in the presence of either 100mM or 0.5mM glucose. Values are Mean ±SEM. (n=3 for each substrate). P-values when < 0.05 are represented by (*), or (**) when the p-value is ≤ 0.01. One IU of enzyme converts one µmole of glucose to glucose 6-phosphate per min per g of liver.

Glucose phosphorylating activity of ovine liver homogenate in the presence of 5mM phosphoryl donors (or blank – inherent substrate only) and either 100mM or 0.5mM glucose. Values are Mean ±SEM. (n=3 for each substrate).

However, the activity of the enzyme is higher for inorganic phosphate donors (P_{700} and Tri-P) 0.64 and 0.61 IU respectively, and becomes maximum when it is used HMP (1.3 IU), to compare the effect of each substrate on PPGKm activity and the accuracy of our results, I used one-way ANOVA Test (Table 3.1-1). The P-value of the activity when using the different nucleosides, compared with the inherent substrate, was as follows: ATP P-value =0.113(non significant).P-value when using ADP was 0.083, AMP P-value was 0.097 , AP4A P-value was 0.177, AP5A was 0.981, GTP was 0.587, non of the substrate tested showed significant increase in PPGKm activity compared to the activity with inherent substrate, PPGKm activity

with UTP was dramatically decreased when compared with inherent substrate and showed a P- value of 0.002 this may indicate an inhibitory effect of the substrate .

The results obtained until now demostrate the presence of an enzyme, PPGKm, in sheep liver extracts, shown for the first time to utilize inorganic polyphosphate as a substrate to phosphorylate glucose at high concentrations, being the first report of an enzyme involved in polyphosphate metabolism identified in mammals (Pavlov et al., 2010).

Substrate	Activity(IU)	P-value
HMP	1.3	0.001
Inherent substrate	0.44	
ATP	0.36	0.113
ADP	0.31	0.083
AMP	0.27	0.097
UTP	-0.023	0.002
AP4A	0.51	0.177
AP5A	0.47	0.981
P_{700}	0.64	0.041
Tri-p	0.61	0.041
CTP	0.32	0.532
GTP	0.31	0.587

Table 3.1–1 Shows the P-value when the activities (*IU) with the inherent substrate and each of the examined phosphate donors where compared, using one- way ANOVA (Analysis of variance). *IU (in which the activity is expressed as µmol.G-6-P produced / min / gram of liver). 'Activity (IU)' is the mean activity obtained from 3 expts per substrate (n=3).

1.4.9 Statistical significance of the inherent substrate as a phosphate donor

To evaluate the significance of that hexamethaphosphate being used as phosphate donor, twenty Ovine livers were checked for the new PPGKm enzyme activity with

ATP, hexamethaphosphate as phosphate donors, and with no substrate (inherent substrate).

ANOVA test (one-way ANOVA):

The results were calculated as the mean ± standard deviation (SD) for the number of experiments (3 times). Statistical significance between samples group were compared by one-way ANOVA. Values with $P < 0.05$ were considered statistically significant.

The p- value for PPGKm activity with inherent substrate vs. activity with hexamethaphosphate was 0.0320, which considered to be statistically significant The P-value was 0.0241 when the PPGKm activities with 5mM ATP and2 mM HMP were compared, these results showed the preference of PPGKm to use HMP over ATP as phosphate donor in all twenty liver samples assayed, this was clear specially when PPGKm activity with inherent substrate was low.Table 3.1-2. below summarizes the statistical results.

substrate	N	Mean	StDev	95% CI
Activity with ATP	20	0.07317	0.04238	0.03285, 0.11349
Activity in the blank	20	0.2445	0.1186	0.1752, 0.3139
Activity with 2mM HMP	20	0.5977	0.1813	0.5284, 0.6670

Table 3.1–2 Summary of statistical analysis used to investigate the significance of HMP as phosphate dono

1.4.10 PPGKm Km for HMP

Because it is difficult to determine the substrate Km for an enzyme in native homogenate, this was one of the major problems in this study to purify the protein and investigate the relevant biochemical characteristics. In this section an

experiment was carried out to roughly estimate the Km regarding HMP substrate, therefore as mentioned in section 3.2.2, liver homogenate was obtained from the previous screening experiment, sample number 7 which showed the lowest activity with the inherent substrate used, and assayed with incresed concentrations of hexamethaphosphate as phosphate donor to phosphorylate glucose, as shown in the Figure3.1-2 the enzyme activity increased with increased HMP concentration at high glucose concentration (100mM) compared to the activity with inherent substrate.

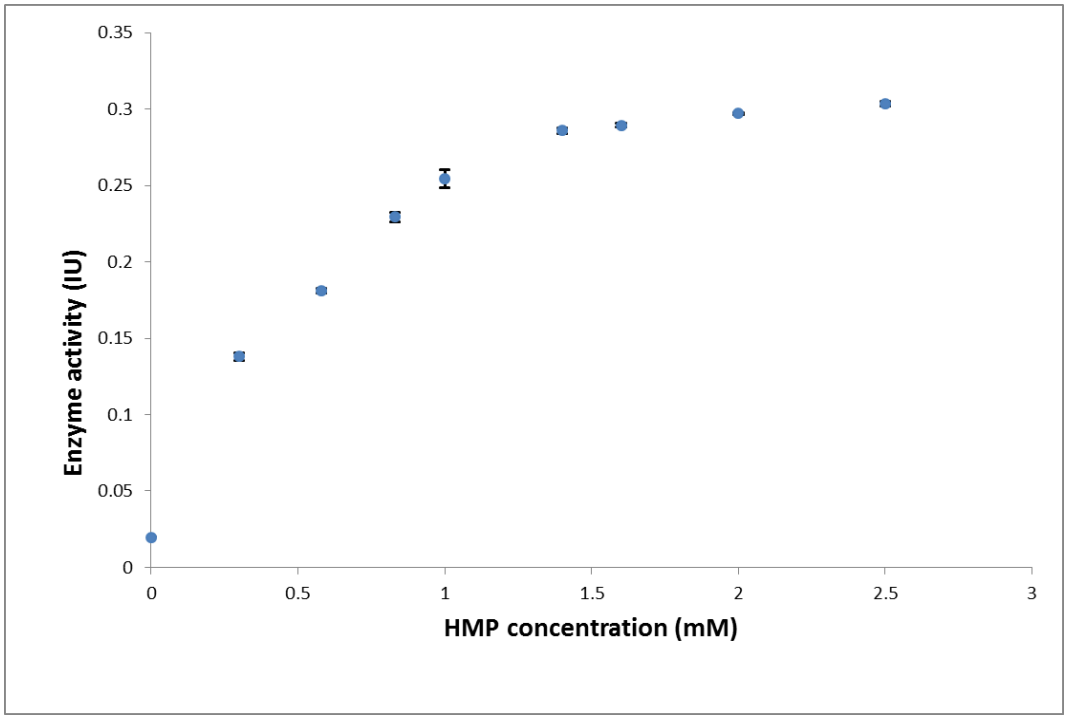


Figure 3.1–2 Assaying Liver sample7 (has lower inherent substrate activity) with increased HMP concentrations (0-3 mM) using either 0.5 mM or 100mM glucose, values were Mean±SEM, n=3.

The results obtained from this experiment suggest that PPGKm is polyphosphate dependent enzyme; the activity increases with the concentration. Follows Michaelis-Menten kinetic being possible to determine Km, it can be observed from the saturation curve that the V_{max} (maximum reaction rate) of the enzyme is roughly estimated as 0.4 µmol.min.g^{-1}. Half maximal velosity is estimated fromthe curve at HMP concentration ($S_{0.5}$) to be close to 0.58µm.

Substrate	Activity (IU)
Inherent substrate	0.030
0.3 µM (HMP)	0.136
0.58 µM (HMP)	0.180
0.83 µM (HMP)	0.227
1mM (HMP)	0.265
1.4 mM (HMP)	0.283
1.6 mM (HMP)	0.287
2 mM (HMP)	0.298
2.5 mM (HMP)	0.310

Table 3.1–3 shows PPGKm activity in Ovine liver homogenate obtained from previous experiment section 3.2.2 sample 7 assayed with increased HMP concentrations and 100mM glucose. 'Activity (IU)' is the mean activity obtained from 3 expts per substrate (n=3).

As shown in the table above, optimum PPGKm is obtained at 2.5mM HMP concentration.

A HMP -saturation curve was produced from these results and is shown in *Figure 3.2.3*.a double reciprocal plot of the same data is shown in *Figure 3.2.4*

From the double reciprocal plot we can use Lineweaver and Burk equation to calculate the Km: 1/v= (Km/Vmax) 1/[S] +1/Vmax.

Intercept is 1/Vmax =1/2.62=0.381

Gradient =Km/Vmax. 1.44=Km/0.381 = Km=1.44*0.381 = 0.549 mM.

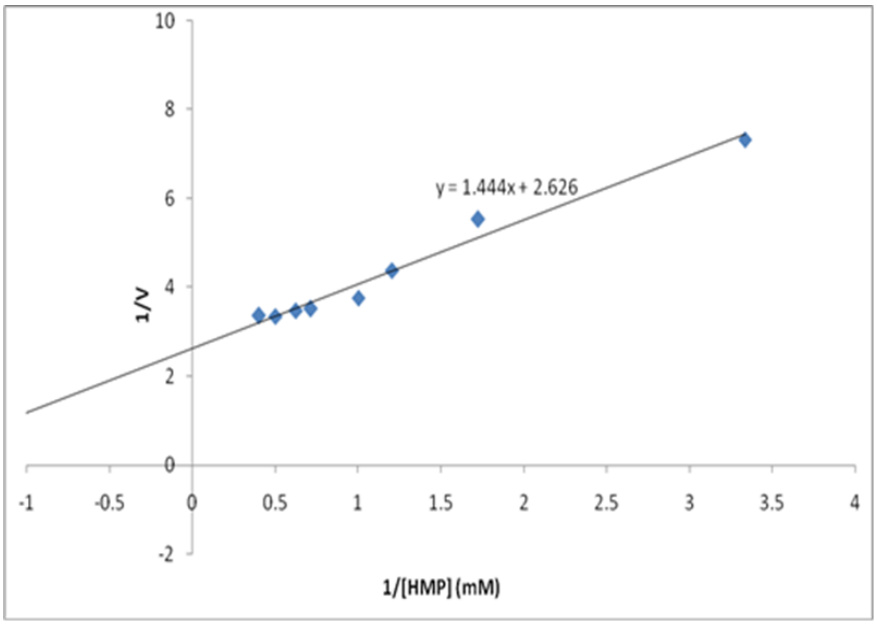


Figure 3.1–3 Double reciprocal plot of the responce of PPGKm to increased concentrations of HMP.

1.4.11 Screening different liver samples for the inherent substrate

To determine the incidence of phosphorylation activity of the glucose at high concentrations in the absence of any common known phosphate donor in ovine liver, twenty different ovine liver samples were assayed for (PPGKm) activity, as described in Section 2.3, Materials and Methods, with 5mM ATP or 2mM Hexamethaphosphate (HMP) as a phosphate donor, and without any phosphate donor (blank –inherent substrate), at high and low glucose concentrations.

The sample that showed the lowest PPGKm activity with inherent substrate (sample number 7), was used to further investigate the response of PPGKm to the increased concentrations of HMP, and roughly estimate the Km for phosphate donor.

Our results showed a variable activity of the PPGKm in all the samples tested, when using high concentration of glucose (100mM) (figure 3.1-4) in opposition to what happens at low glucose concentrations (0.5mM), where no observable significant enzymatic activity was detected (figure 3.1-5), except for hexokinase under 5mM ATP and 0.5mM glucose conditions.

When adding HMP 2mM; the PPGKm activity increases at both sugar concentrations although is very small at low levels and remarkably increased at high

glucose concentrations, especially when the activity with the inherent substrate is low (figures 3.1-4 and 3.1-5) that indicates the higher affinity or certain specificity of the PPGKm for the inorganic donor, HMP.

As shown in the results (figure 3.1-4), the activity of PPGKm with different nucleoside phosphates, and other phosphate donors revealed that the PPGKm activity increased dramatically when HMP used as a phosphate donor compared to the activity of the enzyme with inherent substrate (no phosphate donor added to the assay buffer). Further experiments were performed to elucidate the nature of the enzyme: e.g.coupling the assay in the presence and absence of either glucose or G6PDH and these results are shown in appendix I.To date no previous studies have established the presence of enzymes responsible for polyphosphate synthesis or utilization in mammals. However, many studies investigated the presence of polyphosphate in mammalian cells and its importance and diverse roles in mammals such as cancer cell proliferation (Wang et al, 2003) and protein stabilization by physical interaction with e.g.fibroblast growth factor (FGF-2) (Shiba et al., 2003).

The great variability in activity of PPGKm seen with inherent substrate, could explain the hypothesis that polyphosphate already bound to the enzyme in the native state, the chain lenght of bound polyphosphate then influencing inherent activity as measured in vitro.

Furthermore, no previous information available regarding the nutritional status, age, sex, and breed of the animal, since all samples were obtained from local butchers.

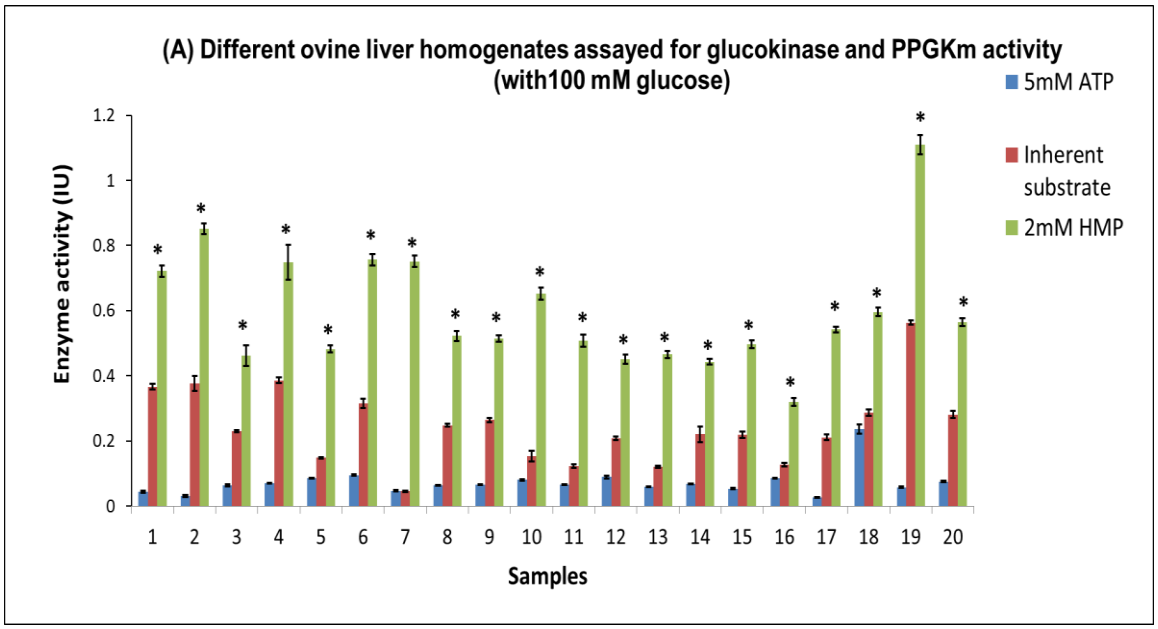


Figure 3.1–4 PPGKm activity assayed in 20 Ovine livers with 2mM HMP, 5mM ATP and inherent substrate with 100mM glucose.(Data represents the Mean ± SEM) n=3. The increase in activity when adding 2mM HMP compared with inherent substrate in the absence of ATP was highly significant in each liver studied (*indicates P- <0.05).

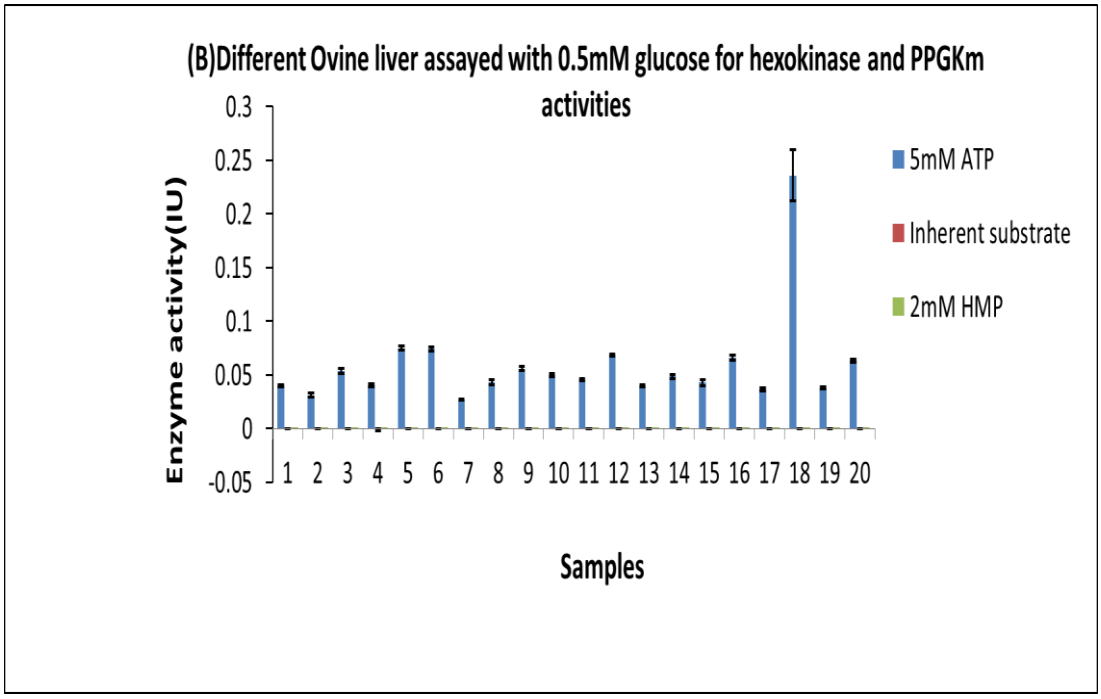


Figure 3.1–5 PPGKm activity assayed in 20 Ovine livers with 2mM HMP, 5mM ATP and inherent substrate with 0.5mM glucose.(Data represents the mean ± SE) n=3. Neither HMP nor inherent, native substrate was capable of significant glucose phosphorylation at 5mM glucose (p<0.001 in all cases).

3.2 Chapter summary

1. Our results clearly show that in ovine liver tissues, there is a constant enzymatic activity at high glucose concentrations (100mM) that varies with the samples It is concluded that the residual activity, observed by other authors (Ballard., 1965) in the high concentrated glucose blanks in from sheep liver extracts, is due to the presence of an enzyme. Due to its biochemical characteristics shown as result of our experiences, we have named this enzyme polyphosphate glucose kinase or PPGKm.

2. The ability of the ovine liver extracts to phosphorylate glucose at high glucose concentration in the absence of added phosphate donors, led to the hypothesis that the phosphate donor is inherent in the liver homogenate. There is a large variability of PPGKm when it is assayed in different liver samples without the addition of exogenous phosphorylating substrate. This variability decreased when HMP is added and much higher activity is returned. A simple explanation for this finding is that the liver sample genuinely contains a variable amount of polyphosphate substrate and that the PPGKm enzyme binds its polyphosphate substrate tightly. Before addition of HMP, livers with plentiful polyphosphate return high enzyme activities and those with lower substrate concentration return lower values of activity.

3. PPGKm activity was highest when HMP was used as a substrate versus other phosphate donors - 1.3 IU with HMP, a less significant activity of 0.6 IU for P700 and Tri-Pi, and under 0.4 IU for AMP, ADP, ATP and the nucleosids, GTP and CTP, being undetectable for UTP.

4. Maximum glucokinase activity in the presence of an inorganic polyphosphate donor has not previously been described in mammals but is commonly in bacteria.

5. The results obtained from the saturation curve with glucose and HMP suggest that PPGKm is a polyphosphate dependent enzyme, its V_{max} (maximum reaction rate) = 1.3 µmol.min.g^{-1} and Km for HMP of 0.58mM.

Chapter 4

Kinetics and Biochemical characterisation of PPGKm

4.1 Introduction

In this chapter a preliminery investigations were carried out to biochemically chracterize PPGKm in Ovine liver, in previous section characterised to be a polyphosphate dependent glucokinase in mammals.

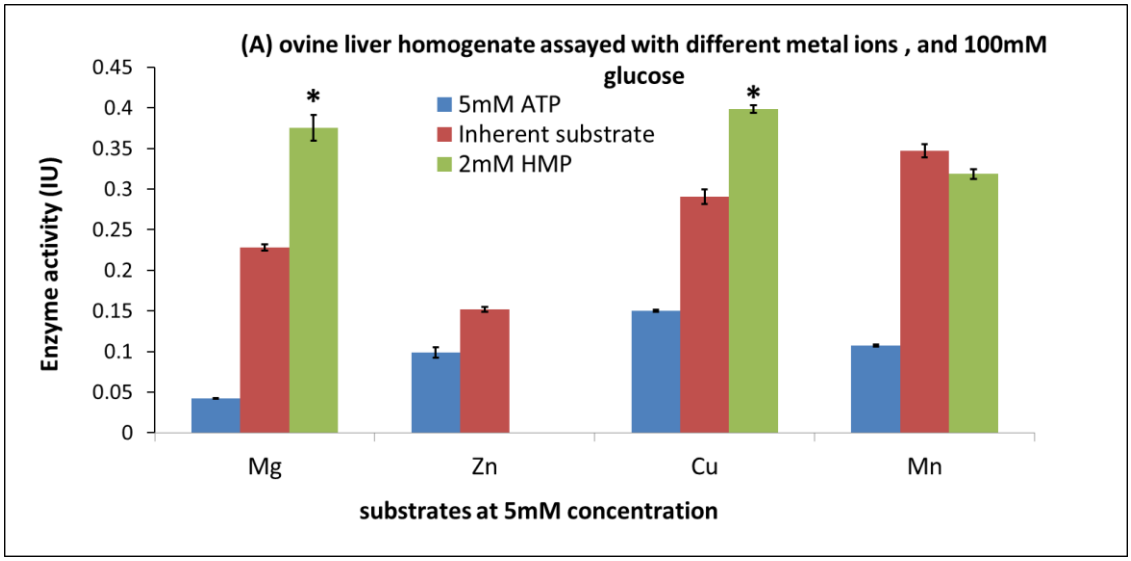
These investigations included Km studies for glucose dependency assay, thermostability of the enzyme, temporal stability, Mg^{2+} concentraions dependency and different metal ions dependency.

4.2 Results and discussion

1.4.12 Metal ions dependency of PPGKm

Ovine liver homogenate assayed using different divalent metal ions substrates at concentration of 5mM for each substrate the PPGKm activity investigated using high and low glucose concentrations (0.5mM and 100mM).
The results showed that hexokinase (enzyme activity with 5mM ATP and 0.5mM glucose) was Mg^{2+} dependent (figure 4.2-1 B). All values calculated were Mean ±SE, the P-value for hexokinase activity with Mg^{2+} compared to the other metal ions was 0.001 using ANOVA one-way test which indicates that hexokinase activity (0.5mM glucose and 5mM ATP) is dependent on Mg^{2+} .

On the other hand PPGKm showed activity with Mg^{2+} as well as other metal ions at 100mM glucose as shown in figure 4.2-1.A.This result is similar to the findings by Tanaka and coworkers, 2003 which indicated the requirement of strictly polyphosphate -dependent glucokinase in *Microlunatus phosphorus*,to 1-10 mM of Mg^{2+} ,Mn^{2+}, Co^{2+} and Zn^{2+} (Tanaka et al., 2003).This indicate that PPGKm and hexokinase might be different in their binding sites for substrates , and allosteric effect of different molecules.



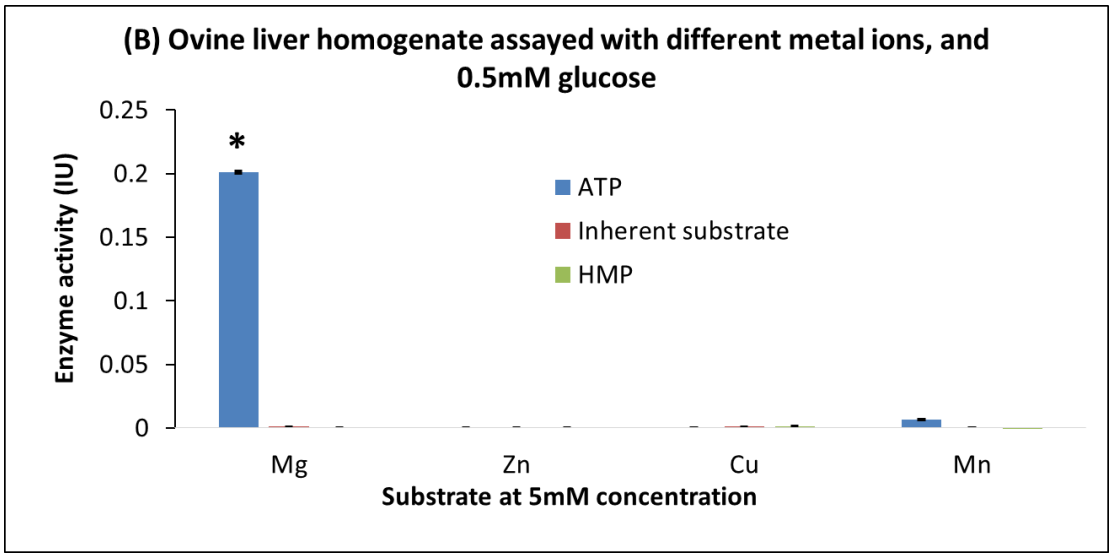
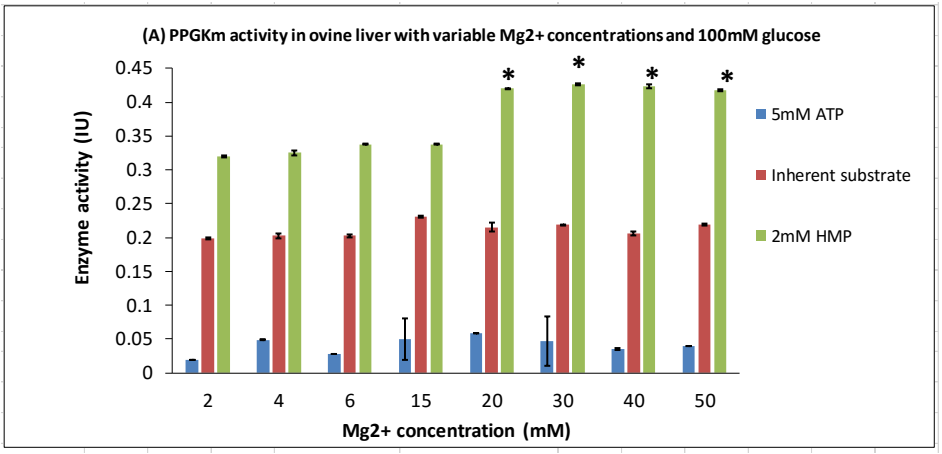


Figure 4.2–1 PPGKm activity assayed with different divalent metal (Mg^{2+},Zn^{2+},Cu^{2+},and Mn^{+2} ions) in the presence of 2mM HMP, 5mM ATP or inherent substrate with high and low glucose concentrations (0.5mM or 100mM glucose). The data represents Mean±SEM, n=3. At 0.5mM glucose only Mg^{2+} was capable of glucose phosphorylation ($p<0.001$ in all cases). At 100mM glucose, Mg^{2+} could be replaced by another maetal ion except Zn ($P<0.001$ for both postulates).



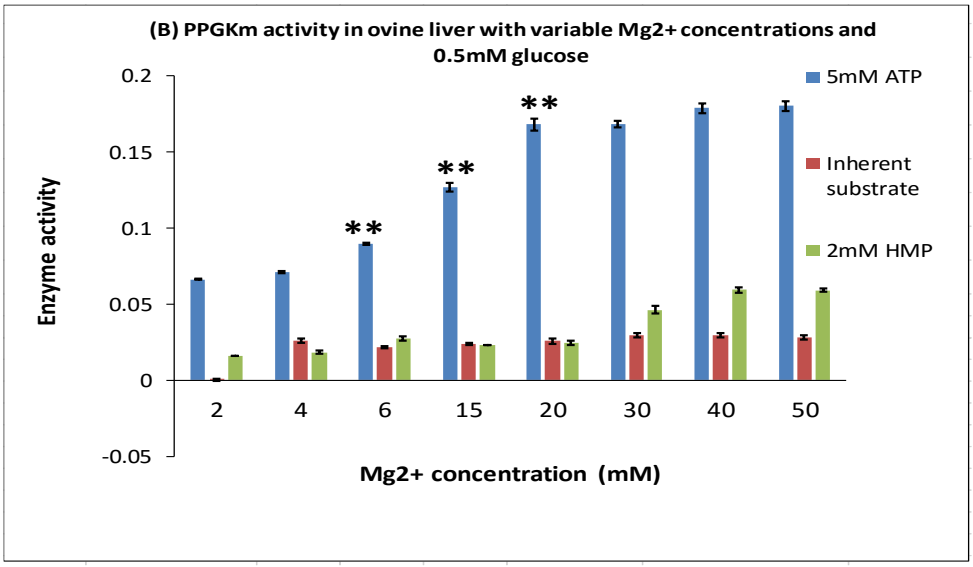


Figure 4.2–2 PPGKm activity assayed with increased Mg^{2+} concentrations with 2mM HMP, 5mM ATP or Inherent substrate at 100mM (figure 4.2-1 A) or 0.5 mM glucose (figure 4.2-1B). The figure plotted from Mean ± SEM (n=3). At 100mM glucose only large changes in Mg^{2+} concentration resulting in significant changes in glucose phosphorylation (* indicates P<0.05 compared to 15mM Mg^{2+} or less). At 5mM glucose the effect of Mg^{2+} on glucose phosphorylation is apparent at lower Mg^{2+} concentration (indicates significant changes (P<0.01) to the concentration preceding – e.g. 6mM Mg^{2+}compared with 4mM Mg^{2+}).**

PPGKm activity was investigated using 2mM HMP and variable Mg^{2+} concentrations, between(2-50 mM) as shown in the figures above with low and high

glucose (0.5 and 100 mM).The results showed an increase in PPGKm activity with Mg^{2+} concentrations at 20,30,40 and 50 mM compared to lower concentrations(2,4,6,16 mM) when 2mM HMP was used as phosphate donor with 100mM glucose. PPGKm activity when assayed with inherent substrate and 100mM glucose was the same with all Mg^{2+} concentrations tested, on the other hand activity with 5mM ATP and 100mM glucose showed the highest activity with 4mM Mg^{2+} with no observable increase with other concentrations, as shown in figure 4.2-2 A.

Figure 4.2-2 B shows the result of the same experiment, but with 0.5mM glucose it can be noted slightly increase of the activity with 5mM ATP,and 20,30,40 and 50mM Mg^{2+}, compared to the activity with lower concentrations ,surprisingly there was an increase in the activity of PPGKm with 2mM HMP, and with inherent substrate with higher Mg^{2+} concentrations, the activity of PPGKm always being detected with 100mM glucose, this result demonestrated the presence of PPGKm activity at 0.5mM glucose with either inherent substrate or when 2mM HMP used as phosphate donor, the possible explanation is that Mg^{2+} might have an allosteric effect on the PPGKm that enhances its affinity for glucose.

1.4.13 Glucose concentration dependency assay

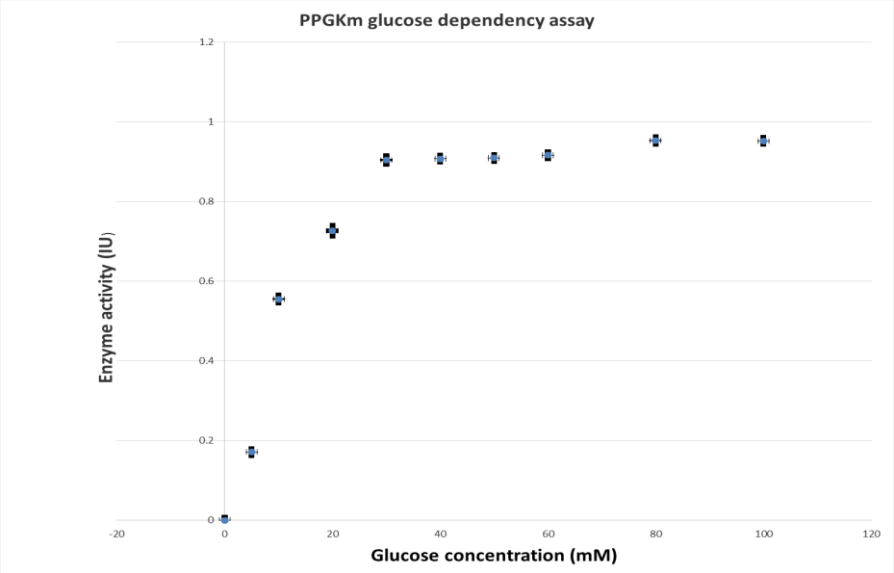


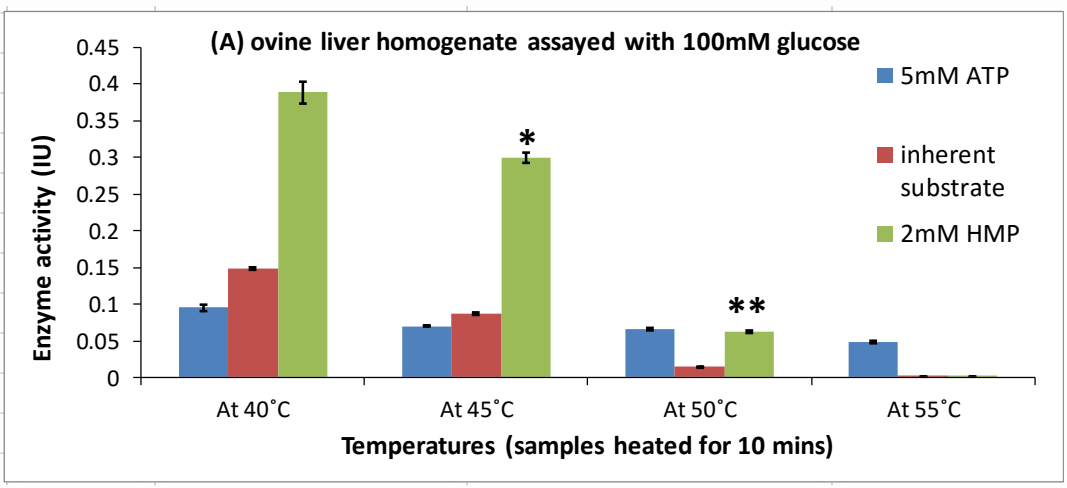
Figure 4.2–3 Glucose saturation curve of PPGKm in Ovine liver

The kinetics parameters for PPGKm regarding glucose were measured in this experiment, by assaying ovine liver homogenate with increased concentrations of glucose (0.5-100mM) as shown in figure 4.2-3. The assay buffer also contained 2mM HMP as phosphate donor. The values were Mean±SE. The experiment carried out in triplicate (n=3).

The graph was plotted and Vmax and Km were estimated .The Km is the substrate concentration at 1/2 Vmax (as shown from the glucose saturation curve above), the V_{max} (maximum reaction rate) of the enzyme is estimated from the saturation curve as 0.5 µmol.min.g^{-1}. Half maximal velocity is estimated from the curve at glucose concentration ($S_{0.5}$) Km estimated to be 15mM.

1.4.14 Thermo stability of PPGK

To study the thermostability of PPGKm, Ovine liver homogenate was prepared as described in section 2.2, and assayed before incubation at different temperatures as described previously in section 2.6. The samples were assayed spectrophotometrically, the activity was calculated for each sample the data obtained plotted as shown in figure 4.2-5(A) which represents the set of samples incubated for 10 minutes and assayed with 100 mM glucose, figure 4.2-5(B) represents set of samples incubated also for 10 minutes, but assayed with 0.5mM glucose. The residual activities were calculated as (%) from the activity values before and after incubation as shown in Tables 4.2-1, 4.2-2, 4.2-3, 4.2-4 in p in pages 99 -100. There was no activity with inherent substrate and 2mM HMP when assayed with 0.5mM glucose, therfore no residual activities for these samples were shown in the tables.



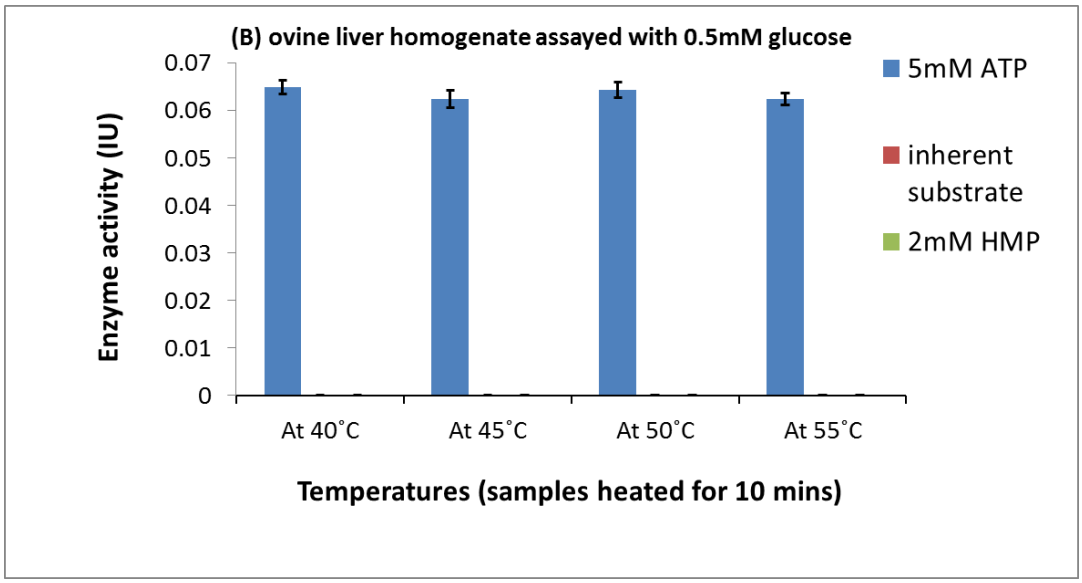
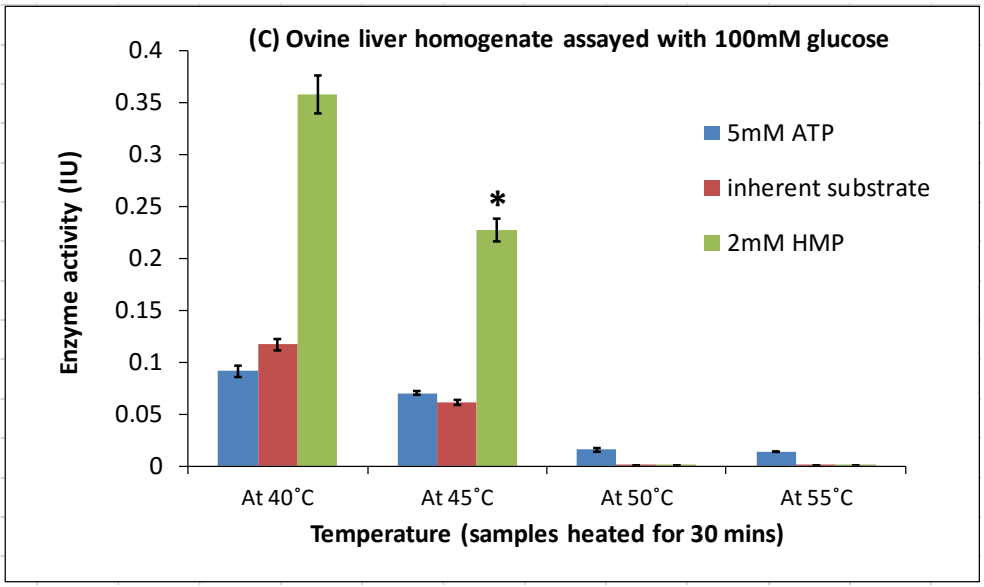


Figure 4.2–4 (A) and (B) shows the effect of heating at different temperatures for 10 minutes, with high and low glucose (0.5 and 100Mm) on the activity of PPGKm, data plotted from the Mean±SEM, n=3. (* indicates p<0.05 and indicates significant changes (P<0.01) to the adjacent lower temperature).**



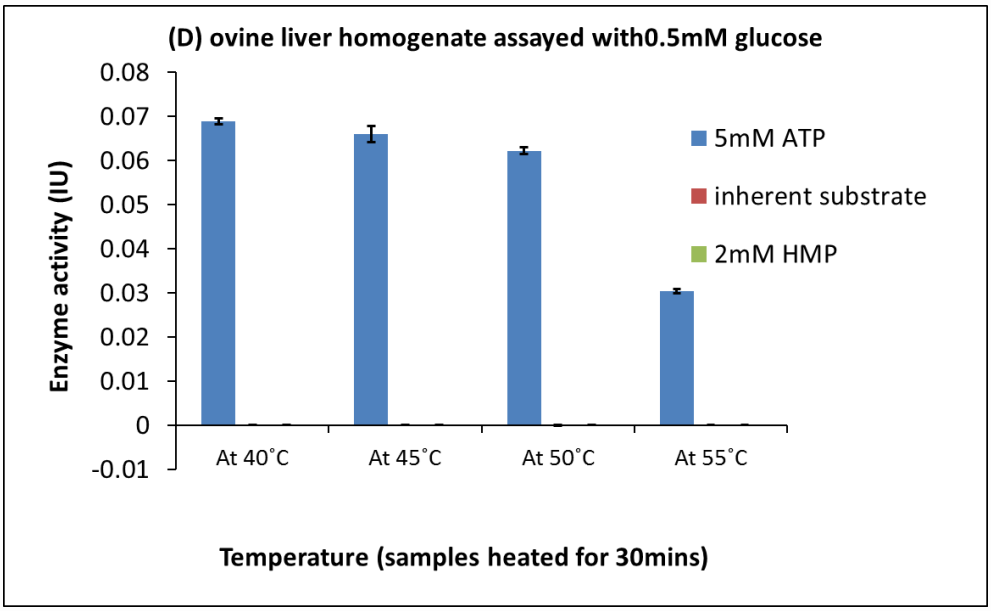


Figure 4.2–5 (C) and (D) shows the effect of heating at different temperatures for 30 minutes, with high and low glucose (0.5 and 100mM) on the activity of PPGKm, data plotted from the Mean±SEM, n=3. (* indicates $p<0.05$ to the adjacent lower temperature).

PPGKm gradually lost its activity when subjected to elevated temperature. This occurred more rapidly the longer period of incubation. Compared to hexokinase, PPGKm was more heat sensitive as it rapidly losts its activity. The presence of HMP appeared to make little difference to the enzyme heat stability.

Temperature	Residual activity with 5mM ATP+0.5mM glucose(heated for 10 mins)
40°C	90.6%
45°C	90.1%
50°C	88.0%
55°C	87.9%

Table 4.2–1 showing the residual activity (%) of thermostability experiment with 5mM ATP and 0.5mM glucose (samples heated for 10 mins).

Temperature (heated for 10 mins)	Residual activity with 5mM ATP+100mM glucose	Residual activity with Inherent substrate +100mM glucose	Residual activity with 2mM HMP+ 100mM glucose
40°C	86.4%	94.4%	97.0%
45°C	63.6%	55.6%	75.0%
50°C	59.6%	9.0%	15.6%
55°C	44.2%	0%	0%

Table 4.2–2 showing the residual activity (%) of thermostability experiment with 100mM glucose (samples heated for 10 mins)

Temperature	Residual activity with 5mM ATP+0.5mM glucose(heated for 30 mins)
40°C	62.6%
45°C	60.0%
50°C	56.5%
55°C	27.6%

Table 4.2–3 showing the residual activity (%) of thermostability experiment with 0.5mM glucose (samples heated for 30 mins)

Temperature (heated for 30 mins)	Residual activity with 5mM ATP+100mM glucose	Residual activity with Inherent substrate +100mM glucose	Residual activity with 2mM HMP+ 100mM glucose
40°C	83.2%	75.2%	89.3%
45°C	63.7%	39.4%	56.7%
50°C	14.1%	0%	0%
55°C	12.2%	0%	0%

Table 4.2–4 showing the residual activity (%) of thermostability experiment with 100mM glucose (samples heated for 30 mins)

1.4.15 Temporal stability of PPGKm

To assess the temporal stability of PPGKm, Ovine liver homogenate was aliqouted, and then some were kept at -20°C, 4°C and at room temperature (22°C).

Figure 4.6.1 represents the data obtained from the follow up enzyme assay through a period of 4 months with 100mM glucose, 5mM ATP or 2mM HMP, samples here were kept at (-20°C). Figure 4.6.2 shows the results of the same experiment, but with 0.5mM glucose.

The temporal stability of PPGKm was longer compared to hexokinase, since the highest activity for hexokinase lasts for about 10 days then declined, while PPGKm activity lasts for a couple of months before any observable decrease in the activity can be observed.

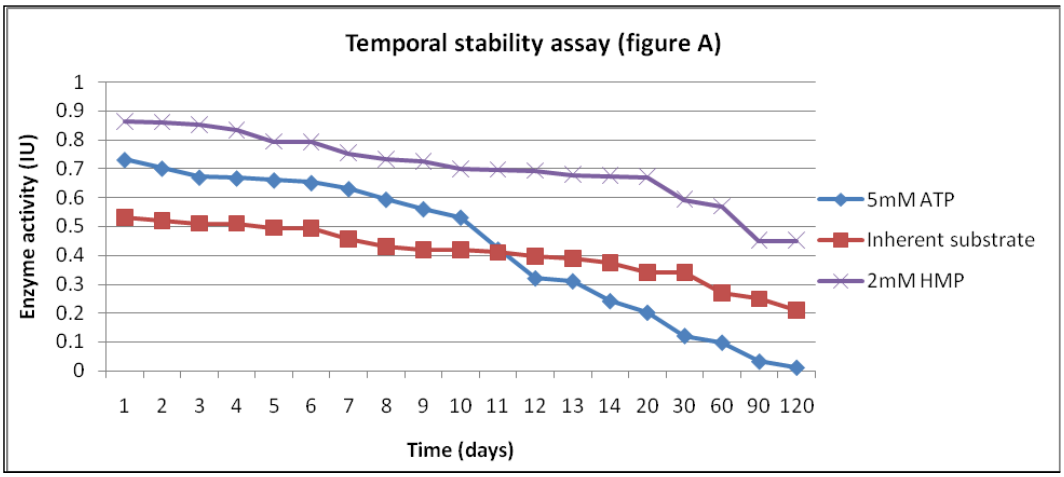


Figure 4.2–6 Temporal stability of PPGKm(inherent substrate or 2mM HMP) and hexokinase (5mM ATP) stored at -20˚C, and assayed with 100mM glucose (n=1).

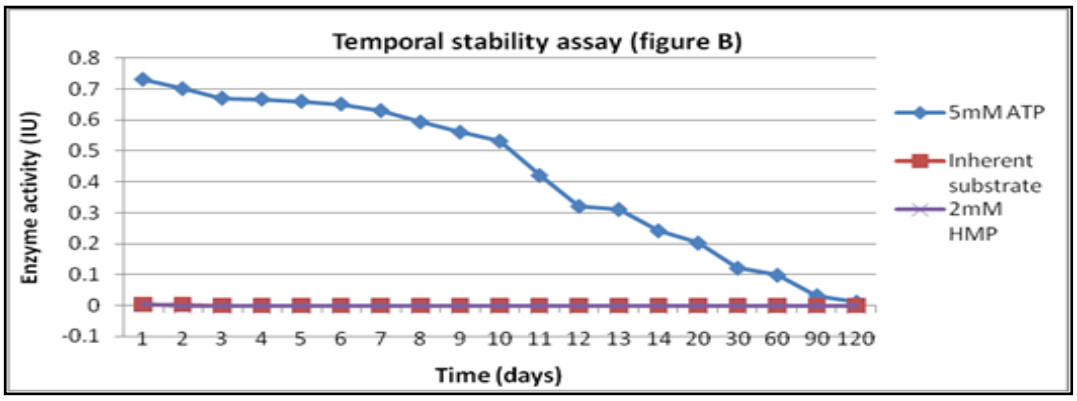


Figure 4.2–7 Temporal stability of PPGKm(inherent substrate or 2mM HMP) and hexokinase (5mM ATP) stored at -20˚C, and assayed with 0.5 mM glucose, (n=1).

Figure 4.2-7 represents the data obtained from the follow up enzyme assay through a period of 5 days with 100mM glucose, 5mM ATP or 2mM HMP, samples here were kept on the bench at room teperature (22°C). Figure 4.2-8 shows the results of the same experiment, but with 0.5mM glucose.

Activity of hexokinase (5mM ATP) declined dramatically at day 2, compared to the activity of PPGKm (2mM HMP) as well as with inherent substrate, which showed a longer life span at 22˚C, since its activity declined at day 3 and disappeared at day 4.

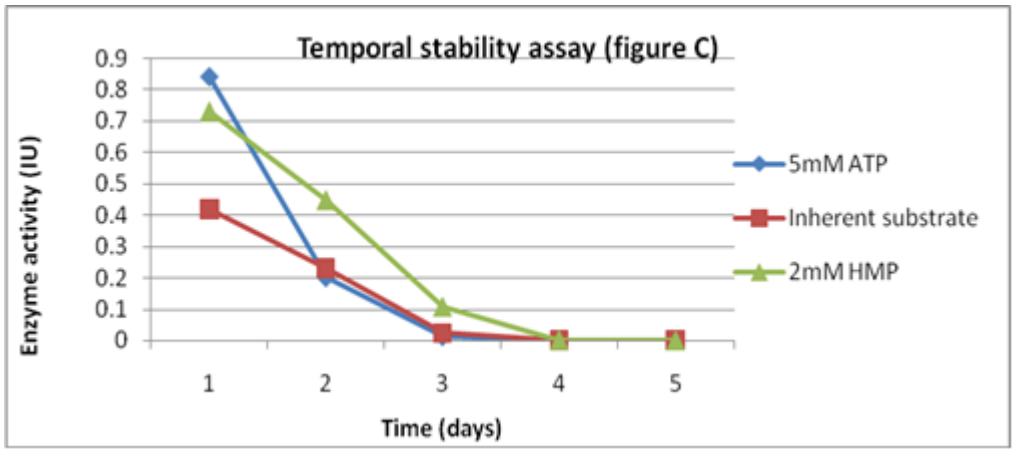


Figure 4.2–8 Temporal stability of PPGKm(inherent substrate or 2mM HMP) and hexokinase(5mM ATP) incubated at 22˚C and assayed with 100 mM glucose, (n=1).

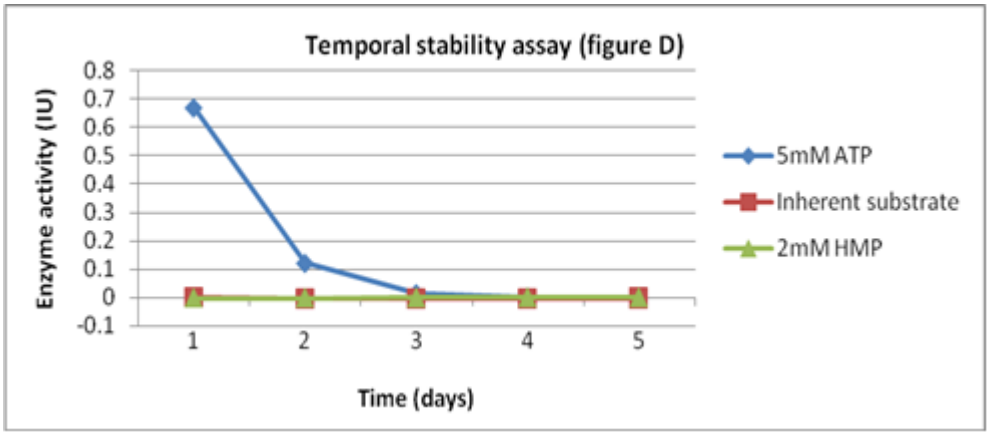


Figure 4.2–9 Temporal stability of PPGKm(inherent substrate or 2mM HMP) and hexokinase (5mM ATP) incubated at 22˚C and assayed with 0.5 mM glucose, (n=1).

Figure 4.2-9 represents the data obtained from the follow up enzyme assay through a period of 10 days with 100mM glucose, 5mM ATP or 2mM HMP, samples here were kept at 4°C in the fridge. Figure 4.2-10 shows the results of the same experiment, but with 0.5mM glucose.

The results demonestrated that PPGKm was more stable than hexokinase at 4˚C, since PPGKm activity with both inherent substrate and 2mM HMP remained up to day 8 on the other hand hexokinase activity (5mM ATP) disappeared at day6.

PPGKm is more stable than hexokinase when stored at -20°C with high activity lasting for 60 days, similar study by Tinsley and colleagues 1993, who reported that PPK purified from *N.meningitidis* stored at -20˚C for up to 4 months with no significant loss of activity.

Hexokinase is clearly quite labile under the conditions of the liver homogenate even when frozen, but it is more resistant to heating for short periods of time. These results suggest that HK and PPGKm undergo quite different mechanisms of activity loss, and suggest they have distinct structural differences or that these differences are due to association with Poly-Pi.

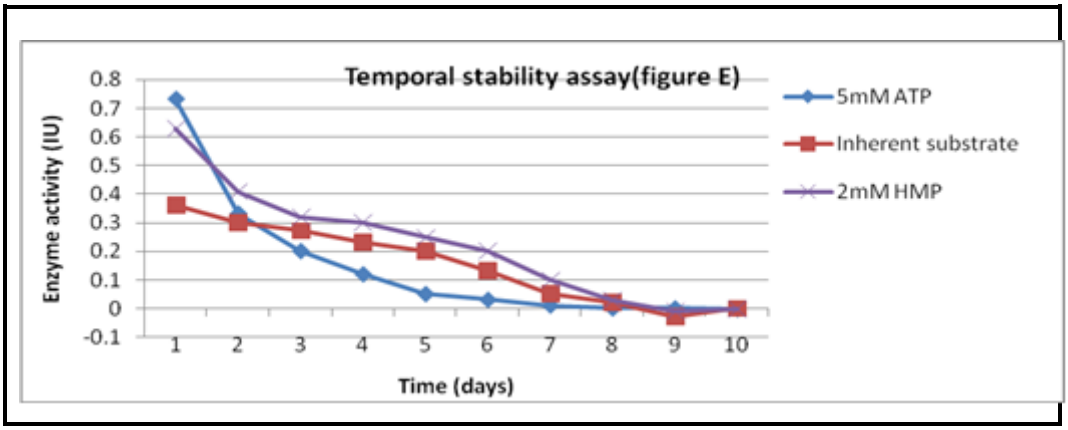


Figure 4.2–10 Temporal stability of PPGKm(inherent substrate or 2mM HMP) and hexokinase(5mM ATP) incubated at 4˚C, and assayed with 100 mM glucose over a period of 10 days, (n=1).

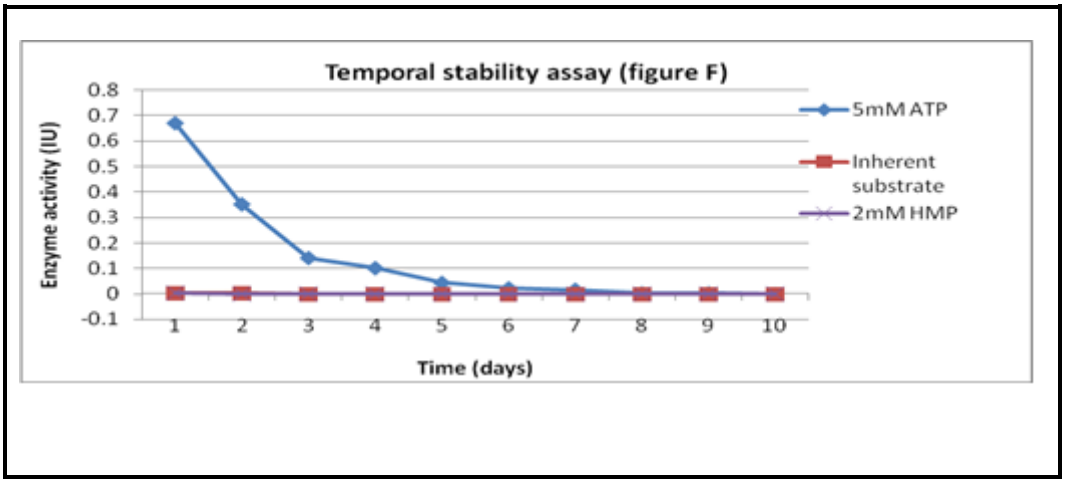


Figure 4.2–11 Temporal stability of PPGKm(inherent or 2mM HMP) and hexokinase(5mM ATP) incubated at 4˚C, and assayed with 0.5 mM glucose over a period of 10 days, (n=1).

4.3 Chapter summary

1-PPGKm in Ovine liver has a high Km for glucose, defining it was estimated to be 10mM, no sufficient studies were performed on sheep GK (hepatic GK or pancreatic GK) since it was previously (erroneously) reported by (Ballard., 1965) that sheep lack GK.

2-Ruminant do not absorb significant amounts of glucose from the gut because ruminal microflora will breakdown the glucose present in the diet and sheep rely almost entirely on glucneogenesis to maintain constant blood glucose.

3- At this time it is unclear how this new enzyme is integrated in to liver metabolism or what its role (if any) is in regulating glycemia. However, it is clear from the studies in this section that the enxyme is a stable hexokinase with a high Km for glucose and a practical Km for HMP, as this substrate is present in some cellular compartments at this concentration in ovine and other mammalian tissues (Kumble & Kornberg., 1995).

4-The high Km ($S_{0.5}$) isoform, ATP-dependent glucokinase, has a specific role in omnivores in both controlling insulin secretion and hepatic glucose uptake and output (Murphy H.C. et al., 2003). ATP-GK achieves this because its half-saturation is in the physiological range such that as blood glucose concentration rises from a fast, more G6P is made in tissues expressing GLUT2 transporters (pancreas and liver) – this signals insulin secretion and hepatic glycogen synthesis which both lower blood glucose in a concerted way back to fasting levels.

5-The Km of the ovine PPGKm is even higher than $S_{0.5}$ of glucokinase (EC2.1.7.2) in humans and rats (about 6mM in human) (Iynedjian., 2009), suggesting its role is sensing glucose concentration above that value. One possibility is that it is part of the gluconeogenic mechnaism and physically proximal to this important ovine biochemical pathway, such that the local glucose concentration is higher than that measured in the blood or liver as a whole. Pregnancy toxemia is a metabolic disorder occurring in pregnant small ruminants (ewe), and it is similar to diabetes mellitus in humans. The main cause of this disorder is abnormal carbohydrate and fat metabolism which affects pregnant ewes toward the end of gestaion period, and particulary if multiparous (Edmondson et al., 2012), PPGKm might be part of the abnormal glucose sensing in such conditions as ATP-dependent GK is absent in this species. Further biocehmical characterization, including intracellular location of the enzyme was undertaken (see below) to begin to address some of these questions. The PPGKm activity was shown to be dependent on divalent metal ions, and it is possible Poly-Pi is bound to the enzyme and it is negatively charged, divalent metal ions may play a crucial role in this binding.

6-PPGKm was more stable over time than than hexokinase, but HK had much better short term stability at high temperatures. These results suggest that inactivation of the enzymes occurs in different ways, and further structural studies will help to

elucidate these intersting features once the gene for the enzyme has been identified such that a pure form of the enzyme can be synthesised recombinantly for X-crystallograpic and NMR studies.

Chapter 5

Screening of different Ovine tissues and different species for the activity of PPGKm

5.1 Introduction

As the role of the PPGK enzyme was completely unknown, studies were undertaken to characterize the enzyme further. Both tissue wide and subcellular location of GK and HK in mammalian tissues are an important features of both the biochemical functioning of these enzymes in health and disease, but also how they are used to physiologically integrate glucose metabolism across the organs and maintain glycemia. The fundamental principles of glycemic control by GK are given in the introduction (1.7.2). The purpose of these studies was to investigate the tissue and organ-wide distribution of the enzyme in ovine tissues but also in other species. The Ovine tissues investigated include: liver, kidney, Lung, Lymph node, bone marrow, cardiac muscle, skeletal muscle, and adipose tissue. Parallel investigation for the presence of PPGKm activity in different animal species was done by comparing liver tissues homogenate from sheep, cow, pig, rat, rabbit and chicken. Since this is the first description of this enzyme's cell, tissue, organ and species distribution to my knowledge, no similar studies are available for comparison, although much is known about the distribution of other hexokiase enzymes.

5.2 Results and discussion

1.4.16 PPGKm Enzyme assay for various Ovine tissues

The tissues homogenate were prepared as described in methodology section 2.2.

The homogenates were assayed using 2mM HMP, 5mM ATP and with no substrate (inherent substrate) at high and low glucose concentrations as shown below in the graphs. The results were Mean±SE. (n=3)

PPGKm was shown to be dominant in liver samples, very little activity was detected in cardiac muscle, skeletal muscle and lung other tissues examined (adipose tissue, bone marrow and kidney) showed no PPGKm activity.

Once the gene for PPGKm is identified, an antibody can be generated and immunohistochemistry and molecular studies could be applied to investigate the espression profile of PPGKm in various tissues and subcellular compartments, a cDNA library could also be generated from cellular mRNA.

PPGKm might be expressed in all tissues, but the presence of certain inhibitors doesn't allow the detection of its activity. Future research can investigate the presence of such inhibitors and their co-localisation, and if they are absent in liver tissue, this will justify the fact that PPGKm has the predominat high activity in Ovine liver.

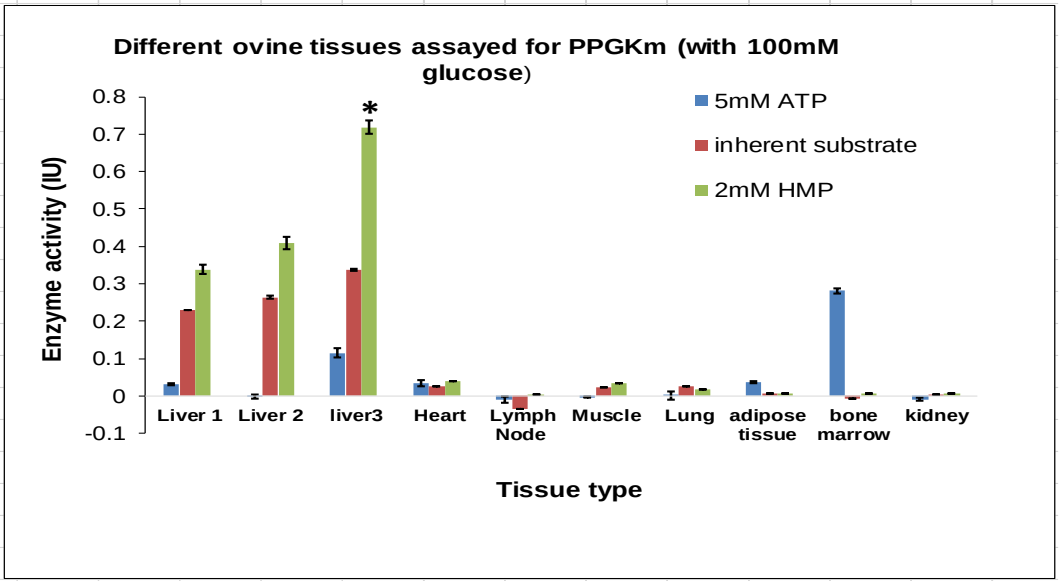


Figure 5.2–1 Different Ovine tissues screened for PPGKm activity with 5mM ATP, 2mM HMP at high glucose concentraion (100mM), data represent Mean±SEM, (n=3). Liver 3 clearly had the highest PPGKm activity measured with either inherent or substrate or HMP (P<0.01)

As shown in figure 5.2-1, no PPGKm activity was detected in inherent substrate or 2mM HMP when assayed with 0.5mM glucose, only hexokinase activity was present (5mM ATP+ 0.5mM glucose).

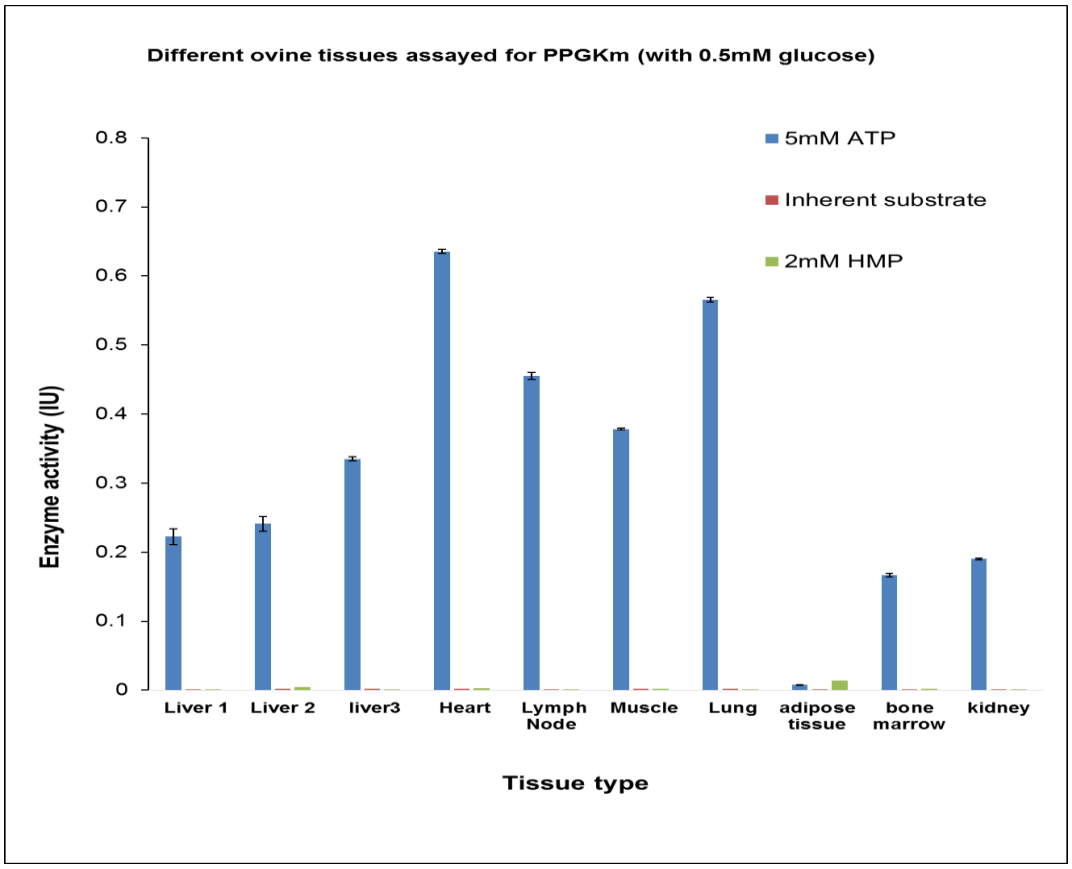


Figure 5.2–2 Different Ovine tissues screened for PPGKm activity with low glucose concentraion (0.5mM), data represent Mean±SEM, (n=3). HK activity was hgighly variable as expected in diffrent tissues types (see Appenndix IV).

1.4.17 PPGKm activity in livers of different species

Livers from different species homogenized as described previously in section 2.2, then assayed with 5mM ATP, 2mM HMP and with no phosphate donor at high and low glucose (0.5mM and 100mM glucose).

Figure 5.2-3 shows the activity of PPGKm with 100mM glucose in livers from different species, it can be observed that the highest PPGKm present in sheep, pig and cow respectively, however there was very little activity in chicken, rabbit and rat.

The possible explanation for this low activity is that the amount of protein expressed in these species is low, due to the presence of certain factors that affect PPGKm transcription and expression in these species, or the protein might be expressed but some inhibitors interfer with its activity and could be investigated in future research.

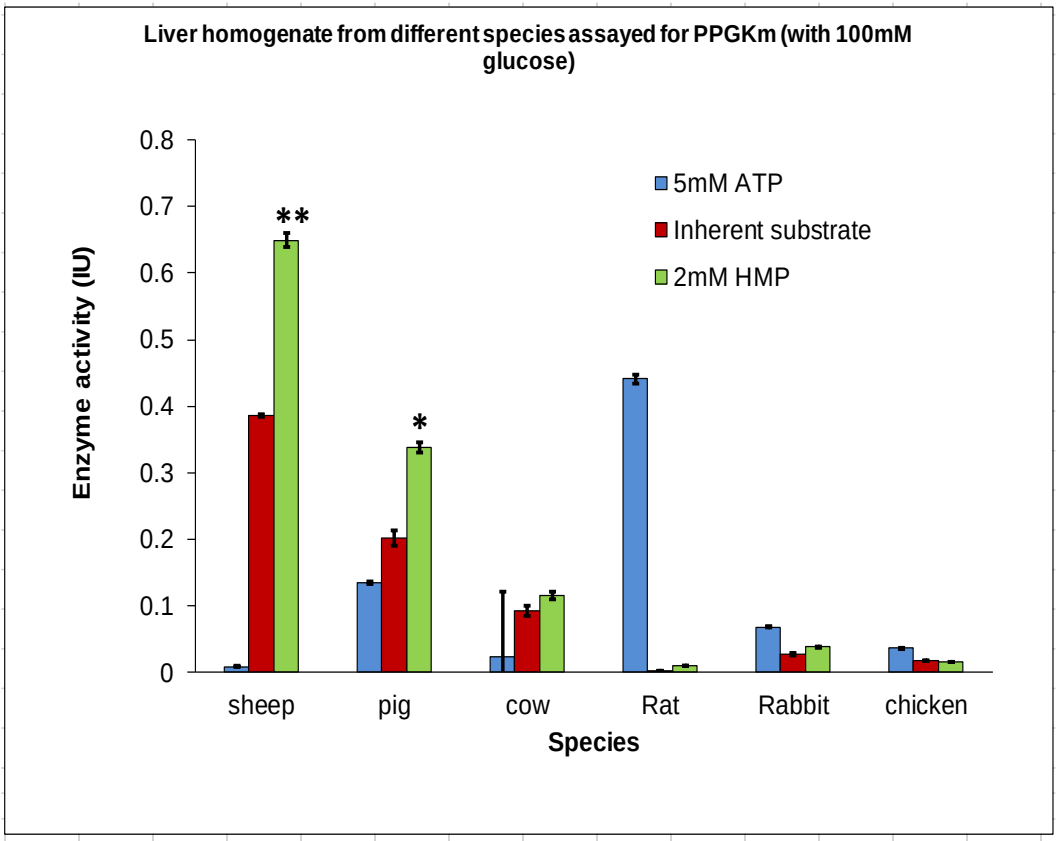


Figure 5.2–3 Screening different species liver tissue for the presence of PPGKm with 5mM ATP, 2mM HMP at high glucose concentration (100mM), data plotted from Mean±SEM, (n=3). Sheep had the highest PPGKm activity (with HMP) compared with other species (indicated P<0.01), porcine activity was higher than bovine (*<0.05).**

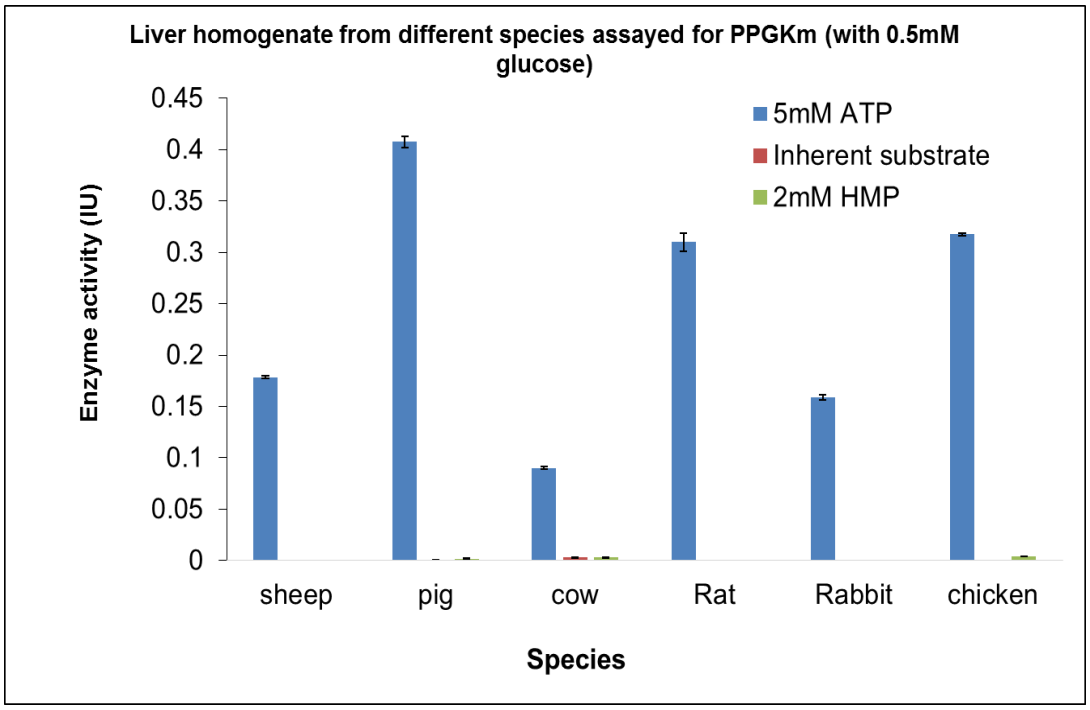


Figure 5.2–4 Screening different species liver tissue for the presence of PPGKm with low glucose concentration (0.5mM)), data plotted from Mean±SEM, (n=3).

Figure 5.2-4 shows the data from assaying different species livers assayed with 5mM ATP, 2mM HMP or inherent substrate and 0.5 mM glucose .The data showed no activity in inherent substrate and 2mM HMP,only activity with 5mM ATP was detected.

5.3 Chapter summary

1-PPGKm activity is present at very high activity in Ovine liver, comparable to the ATP depdendent glucokinase activity found in man and rats that is capable of synthesisng 100g of glycogen following a meal in a few hours (Murphy et al., 2003) In the other ovine tissues investigated activity is present at lower amounts (e.g. muscle) or activity appears absent.

2- One of the limitations of this project is that no information regarding nutritional status of the animal, age and sex, breed etc was available and this may provide further understanding in future studies. However, it can be interpreted that tissues that are involved in glucose metabolism appear to express the enyzyme (Liver, muscle, heart). It was not possible to obtain pancreatic tissue from sheep and this

would be an obvious tissue to study in future because of the role of high Km GKs in insulin secretion.

3-Data from the liver of different species showed the presence of high PPGKm activity at 100mM glucose, in sheep, pig and cow. Low PPGKm activity was detected in chicken rabbit and rat, and although the activity is low (about 5% of ATP-GK) this enzyme may be an important point of study since much is known about liver glucose metabolism with respect to high Km GKs.

4-The absence of PPGKm activity does not completely rule out its presence in these tissues: ATP-GK is bound and inactivated in the hepatcoyte nucleus during starvation and it is possible that such inhibitory processes may occur for PPGKm in these tissues. Once the gene for PPGKm is identified, an antibody can be generated and immunohistochemistry and molecular studies could be applied to investigate the espression profile of PPGKm in various tissues and subcellular compartments, a cDNA library could also be generated from cellular mRNA.

Chapter 6
Sub- cellular activity of PPGKm

6.1 Introduction

Because HKs and GKs are bound in discreet cellular locations which is helping to elucidate why cells need 5 or more enzymes to phosphorylate glucose, it was considered a potentially valuable investigation to study the sub-cellular distribution of PPGKm. Subcellular fractionation techniques are well established and routine well-described methodology methods was followed.

6.2 Results and discussion

Figure 6.2-1 shows different fractions obtained from subcellular fractionation experiment, assayed with 100mM glucose, 5mM ATP or 2mM HMP and inherent substrate. No PPGKm activity was observed with 0.5mM glucose as seen in figure 6.2-1

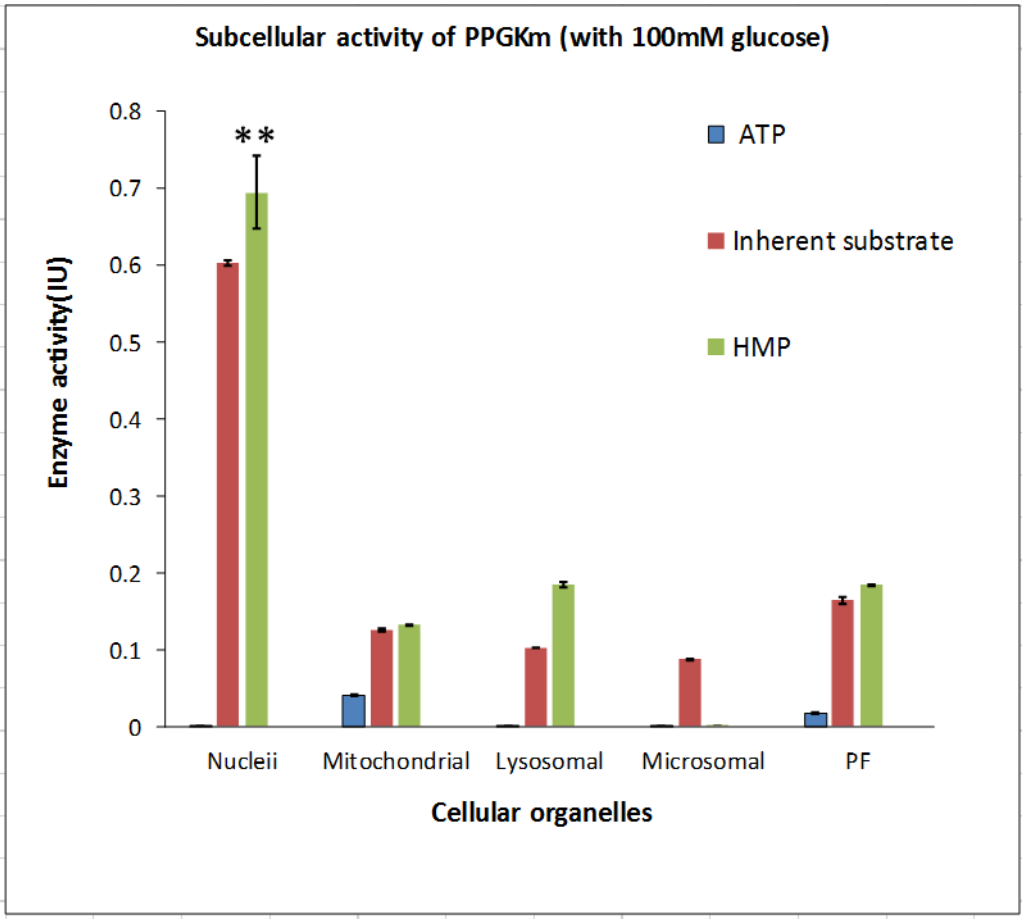


Figure 6.2–1 Subcellular activity of PPGKm in Ovine liver (fractions assayed with 100mM glucose) data plotted from the average of 3 independent experiments, Mean ± SEM (n=3). PPGKm was highly localized to the nucleus (indicates P<0.01 compared with other fractions). (*PF = particle free supernatant).**

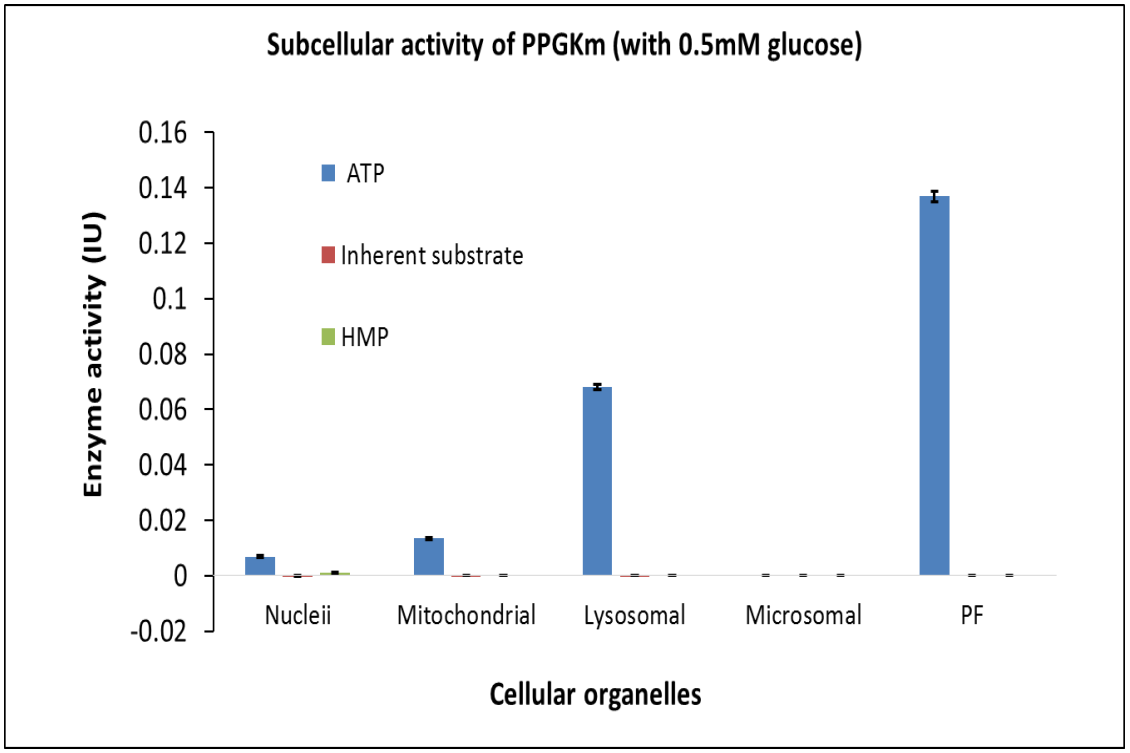


Figure 6.2–2 Subcellular activity of PPGKm in Ovine liver (fractions assayed with 0.5 mM glucose) data plotted from the average of 3 independent experiments, Mean ± SEM (n=3). (*PF= particle free supernatant).

6.3 Chapter summary

It was clearly observed that nuclear fraction has the highest PPGKm activity; however other cellular organelles contained some PPGKm activity, this is not necessarily that PPGKm is already present in these organelles, but it might be a contamination during the separation processe.

This might be overcome by using commercially available kit to separate the intact cellular organells, for more efficient identification and study of protein of interest.

Presence of specific antibody represents a useful tool to precisely detect the cellular localisation of PPGKm; uses of flourecent dyes in immunohistochemistry can provide a conclusive answer.DAPI stain can also be used to stain polyphosphate, and in this case it could be used together with a specific flourecent antibody for PPGKm, once it becomes available to show if the enzyme and substrate are bound.

Chapter 7

Partial purification and SDS gel electrophoresis of PPGKm

7.1 Introduction

Gel filtration chromatography is a technique that allows protein separation on the basis of size (size exclusion).

In this chapter the molecular weight of PPGKm was roughly estimated from native liver homogenate as well as the nuclear fraction obtained from the subcellular fractionation, by using size exclusion chromatography (sephacryl -200 gel).

7.2 Results and discussion

In figure 7.2-1 showing Fig. 7 shows the size exclusion chromatography on 1mL of Ovine liver homogenate: fractions were eluted using homogenisation buffer described in section 2.1.1. Fractions were then collected in 0.5ml eppendorf tubes. All fractions were assayed using 5mM ATP or 2mM HMP and without phosphate donor (blank), the data obtained from the spectrophotometric measurement of the coupled enzyme assay was plotted as shown in figure 7.2-1 below.

Size exclusion chromatography allows estimation of protein size, repeated gel filtration experiments using 200-Sephacryl gel have clearly shown that PPGKm enzyme is slightly larger than Hk1, which has a known weight at 100 kDa. The PPGKm was 115 kDa at a rough estimation (bovine albumin and vitamin B_{12} were used as standard). Unusually large Hk1 enzymes have been reported, Naked rat mole Hk1: G5B184, along with the two others described by (Rajesh et al., 1996) and (Kalab et al., 1994) were both seen to be around the 115 kDa estimated weight.

It is obvious that PPGKm enzyme eluted few fractions before hexokinase, which confirmed previous unpublished data showing it had a higher Mw than ATP-HK (probably mostly HK1).

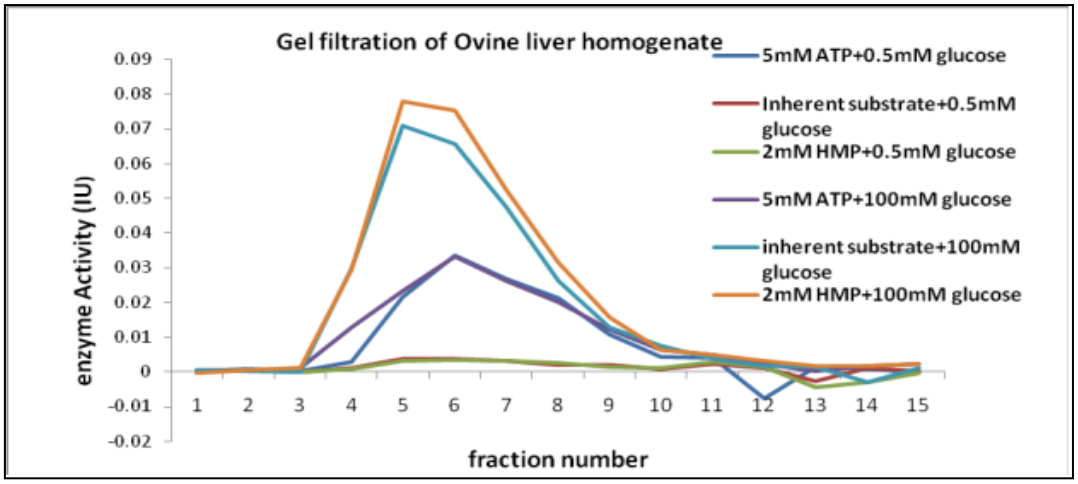


Figure 7.2–1 Fractions from Size exclusion chromatography of Ovine liver homogenate assayed for PPGKm, and hexokinase activities. Data plotted from the average of 3 independent experiments (mean ± SEM) n=3.

Figure 7.2-1 demonstrate the results obtained from size exclusion chromatography experiment, in which the nuclear fraction from subcellular fractionation was loaded on 200-Sephacryl gel, and eluted using homogenization buffer the fractions were collected in 0.5mL eppendorf tubes, and then assayed for PPGKm activity.

As shown in the figure below it is clear that only PPGKm was present, no hexokinase activity was detected (5mM ATP+ 0.5mM glucose), which also confirms that PPGKm is totally different from hexokinase, it is also clear that ATP has an inhibitory effect on PPGKm, as we can see from figure 7.2-1 the activity with 5mM ATP+ 100mM glucose is lowered compared to the activity with inherent substrate and 100mM glucose. On the other hand the same figure shows that HMP has been used as phosphate donor since the activity with 2mM HMP is much higher than inherent substrate or 5mM ATP.

PPGKm appears to be a nuclear enzyme and hexokinase known to be a cyosolic enzyme, cellular localisation of proteins provides a useful tool to address their function/s in the cell**.** The exact function/s of PPGKm is yet to be identified; extensive research is needed to map the metabolic pathways in which the enzyme is involved in.

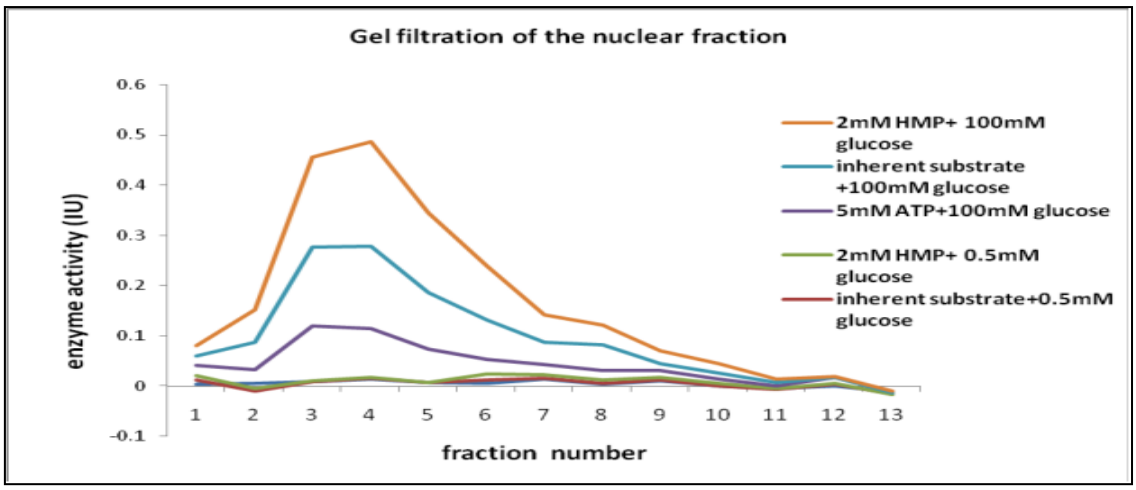


Figure 7.2–2 Fractions from Size exclusion chromatography and cell fractionation experiment, (nuclear fraction) assayed for PPGKm activity. Data plotted from the average of 3 independent experiments (mean ± SEM) n=3.

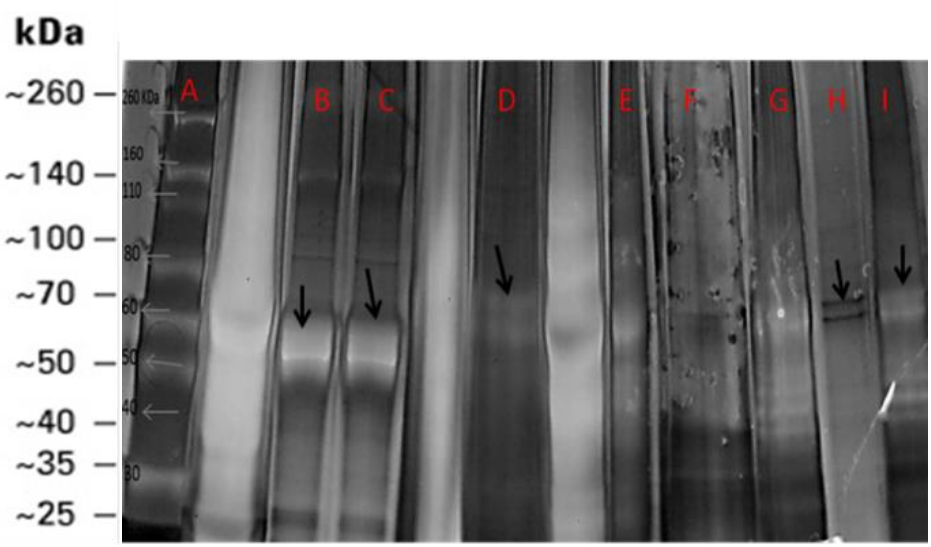


Figure 7.2–3 Silver stained SDS-PAGE of different fractions containing PPGKm obtained from previous chromatography experiments.

Lane (A) is the molecular weight marker; the size is shown on the left in kDa.

Lane (B) Recombinant sheep glucokinase isoform 1, Lane (C) Recombinant sheep glucokinase isoform 2, Lane (D) nuclear fraction obtained from sub cellular fractionation assay, Lanes (E, F and G) samples from previous anion exchange/ size exclusion chromatography, Lanes (H, I) samples from size exclusion/ anion exchange followed by inherent substrate depletion for 24 hrs (these samples were obtained from previous student project).

The amount loaded for all samples was 10µL, on 16% SDS-PAGE.

Samples B, C, D, and H were cut, and used for MS/MS and finger printing, the first trial to identify these bands only was succssful to identify recombinant sheep glucokinases but not other bands, the possible explanation for this the band contains a complex of proteins / the Poly-Pi bound to the protein, therefore they didn't show any match when compared against data base. To overcome this problem an alternative strategy to investigate the fingerprinting, was suggested by protein service team at York University.

The result obtained by LC-MS/MS is shown in appendix II, and no match has been found to any of polyphosphate kinases. One protein identified, that might be important for future investigation is the HSP70 (Heat shock protein70), which also called molecular chaperon it has a common ATPase binding domain with hexokinase family. HSP70 is an important protein involved cellular protection against stress, and has a role in protein folding.

- This experiment needs to be repeated in the future research using affinity chromatography columns (Poly-Pi Zirconia beads columns) as described by Lorenz et al; 1994a to purify bacterial polyphosphate dependent glucokinase. The fractions that show PPGKm activity could be loaded on SDS-PAGE, the band/bands then can be further analysed using LC-MS/MS, and finger printting. Because of time limittation this approach could not been attempt.

7.3 Chapter summary

1- Size- exclusion chromatography revealed that PPGKm has a slightly bigger size 115 kDa compared to Hk1 which is 100kDa, this was established from a nmuber of experiments, in which both activities were consistently detected in a different fractions: PPGKm activity appeared few fractions before HK1, Variable size polyphosphate dependent glucokinase was previously reported by Szymona et al., 1964, in *M.pheli*, as well as it was mentioned in the literature (section 2.5.4) that polyphosphate was able to form complexes with many different proteins (discussed in chapter 9.1.4).

2- Analysis was performed by LC-MS/MS post in-gel digestion with trypsin, this was an alternative approach to MS/MS, since the later does not show any match against expected protein database the *Ovis* subset of the NCBI database. The explanation for this is that our

protein of interest is forming a complex with polyphosphate, and accordingly it is difficult to show any similarity with other proteins in the data base. Therefore LC-MS/MS provided the advantage of physical separation by liquid chromatography, as well as, the capability of mass analysis by mass spectrometry, it is commonly used technique to identify a complexed mixtures.

Peptides obtained by LC-MS/MS, were identification by searching tandem mass spectra against the expected protein database the *Ovis* subset of the NCBI database (147,277 sequences; 77,737,218 residues, using the Mascot search program. Matches were filtered to accept only peptides with expect scores of 0.005 or better. Protein inferences required a minimum of two unique peptide sequences. LC-MS/MS results does not show any match with the known polyphosphate metabolising enzymes (results shown in appandix II). However one interesting finiding was the presence of HSP70 (heat shock protein70), this protein has a structural similarity with actin and hexokinase family of enzymes. Three-dimensional structures of actin, hexokinase, and HSP70 protein families included a common ATP binding motifs which are the "Phosphate-1" and "Phosphate-2" motifs joining with β- and Ɣ-phosphates of ATP and the "Connect-1" and "Connect-2" motifs at the interface between the subdomains(Bork et al, 1992, Bork et al, 1993). Residues present in these motifs which play a role in the interaction of ATP, are highly conserved in many glucokinases; these residues are Asp and Gly (in Phosphate-1), Asp (in Connect-1), Gly and Thr (in Phosphate-2), and Gly (in Connect-2) (discussed in chapter 9.1.4). This would provide an idea for future research and investigation.

Chapter 8

NMR studies of PPGKm

8.1 Introduction to (NMR) ^{31}P Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy has been used extensively to study metabolism (Glonek et al., 1971; Salhany et al., 1975; Burt et al., 1977; Ugurbil et al, 1978; Navon et al, 1977a, b, 1979; Ferguson et al, 1978; Ostrovsky et al, 1980; Gadian, 1982; Sianoudis et al, 1986; Roberts, 1987; Shanks and Bailey, 1988; Chen., 1999).

In vivo and *in vtiro* ^{31}P NMR spectroscopy, referred to as MRS and NMR repectively are powerful methodologies which are quantitative for many phosphorylated metabolites and causes no disruption to cellular or tissue functions when used *in vivo* (Gadian., 1982; Roberts, 1987; Chen, 1999).

The basic principle of the nuclear magnetic resonance (NMR) spectroscopic technique involves measurement of the radio frequency of the energy absorbed by magnetic nuclei (Roberts, 1987; Chen., 1999). Phosphorus compounds of living cells include phosphates, phosphonates and various esters of phosphates and phosphonates. The chemical shift of ^{31}P atoms in these compounds can span over a 30ppm range, making ^{31}P-NMR spectroscopy an attractive tool for examining phosphorus metabolites in microorganisms, plants and animal tissues (Burns S.P. et al., 1997) The singular weakness of *31*P NMR or MRS is sensitivity, and can only measure in the mM range (Burns S.P. et al., 2000).

^{31}P NMR spectrum of living cells or cell extracts is fairly simple with about 10 peaks from relatively the ‘mobile’ compounds. Insoluble or immobilized compounds, including membrane phospholipids, may give broad signals that are partly ‘NMR-invisible’ or appear as broad humps underlying the narrow metabolite signals, especially but not only *in vivo* (Burns S.P. et al., 1996). It is possible that some of the Poly-Pi bound to enzymes or protein systems are bound and partially invisible.

NMR spectroscopy can sometimes provide information on cellular compartmentalization of metabolites, for example the signal for Pi in yeast is split in two probably as a consequence of pH difference between cell compartments (Navon et al., 1979; Gillies et al, 1981; Nicolay et al., 1982, 1983). This is caused because of the pKa of Pi in the

physiological range (pKa =6.8) and undergoes pH-induced shifts of the peak – e.g. in mitochondrial matrix – high pH and cytoplasm of muscle cell (low pH). This was elegantly demonstrated in a study of live cell heterogenity, with some hepatocytes working at pH 7.8 and others at pH 7.2 (Burns S.P. et al., 2001).

NMR can be used to study the structure of phosphate compounds and their metabolism in biological samples. Poly-Pi NMR spectra have been widely investigated (Glonek et al., 1971; Salhany et al., 1975; Burt et al., 1977, Navon et al, 1977a, b; Ugurbil et al., 1978; Ferguson et al., 1978; Ostrovsky et al., 1980; Tijssen and Van Steveninck., 1984; Roberts., 1987; Chen., 1999). Poly-Pi has three principle peaks: terminal P, at about −6.62 to −7 ppm; penultimate P at about −20.17 to −21.7 ppm; middle P at about −22.37 to −22.5 ppm. at −5.39 ppm assigned to the γ -phosphate groups of nucleoside triphosphate and the β-phosphate groups of nucleoside diphosphate, at −10.05 ppm to the α-phosphate groups of nucleoside phosphates, and at −18.90 ppm to the β-phosphate groups of nucleoside triphosphate, respectively. The peak at −10.66 ppm includes both NADP and NADP (H). The peak at (−6.62 ppm) is the sum of PPi and the terminal phosphate groups of Poly-Pi. The peaks at (−18.00 ppm) and (−20.17 ppm represent) are penultimate phosphates from Poly-Pi, while peaks at (−22.37 ppm) represents the middle phosphate from longer Poly-Pis.

The concentration or chain length of the linear Poly-Pis can be calculated using the peak area, provided the Poly-Pi is not bound to proteins, which is unlikely to be completely accurate in biological samples. The quantitative determination of Poly-Pis by using the NMR shows some anomalies, (Krupyanko et al., 1998) with the total area of the core phosphate groups proportional to the concentration of each individual Poly-Pi with a defined chain length, but no proportional correlation between Poly-Pis with a small number of phosphate groups and Poly-Pis with a large number of these groups. Poly-Pi which can be detected by NMR spectroscopy are called ‘NMR-visible’, and represent a more mobile fraction of the total Poly-Pi content. Lack of an ‘NMR-visible’ Poly-Pi signal does not indicate the absence of Poly-Pis in a sample. In addition the peak positions are influenced by the divalent cations concentration in the sample further complicating clear interpretation (Pilatus et al., 1989). The detection of a Poly-Pi signal indicates their presence in the sample, but their absence cannot be concluded with NMR as described in

experiments using Chlorella fusca (Sianoudis et al., 1986). NMR spectroscopy therefore provides a unique but sometimes incomplete picture of Poly-Pi metabolism in biologcal samples.

^{13}C NMR spectroscopy has also been used widely in biosicence studies but is greatly limited by the very low natural abundance (c. 1.1%) of ^{13}C in living systems (Murphy H.C. et al., 2003). ^{12}C does not produce an NMR signal and so the total observed signal arises from the presence of ^{13}C. However, this gives the opportunity to add a labelled ^{13}C compound to a biological system and to obseve which metabolites and structures it enters (Murphy H.C. et al., 2003). In the present work $^{13}C_1$ labelled glucose was used in ezyme assays conducted inside the NMR spectrometer to look at the evolution of the label from the C_1 of glucose. It was anticipated that following the action of G6PDH on $^{13}C_1$-G6P made by PPGK a new NMR signal would appear in the NMR sepctrum at the position of 6-phosphogluconate (6PG) the product of G6PDH action on glucose-6-phosphate (G6P). This would only occur if PPGK was converting glcuose to G6P and in the absence of G6PDH, the G6P would accumulate. G6PDH conducts a dehydrogenation reaction of G6P converting its C_1 to a carbonyl causing a shift from 99 and 97ppm for G6P (not visible because the very large glucose peaks in a very similar position) to 178ppm because of the chemical shift exerted by the new carbonyl group at C_1. This would provide definitive and unequivocal evidence that in the absence of ATP that PPGK was using Poly-Pi to convert glucose to G6P – i.e. defining it as a high Km Polyphosphate dependent glucokinase in mammalian tissue.

8.2 Results and discussion

Phosphorus NMR spectra of Ovine liver homogenate assayed with 2mM HMP, and 100mM glucose, spectra A, B and C in which the sample ran for 3hrs with no G6PDH was added to the assay mixture, the experiment stopped by taking the NMR tube out of the NMR machine, and 10µL of G6PDH was added and sample ran again for one hour, spectra D shows the disappearance of G6P after addition of G6PDH enzyme, and the increased appearance of 6-phosphogluconate

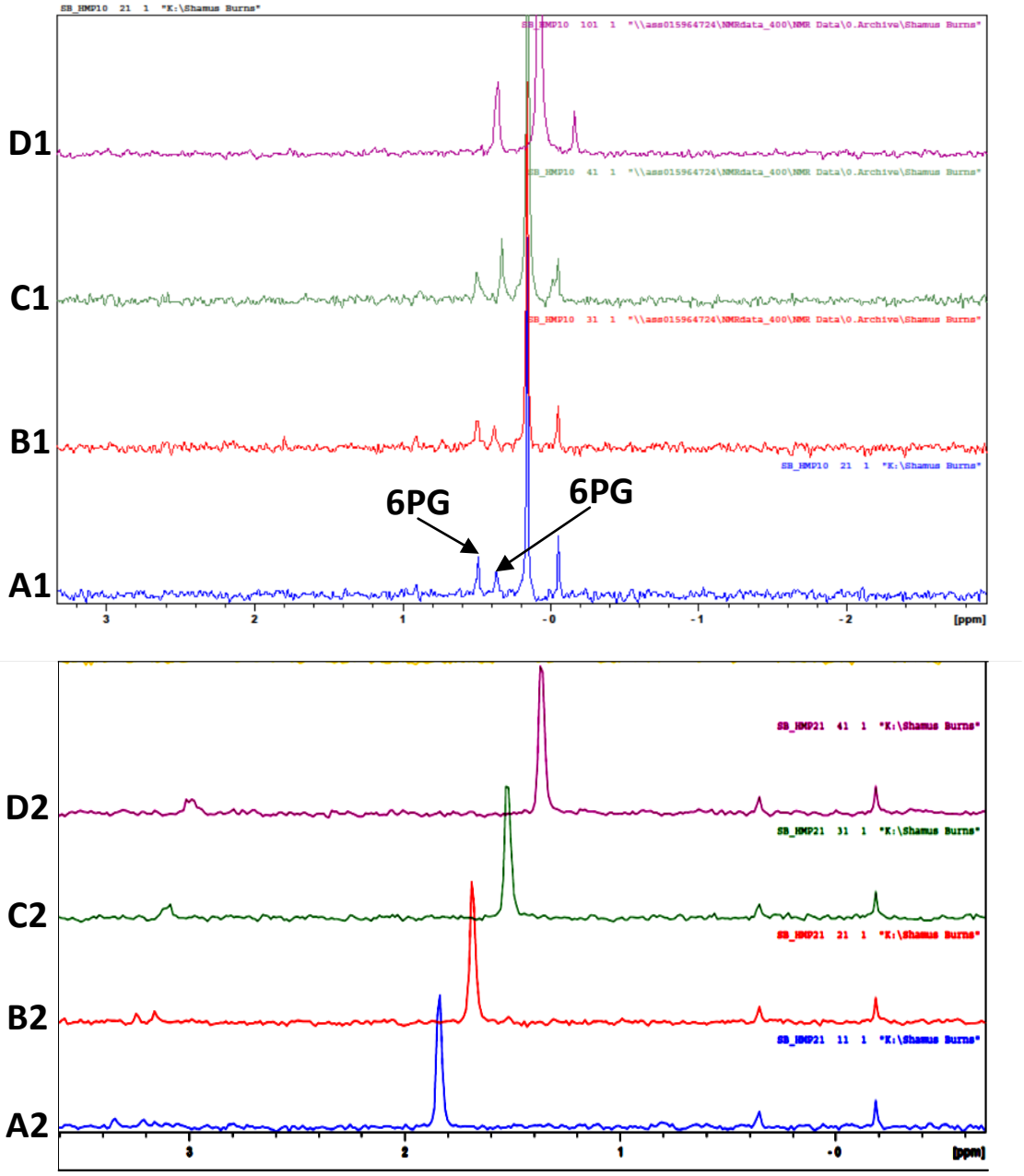


Figure 8.2–1 Shows the 31P NMR spectra obtained from ovine liver homogenate assayed for PPGKm activity over a period of 3hrs without G6PDH (A=from 0-1h, B=1-2h and C=2-3h), spectra (D) ran for 1hr after the addition of G6PDH. The first panel (A1-D1) represents the experiment where HMP was added, showing the appearance of G6P (glucose 6-phosphate), and 6PG (6phosphogluconate), the second panel (A2-D2) shows results but intnhe absence of HMP: G6P and 6PG were not detected.

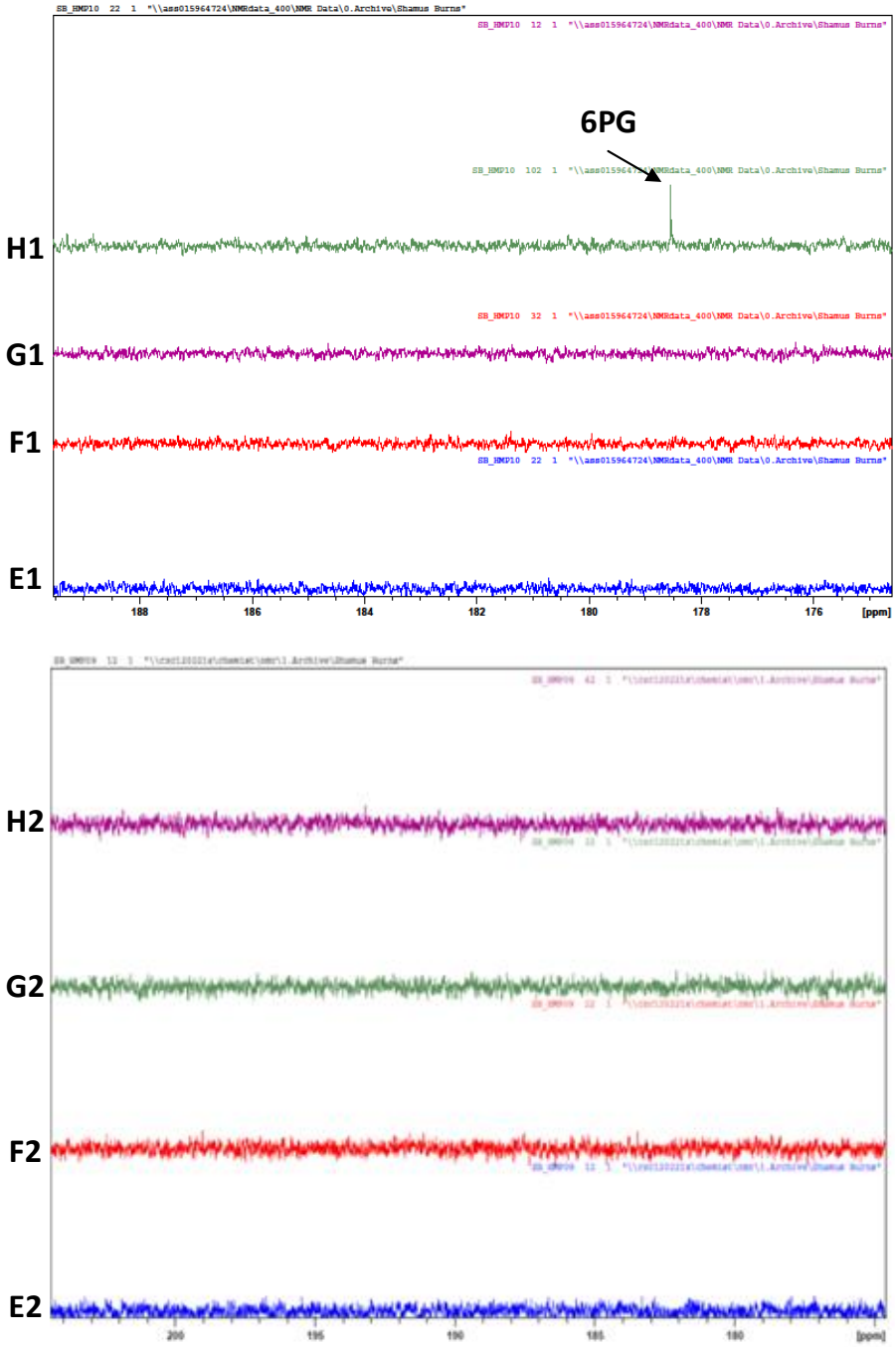


Figure 8.2–2 Shows the 13C spectra obtained from liver homogenate assayed for PPGKmactivity over period of 3hrs without G6PDH, (E=from 0-1h, F=1-2h and G=2-3h), Spectrum H1 is the sample from G1 run in the presence of G6PDH for 1h. The first Panel (E1-H1) represents the experiment where HMP was added, and the second panel was an indenitcal experiment in the absence of HMP.

Carbon spectra of the same experiment explained above (E, F, and G) before addition of G6PDH, (H) after adding G6PDH.

The sudden appearance of the signal for 6-phosphogluconate (6PG) occurs because the ^{13}C1 glucose which has been steadily converted to glucose 6-phosphate by PPGK is rapidly converted to 6PG by the addition of exogenous G6PDH. The data provide definitve and supporting evidence that PPGK is synthesising G6P from glucose and is a high Km ATP independent and Poly-Pi dependent glucokinase in mammalian tissues.

8.3 Chapter summary

It is clear from the NMR data that the novel Poly-Pi dependent GK converts glucose to G6P in the absence of ATP. The appearance of G6P in the ^{31}P NMR spectrum when G6PDH is not present and then its conversion to 6-phosphogluconate provides definitive evidence for the action of the novel enzyme as a high Km Polyphosphate dependent glucokinase in mammalian tissue. This data is reinforeced by the use of $^{13}C_1$ glucose in this assay because only in the presence of G6PDH does a new peak coincident with 6-phosphogluconate appear in the ^{13}C NMR spectrum (confirmed with a standard sample of 6PG). This 6PG is synthesised from G6P made by the PPGK in the homogenate. The only ATP that could have been present in the NMR experiment run in the absence of ATP would have arisen from the original tissue sample and estimated to be less than 100µM following homogenization and dilution. This absence of ATP powerfully supports the hypothesis that the PPGK synthessies G6P from glucose in the reaction using the Poly-Pi substrate.

Chapter 9
Concluding discussion

9.1 Major findings and contributions

1.4.18 PPGKm substrate specificity

It is proposed a novel kinase that is present in Ovine liver but also in other mammal tissues. This novel enzyme is able to phosphorylate glucose using polyphosphate as donor, which I proposed to be HMP, and shows a very high activity at high glucose concentration in comparison to the ATP dependent glucokinase activity found in man and rats. The high Km of the enzyme regarding glucose, suggesting its role is sensing glucose concentration above normal values, however the enzyme showed a very low activity at low glucose concentrations. Therefore, I proposed for this enzyme the name, PolyPhosphate-Glucokinase (mammalian) or PPGKm.

PPGKm activity was determined in ovine liver homogenate in response to increased HMP concentrations with 100mM glucose. The results obtained from the saturation curve suggest that PPGKm is a polyphosphate dependent enzyme, being its V_{max} (maximum reaction rate)= 0.4 µmol.min.g^{-1} and Half maximal velocity at HMP concentration ($S_{0.5}$) of 0.58mM (in this experiment liver sample number 7, that showed the lowest inherent activity was used in this assay see section 3.2.4). These data suggest a possible role in the mechanism of glucose-regulation in a similar way to the ATP-dependent glucokinase (Murphy H.C. et al, 2003). Whether this enzyme is involved in insulin secretion awaits investigation of its presence in pancreas which will need a molecular probe for mRNA or protein, this in return needs the DNA sequence to be established.

PPGKm activity with different phosphate donors shows a maximum activity (1.3 IU) with 2mM HMP, a lower activity (0.6 IU) with long phosphate chains, P700 and Tri-P and under 0.4 (IU) for AMP, ADP, ATP and the nucleoside, GTP and CTP, being undetectable for UTP.

Polyphosphate glucokinase being discovered and studied only in bacterial species (Szymona.,1957, Szymona et al., 1962), in some bacteria polyphosphate -dependent glucokinase in *M.tuberculosis* was demonstrated to use both ATP and polyphosphate to

phosphorylate glucose (Hsieh et al., 1996), however a strictly polyphosphate-dependent glucokinase was found in *Microlunatus phosphovorus*, which was reported to use polyphosphate of long chain and short chain the Km for both polyphosphates was 0.06µM, and 3.8µM respectively (Tanaka et al, 2003),it could be observed that the affinity to ployphosphate depends on chain length: the enzyme has a high affinity to polyphosphate with long chains, await research would investigate the affinity of PPGKm to variable Poly-Pi chains, in the recent project only long chains Poly-Pi were investigated since no short chain Poly-Pi were available to purchase.

The results of the recent project showed that both ADP and AMP when used as phosphoryl donors the activity of PPGKm was decreased, which suggest that they have an inhibitory effect on the enzyme, this in agreement with the findings of Hsieh and colleagues 1996, in which they demonstrated the inhibitory effect of both, AMP and ADP, on bacterial polyphosphate dependent glucokinase.

Twenty ovine livers were screened for PPGKm activity with inherent substrate to see if there is a variability, all livers examined they showed variable degree of activity with inherent substrate, however when the homogenates were assayed with 2mM HMP the activity dramatically increased, this could be justified by the amount of inherent substrate present in the native state could be different according to the nutritional status of the animal, age and sex, all these, variables needs to be clarified in future research.

1.4.19 PPGKm activity in different ovine tissues and various species

As I am aware this is the first study to describe a mammalian polyphosphate dependent enzyme, no similar enzymes were studied or found in animals, although polyphosphate was found to be distributed in some animals (Imsiecke et al., 1996), the relation between Poly-Pi and RNA synthesis was studied in the embryos of frog (Shiokawa and Yamana., 1965), different mammalian tissues were shown to contain Poly-Pi, but in a concentrations less than those found in microorganisms (Kumble and Kornberg, 1995; Kornberg, 1999;and Schröder et al., 2000).

These findings demonstrate the wide spread of polyphosphate in many living organisms at different stages of evolution, depending on growth conditions, type of tissue or cellular compartment being studied.

The sub-cellular fractionation results demonstrated the presence of PPGKm in the nuclear fraction of liver tissue homogenate, the enzyme in the nuclear fraction showed high activity with inherent substrate compared to the activity with inherent substrate when total liver homogenate was used, this clearly shows that the inherent substrate (polyphosphate) abundantly present in the nucleus, this is supported by the findings of Kornberg., 1999 in which he demonstrated the presence of high concentrations of polyphosphate in the nucleus of rat liver (89µM) compared to the cytosol containing (12µM) (Kornberg., 1999).

The screening of liver of different species revealed the presence of high PPGKm activity at 100mM glucose in sheep, pig and cow livers whereas low activity (about 5% of ATP-GK) was detected in chicken, rabbit and rat, constituting an important point of study to understand liver glucose metabolism with respect to high Km GKs.

In ruminants glucose metabolism is different from monogastric animals, in this regard dietary carbohydrates are fermented by ruminal micro-flora to short chain fatty acids, therefore only little glucose, if any, absorbed from small intestine (Bergman et al., 1974), in contrast monogastric animals they absorb glucose directly from small intestine. Thus in ruminants blood glucose has to be generated nearly exclusively from gluconeogenesis (Elmahdi et al., 1997), this explain the markedly lower blood glucose level in most ruminants (2.5-3.5 mM/l) compared to monogastric animals (3.5-5.0 mM/l), with the exception of camel that has a higher blood glucose level (7.0 mM/l) (Elmahdi et al., 1997).

According to the unique glucose metabolism in ruminants, PPGKm may play a distinctive role in this regard specially in glucose sensing to avoid hypoglycaemia. Liver lobules have a unique structure, the periportal hepatocytes, are specialised to perform gluconeogenesis, on the other hand perivenous hepatocytes has the key enzymes for glycolysis (Keppens and Wulf., 1988). The intra lobular distribution of PPGKm would provide an important area of research in future investigations.

The screening of different ovine tissues showed PPGKm to be dominant in liver samples, However, activity was detected in cardiac muscle, skeletal muscle and lung. Other tissues examined (adipose tissue, bone marrow and kidney) showed undetectable PPGKm activity. This absence of PPGKm activity does not completely rule out its presence in these

tissues, and future experiments will need to investigate these when molecualr probes become available.

1.4.20 Biochemical properties of PPGKm

The results of this study confirmed that PPGKm has high Km for glucose, estimated to be 20mM, as high activity rates of the enzyme shown with increased glucose concentrations. On the other hand, no detectable activity was observed with low glucose concentrations. The Km of HMP was estimated to be 0.6µM. These findings demonstrate that PPGKm has low affinity to glucose, but its affinity to HMP is high, this might highlight that PPGKm has a role in glucose sensing in the nucleus, and generally in hepatic energy metabolism. As described previously ruminants depends on continuous gluconeogenesis to maintain constant blood glucose this sustained process needs a highly controlled mechanism as well as a ceaseless source of energy, the presence of high km glucokinase dependent on polyphosphate might provide these requirements. The binding of polyphosphate to the enzyme may play a role in its regulation, since it was demonstrated that sheep lack a glucokinase regulatory protein due to mutation in the gene, but no mutations being identified in their glucokinase gene (Irwin and Huanran, 2014). Recombinant sheep glucokinase 1 and isoform 2 where purchased and assayed for activity, both demonstrated activity with high glucose and 5mM ATP, but no activity was observed with 2mM HMP (data not shown), this proves the presence of functional high km glucokinase in sheep.The sequences were obtained from the *ovis aries* protein sequence at NCBI data base, as it was mentioned in the data base the sequences belong to sheep pancreatic glucokinase isoform, this suggest that PPGKm is the hepatic glucokinase isoform and accordingly it is different in its biochemical properties and function, this would be investigated in the future studies.

The PPGKm activity has shown to be dependent on divalent metal ions, suggesting possible Poly-Pi binding to the enzyme, since Poly-Pi is negatively charged this allows the interaction with divalent metal ions.

On the other hand hexokinase showed highest activity only with 5mM ATP, and 4mM Mg^{2+}, the positively charged Mg^{2+} interacts with negatively charged phosphate oxygen atoms of ATP, providing charge compensation and allows the favorable conformation of

ATP at hexokinase active site. This demonstrates that PPGKm has adiscriminated substrate binding sites compared to hexokinase.

It was demonstrated by Belozersky and Kulaev, 1970 that Poly-Pi and RNA they were tightly bound and difficult to separate due to the interaction with Ca^{2+} and Mg^{2+} ions, this was further investigated to see if there is any presence of covalent or hydrogen bonds associated with Poly-Pi and RNA complex, but it was confirmed that both types of bonding were absent and the only explanation for this tight binding is the presence of electrostatic interaction mediated by divalent cations (Belozersky and Kulaev., 1970).

The results also showed an interesting observation in which there was PPGKm activity with increasing Mg^{2+} concentrations, at low glucose concentration (0.5mM) with both inherent and 2mM HMP. PPGKm activities were persistently reported throughout the study with high glucose concentrations, this might be explained by that Mg^{2+} might have an allosteric effect on PPGKm to enhance its affinity to glucose.

Thermostability assays suggest that PPGKm is more stable over time than hexokinase, but higher short term heat lability. These results suggest that inactivation of the enzymes occurs in different ways, and further structural studies will help to elucidate my findings. The recognition of sequences participating in polyphosphate binding is not clear yet. Previously, Bork and co-workers (1992), showed that amino acid tryptophans residues in the peptide, KNDWTYPKWAKQ, of Poly -P-glucokinase from H_{37} Ra strain (*M.tuberculosis*) were found to be particularly oxidized by *N*-bromosuccinimide with associated loss of enzymatic activity (Hsieh et al., 1993). Tetrapolyphosphate or long chain polyphosphate substrate sustains protection against this oxidation and the loss of activity. Residues 177-216 of the Poly-Pi-glucokinase sequence from H_{37} Rv encode a closely related sequence, RKDWSYARWSEE. Therfore this region might be a binding site for polyphosphate, in addition to Phosphate 1 and 2, which specifically enables the Poly-Pi-glucokinase to utilize polyphosphates. Several charged groups around this region as Lys_{188}, Glu_{189}, Lys_{190}, Asp_{192}, Lys_{197}, and Lys_{200} may play a crucial role in Poly-Pi binding mechanism (Bork et al, 1993). Therefore I would suggest looking for the homology of these residues in mammalin glucokinase in general, and ovine in specific.

1.4.21 PPGKm size estimation and partial purification

Size exclusion chromatography revealed that PPGKm has a slightly bigger size than hexokinase, this could be interpreter by the binding of polyphosphate with the enzyme, the ability of Poly-Pi to bind with other proteins demonstrated in many studies. (Szymona et al, 1964; Lorenz et al, 1994a; Kusano and Ishihama, 1997; Andreeva et al., 2001, 2004). There have been numerous reports on the occurrence of various isoenzymes of polyphosphate glucokinase in different microorganisms and on differences in the molecular weights of the enzyme in the same organism (Szymona et al, 1977; Kowalska et al, 1979; Pastuszak and Szymona., 1980). Szymona et al., (1977) found that the molecular mass of native enzyme from *M. tuberculosis* was 118 kDa using sephacryl-200 size exclusion chromatography, while Pastuszak and Szymona (1980) found a larger form of the enzyme. The enzyme from M. phlei was found to be a protein of 113 kDa (Szymona and Ostrowski, 1964) or 275–280 kDa (Girbal et al., 1989). The native enzyme from P. shermanii was reported to have a molecular mass of 31 kDa (Clark., 1990). The purified enzymes from *P. shermanii* (Phillips et al., 1993) and *M. tuberculosis* (Hsieh et al., 1993b) also showed multiple proteins by HPLC gel filtration, native PAGE and isoelectric focusing (lEF)–PAGE, although a single band was observed by SDS–PAGE.One explanation for the existence of varying molecular masses of these glucokinases, is variable chain lengths of strongly bound Poly-Pis (Phillips et al., 1999). Although this remains speculative while plausible, is strongly supported by the evidence presented in this thesis. An obvious outstanding question is how the enzyme might go about using bound Poly-Pi, and also whether the chain length influences factors like strength of binding, or maximum enzyme activity.

Size exclusion gel filtration of the nuclear fraction showed no hexokinase activity, which confirms that the PPGKm is not the same protein, furthermore the size exclusion of total liver homogenate revealed both hexokinase activity and PPGKm activity, and they were eluted in a different fractions. The nuclear localization of the enzyme together with its substrate, polyphosphate, could assist in the future studies to highlight the possible functions of PPGKm like apoptosis, gene transcription, and DNA repair. As it was mentioned previously in the literature the vital roles of polyphosphate in mammalian system when it form complexes with proteins important in cell regulatory processes, like

RNA polymerase isolated from the stationary -phase cells of *E.coli* (Kusano and Ishihama., 1997), also the ATP-dependent protease Lon forming a complex with Poly-Pi under degradation of ribosomal proteins during amino acid starvation (Kuroda et al., 2001), furthermore Poly-Pi was shown to compete with DNA at its histones binding sites (Schröder et al, 1999). Poly-Pi also can bind with non-histone proteins in the nucleus (Offenbacher and Kline., 1984).

LC-MS/MS of the silver stained protein band obtained from nuclear fraction (cell fractionation experiment), and from chromatography protein purification (anion exchange and depletion assay), didn't exclusively confirm the presence of any protein that might share any homology to the known bacterial enzyme involved in Poly-Pi metabolism. The only observation that might be interesting is the presence of HSP70 a molecular chaperon, recombinant HSP70 was assayed with 2mM HMP and 5mM ATP at high and low glucose concentration, the protein was also incubated with 2mM HMP for 24 hours, but no activity was detected (data not shown)

It was established by Bork and colleagues (1993), that the three-dimensional structures of actin, hexokinase, and Hsp70 protein families included a common ATP binding motifs which are the "Phosphate-1" and "Phosphate-2" motifs joining with β- and Y-phosphates of ATP and the "Connect-1" and "Connect-2" motifs at the interface between the subdomains(Bork et al., 1992, Bork et al., 1993). Residues present in these motifs which play a role in the interaction of ATP, are highly conserved in many glucokinases; these residues are Asp and Gly (in Phosphate-1), Asp (in Connect-1), Gly and Thr (in Phosphate-2), and Gly (in Connect-2). Analysis of the deduced amino acid sequences of the *ppgk* gene demonestrated that this enzyme involves regions that are homologous to Phosphate-1 and Phosphate-2 regions of yeast glucokinase. Moreover, sequence alignment studies on other prokaryotic glucokinases sequences also revealed the exsistance of phosphate binding motifs. These phosphate binding sites found to be conserved from eukaryotic hexokinases to prokaryotic glucokinases.

I tried also to predict and assay some other proteins throughout my research, in addition to ovine heat shock protein, a recombinant human HKDC1 (hexokinase containing domain 1) was also assayed with high and low glucose in the presence of 2mM HMP or 5mM ATP, it was incubated over night with the substrate, but no activity was detected (data not

shown), HKDC1 its amino acid sequence contains all of the known functional active residues as hexokinase (Irwin and Huanran., 2014).

^{13}C NMR studies confirmed the formation of 6-phosphogluconate in the presence of high glucose concentration (100mM), 2mM HMP and NAD, and this provides powerful evidence for PPGKm. It is concluded that the novel enzyme is a high Km Polyphosphate dependent glucokinase, uses HMP to phosphorylate glucose producing G6P. These data confirm the presence of this type of enzyme for the first time in mammalian tissues.

9.2 Future directions

In this section I suggest some methods that would assists in future studies and investigations. Also a number of factors have to be considered also such as: nutritional status of the animal, age and sex variations .Tissues that are involved in glucose metabolism, such as liver, muscle, heart, or pancreas, must have more focus of attention, specially pancreas this obvious tissue to study in future because of the role of high Km GKs in insulin secretion.

PPGKm is a novel enzyme and its role in cellular functions and metabolism as general has yet to be elucidated. Therfore it opens up a wide area for future research.Investigation of phosphate donor specificity *in vitro* revealed the preference of PPGKm to HMP, P_{700} and Tri-P but the inherent native substrate needs to be further investigated using for example 4', 6-diamidino-2-phenylindole (DAPI) which is a fluorescent dye that is widely used to stain DNA (or RNA), e.g. for the *in vivo* visualization of cell nuclei or bacterial nucleoids. DAPI has also been shown to bind to Poly-Pi (Tijssen et al., 1982). The excitation and emission wavelength of Poly-Pi-DAPI complexes (ex. 415nm, em. 550nm) are different from those of DNA-DAPI complexes (ex. 358nm, em. 461nm) and RNA-DAPI complexes (ex. 358nm, em. 500nm), which allow the discrimination of Poly-Pi signals from DNA and RNA signals (Tanious et al; 1992, Kubista et al., 1987).Its high sensitivity and specificity make DAPI an attractive reagent for the staining of Poly-Pi in cells, in solution and on polyacrylamide gels (Smith &Morrissey, 2007, Aschar-Sobbi et al., 2008).

The sub-cellular fractionation results showed the highest PPGKm activity in the nuclear fraction, however other cellular organelles like mitochondria and particles free contained some PPGKm activity, in order to elucidate this point, we propose more efficient cellular

fractionation by using commercially available kit for the separation of each cellular organelles to minimize cross-contamination.

The gene for PPGKm was not identified yet, therefore approaches as Native gel electrophoresis and in -situ enzyme assay would be usefulthis method was described by Szymona et al, 1977,in which the protein sample loaded in 12.5% native SDS- PAGE, and subject to electrophoresis , the gel then will be stained for enzyme activity using activity stainig.The activity staining contains the same components of HMP enzyme assay mixture in addition to p-nitro blue tetrazolium (0.35 mg/ml, final concentration), and phenazine methosulphate (0.035 mg /ml, final concentration). Band that show activity could be further analyzed for sequence identification.

Protein purification using affinity chromatography is another technique that would provide good opportunity for future studies to sucssesfuly purify PPGKmAs described in section 2.5.5, many Poly-Pi binding proteins were detected in crude cell extracts from different organisms, including yeast and animals, by using filter-binding technique or affinity chromatography on Poly-Pi Zirconia beads (Lorenz et al., 1994a).

PPGKm was shown to be in liver tissue, more specifically it is a nuclear protein For future research directions when the gene for PPGKm is identified, a specific antibody can be generated and produce to be applied in a various immunohistochemical approaches for further quantitative and qualitative studies, this will allow to design more studies to understand the mechanism by which the protein expression is up or down regulated or if there is any post translational modifications that may differ in various tissues and species. Tissue and cell culture techniques would provide a suitable tool in this regard**.**

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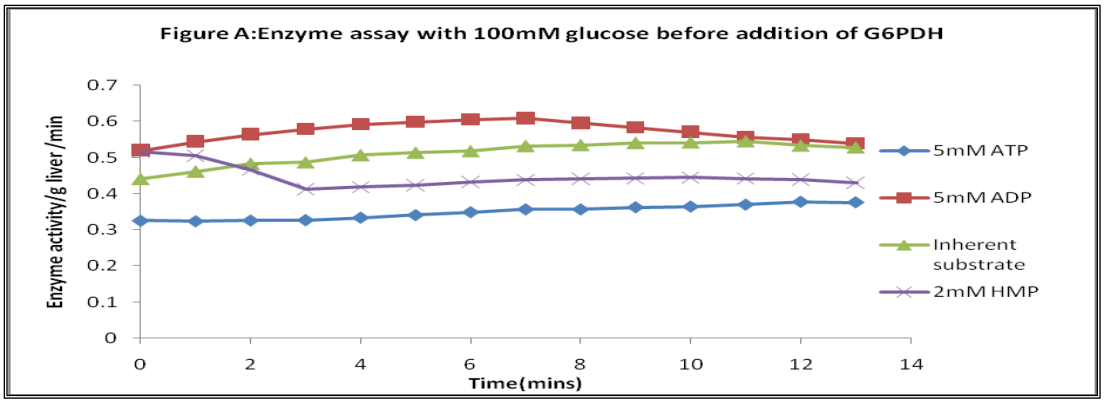
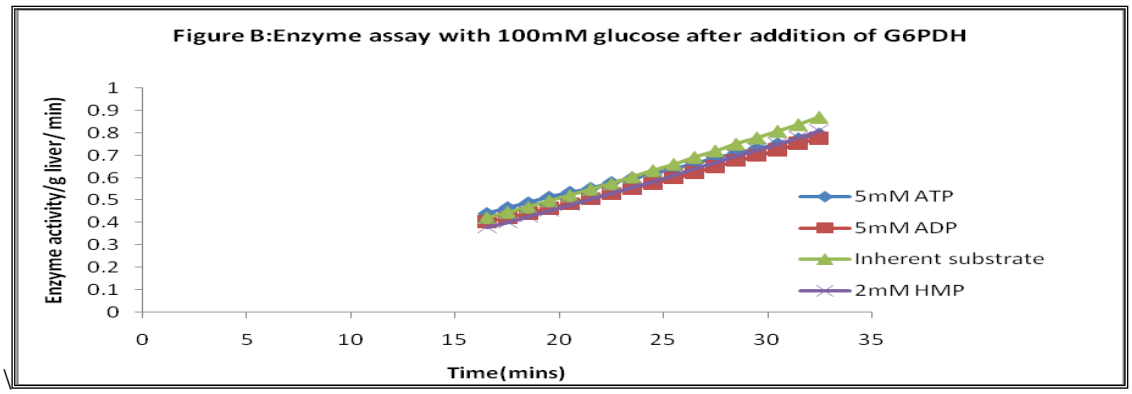
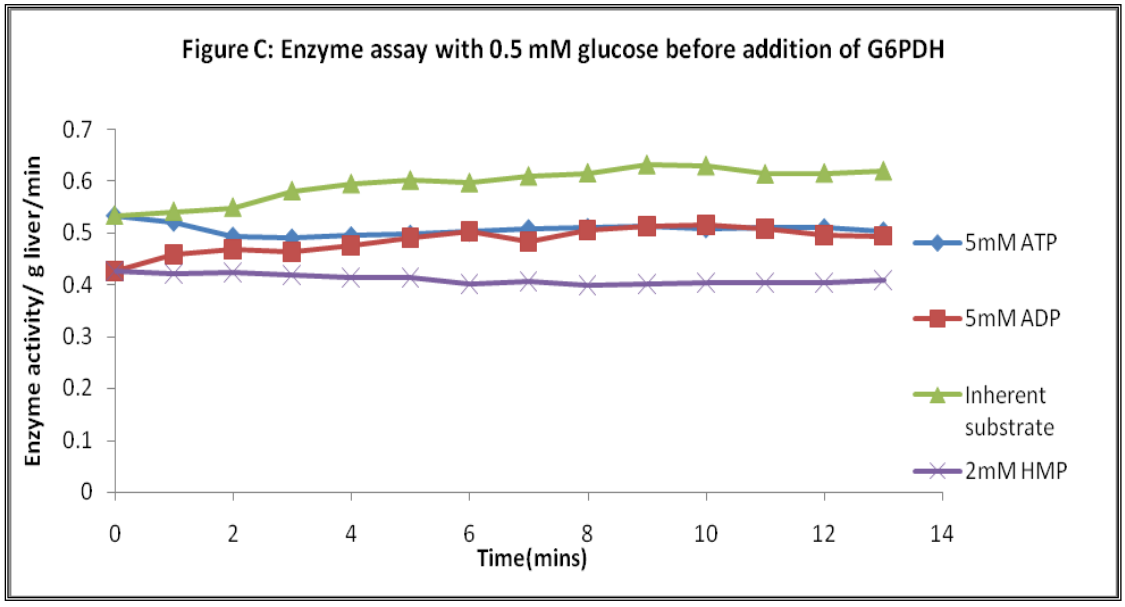
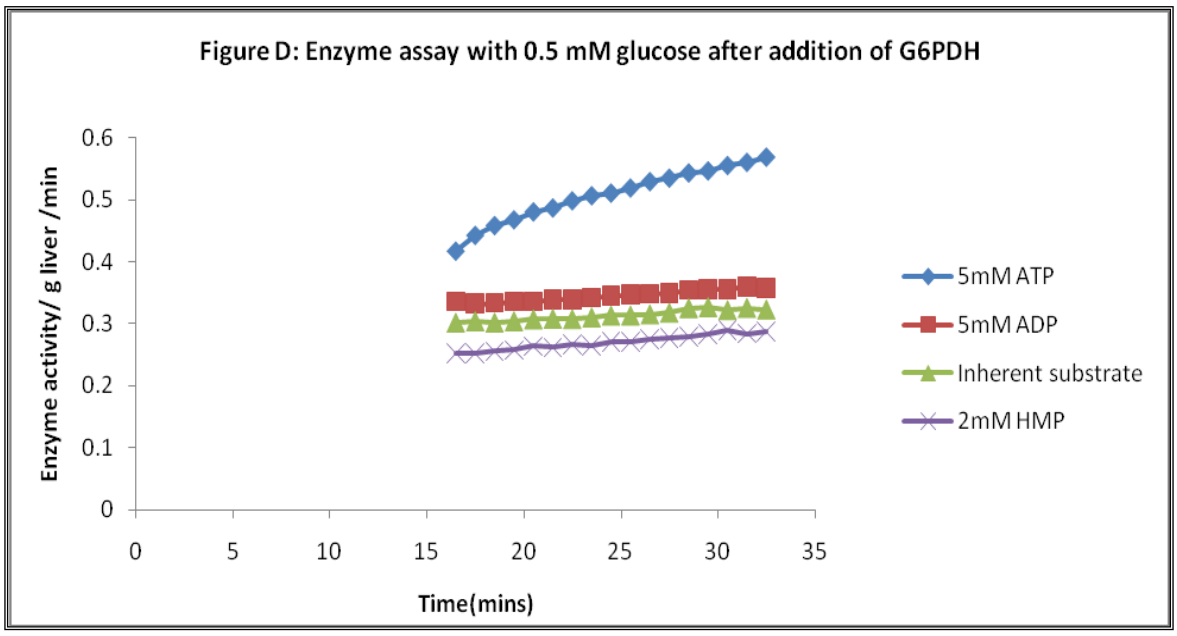
Appendix I: PPGKm enzyme assay in the presence and absence of G6PDH/ glucose.

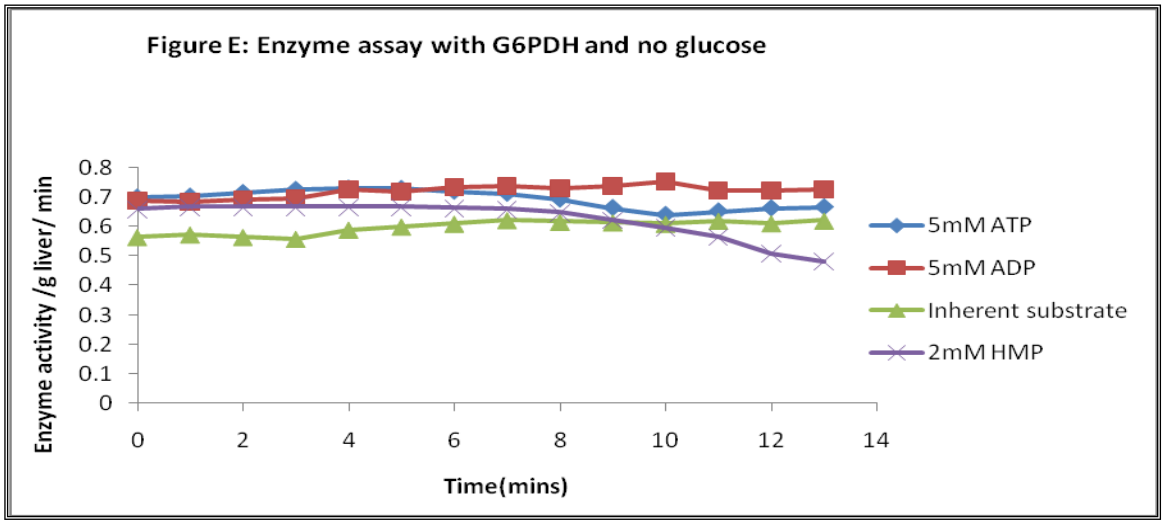








In this experiment the assay ran for 15 minutes, no G6PDH was added to the assay buffers, then the spectrophotometer paused, 1µl of G6PDH was added to each well, and the absorbance measured again. The assay performed with high and low glucose concentrations (0.5mM and 100mM glucose) as showen above in figures A,B,C and D.Another assay carried out with NO glucose, but with G6PDH added to each assay buffers the absorbance measured for 15 minutes then paused, then100mM glucose added to each well, and absorbance measured again (Figures E and F) below.



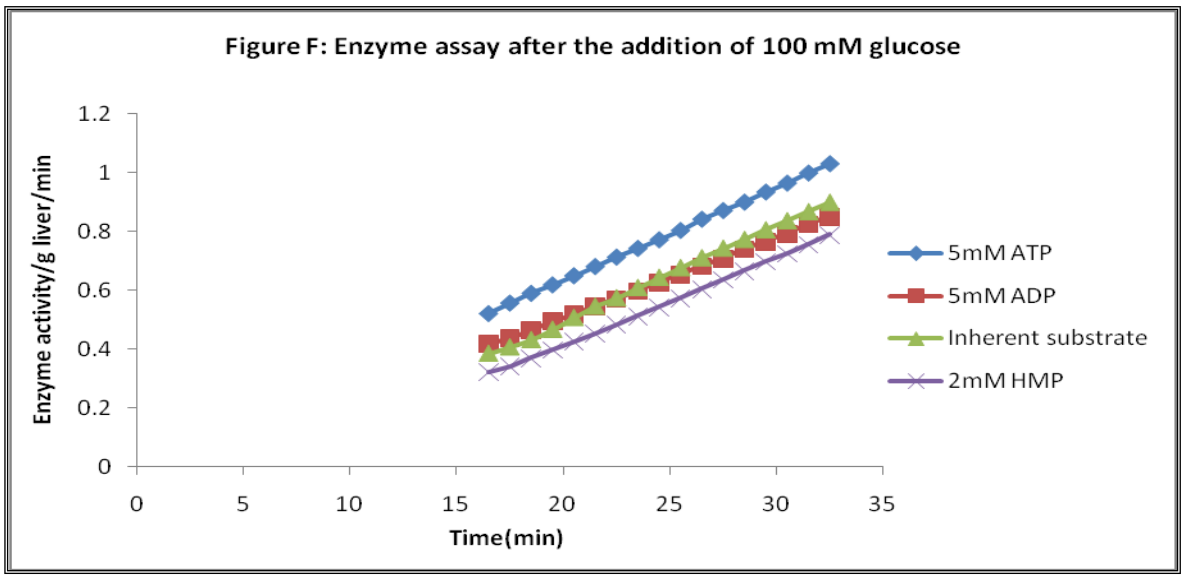
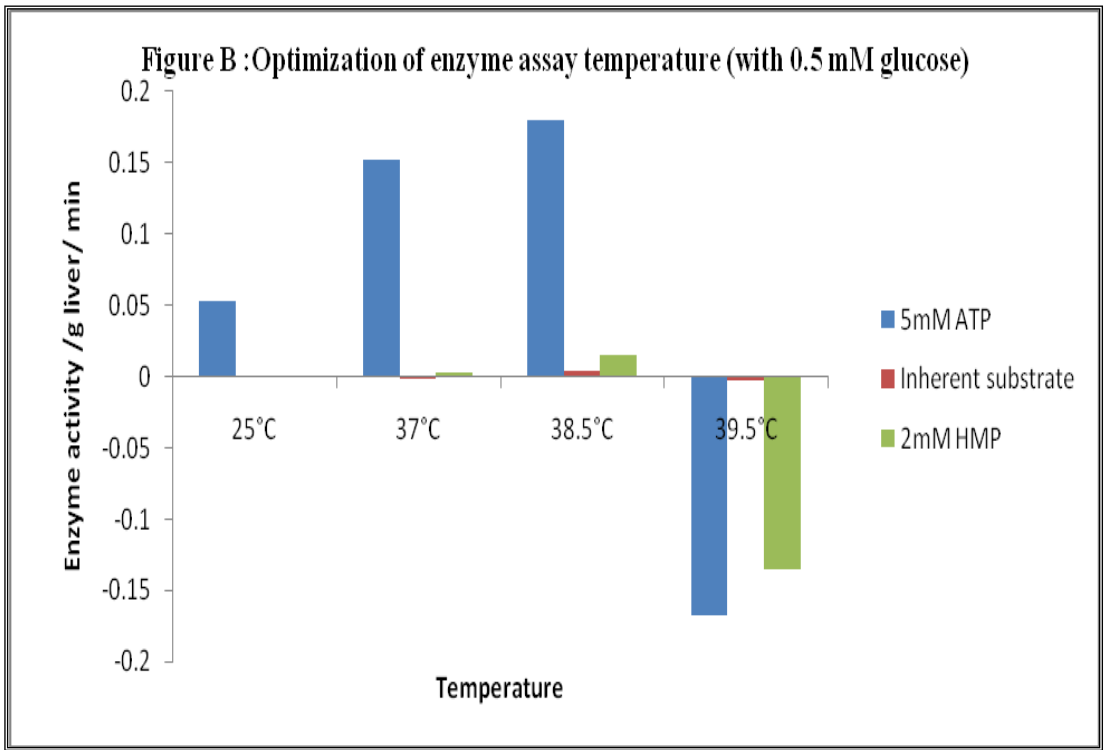


Figure F: Enzyme assay after the addition of 100 mM glucose
Enzyme activity/g liver/min
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
30
35
Time(min)
5mM ATP
5mM ADP
Inherent substrate
2mM HMP

Appendix II: Summary of LC/MS results for nuclear fraction.

NO	Accession number	Description
1	gi|514388105	glutamate dehydrogenase 1, mitochondrial [Ovis aries]
2	gi|426242361	PREDICTED: liver carboxylesterase-like isoform X1 [Ovis aries]
3	gi|4501885	actin, cytoplasmic 1 [Homo sapiens]
4	gi|426238347	PREDICTED: protein disulfide-isomerase isoform X1 [Ovis aries]
5	gi|426245288	PREDICTED: catalase isoform X1 [Ovis aries]
6	gi|426239663	PREDICTED: dimethylaniline monooxygenase [N-oxide-forming] 1 [Ovis aries]
7	gi|251823897	protein disulfide-isomerase A3 precursor [Ovis aries]
8	gi|426239655	PREDICTED: dimethylaniline monooxygenase [N-oxide-forming] 3 [Ovis aries]
9	gi|426239774	PREDICTED: alpha-enolase isoform X3 [Ovis aries]
10	gi|803289802	PREDICTED: carbamoyl-phosphate synthase [ammonia], mitochondrial isoform X2 [Ovis aries musimon]
11	gi|426224929	PREDICTED: ATP synthase subunit beta, mitochondrial [Ovis aries]
12	gi|392583892	heat shock 70 kDa protein 1A/1B [Ovis aries]
13	gi|426221286	PREDICTED: 60 kDa heat shock protein, mitochondrial [Ovis aries]
14	gi|426228900	PREDICTED: calreticulin [Ovis aries]
15	gi|426247368	PREDICTED: aldehyde dehydrogenase, mitochondrial [Ovis aries]
16	gi|57164373	serum albumin precursor [Ovis aries]
17	gi|350535721	cytochrome P450 2D6 [Ovis aries]
18	gi|57164387	corticosteroid 11-beta-dehydrogenase isozyme 1 [Ovis aries]
19	gi|426256884	PREDICTED: amine oxidase [flavin-containing] B isoform X1 [Ovis aries]
20	gi|548484074	PREDICTED: methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial [Capra hircus]
21	gi|803112201	PREDICTED: propionyl-CoA carboxylase alpha chain, mitochondrial isoform X1 [Ovis aries]
22	gi|803277851	PREDICTED: protein disulfide-isomerase A4 isoform X2 [Ovis aries musimon]
23	gi|326937428	UDP-glucuronosyltransferase 2B7 precursor [Ovis aries]
24	gi|426218256	PREDICTED: propionyl-CoA carboxylase beta chain, mitochondrial [Ovis aries]
25	gi|326937422	UDP-glucuronosyltransferase 1-3 precursor [Ovis aries]
26	gi|426239537	PREDICTED: epoxide hydrolase 1 [Ovis aries]
27	gi|426253779	PREDICTED: ATP synthase subunit alpha, mitochondrial [Ovis aries]
28	gi|164414449	myoglobin [Ovis aries]

Appendix III: Optimization of enzyme assay temperature



To investigate the optimum temperature for the PPGKm activity, enzyme assay ran at different temperatures (25˚C, 37˚C, 38˚C and 39˚C) with high and low glucose concentrations (0.5 mM and 100mM) in the presence of 5mM ATP or 2mM HMP and blank (inherent substrate).

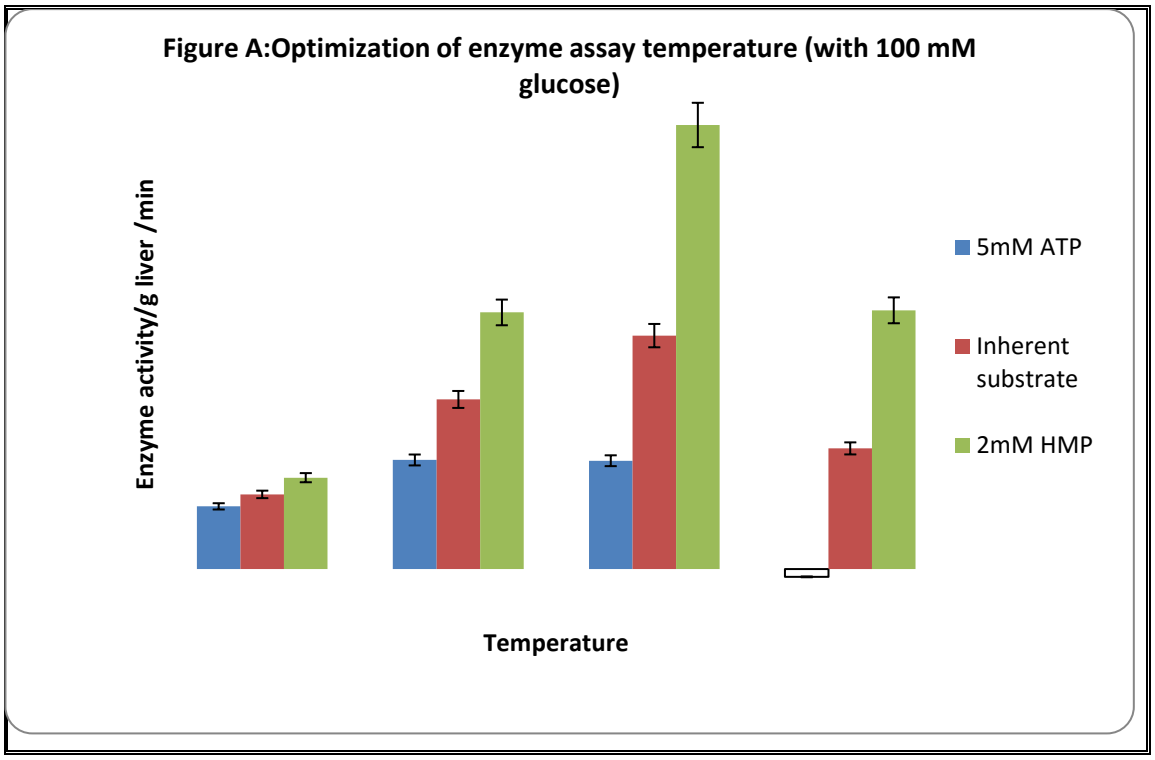
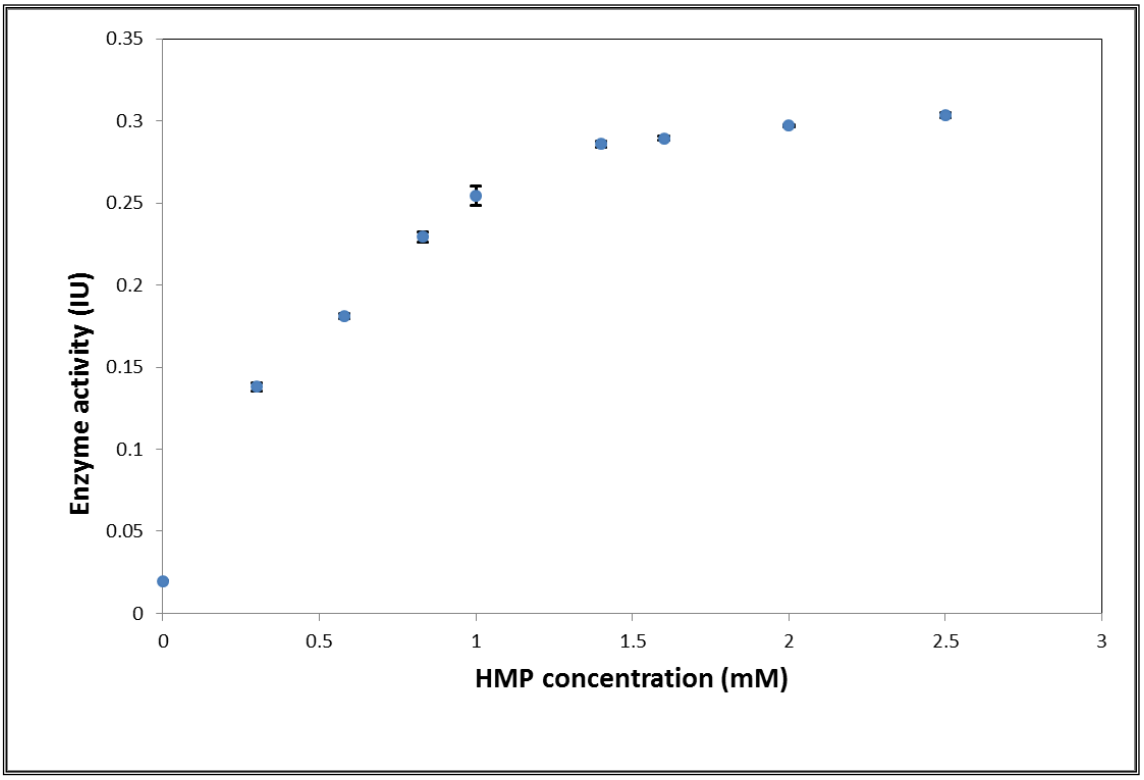


Figure A:Optimization of enzyme assay temperature (with 100 mM glucose)
Enzyme activity/g liver /min
5mM ATP
Inherent substrate
2mM HMP
Temperature

Appendix IV (Raw numerical data)

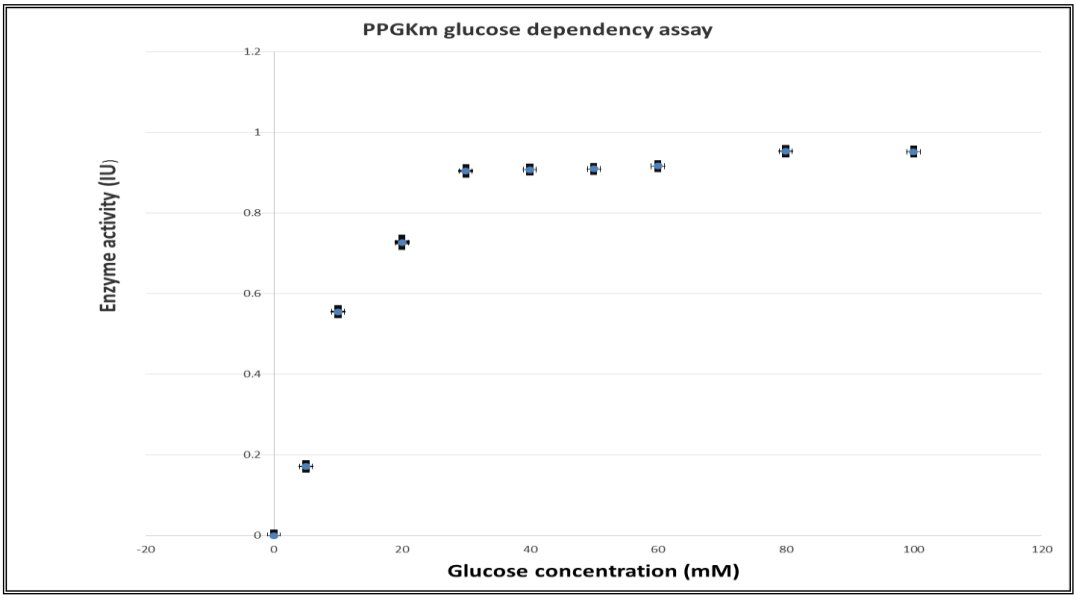
Variable HMP concentrations

Ovine liver with different HMP concentrations					
substrate	Activity			Average	sem
0	0.02010	0.01980	0.01960	0.01983	0.00015
0.3µM	0.13641	0.14310	0.13460	0.13804	0.00259
0.58µM	0.18080	0.17900	0.18400	0.18127	0.00146
0.83µM	0.22785	0.23500	0.22500	0.22928	0.00297
1mM	0.26555	0.24600	0.25100	0.25418	0.00586
1.4mM	0.28382	0.29000	0.28400	0.28594	0.00203
1.6mM	0.28750	0.29100	0.29000	0.28950	0.00104
2mM	0.29806	0.29700	0.29600	0.29702	0.00060
2.5mM	0.30610	0.30300	0.30100	0.30337	0.00148



Different glucose concentrations

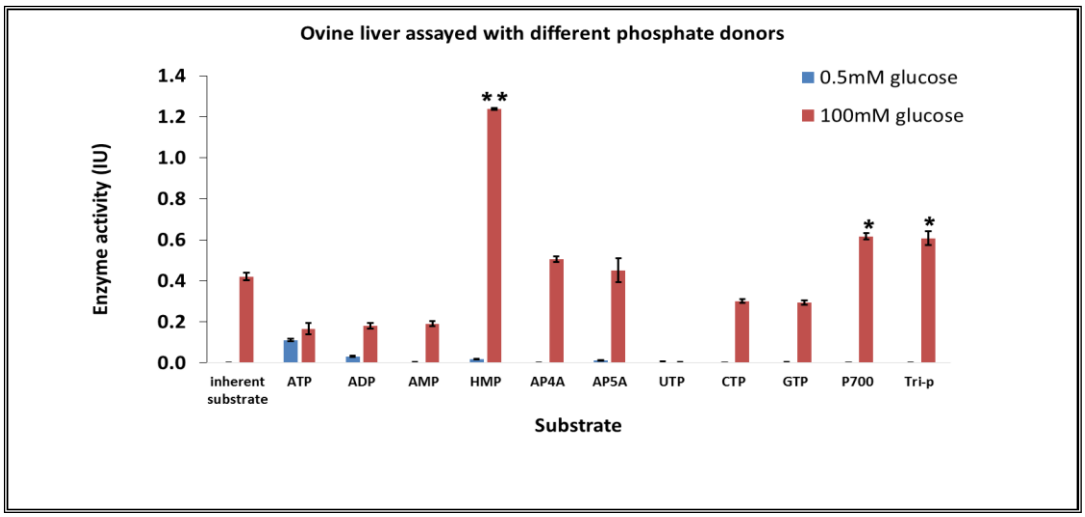
			PPGKm glucose Km				
Glucose concentration					AVERAGE	stdev	SEM
0		0.0001	0.0001	0.0001	0.0001	0.0000	0.0000
20		0.1714	0.1684	0.1710	0.1703	0.0016	0.0009
30		0.5555	0.5631	0.5573	0.5586	0.0040	0.0023
40		0.7270	0.7321	0.7151	0.7247	0.0087	0.0050
50		0.9044	0.8961	0.9044	0.9016	0.0048	0.0028
60		0.9080	0.9065	0.9076	0.9074	0.0008	0.0004
70		0.9095	0.9103	0.9087	0.9095	0.0008	0.0005
80		0.9166	0.9175	0.9165	0.9169	0.0006	0.0003
100		0.9532	0.9531	0.9487	0.9517	0.0026	0.0015
120		0.9525	0.9497	0.9510	0.9511	0.0014	0.0008



Different phosphoryl donors

Activity of PPGKm with 100mM glucose and different phosphoryl donors												
	inherent substrate	ATP	ADP	AMP	CTP	GTP	HMP	AP4A	AP5A	P700	Tri-p	UTP
	0.4566	0.2000	0.2200	0.1700	0.3100	0.2740	1.3023	0.5100	0.4700	0.6400	0.6300	0.0033
	0.3950	0.2300	0.1770	0.1910	0.2930	0.3210	1.2000	0.4870	0.4530	0.5900	0.6500	0.0030
	0.4100	0.2200	0.2010	0.2110	0.3010	0.2890	1.3000	0.5200	0.4310	0.6200	0.5400	0.0031
AVERAGE	0.4205	0.2167	0.1993	0.1907	0.3013	0.2947	1.2674	0.5057	0.4513	0.6167	0.6067	0.0031
STDEV	0.0321	0.0153	0.0215	0.0205	0.0085	0.0240	0.0584	0.0169	0.0196	0.0252	0.0586	0.0001
SEM	0.0186	0.0088	0.0124	0.0118	0.0049	0.0139	0.0337	0.0098	0.0113	0.0145	0.0338	0.0000

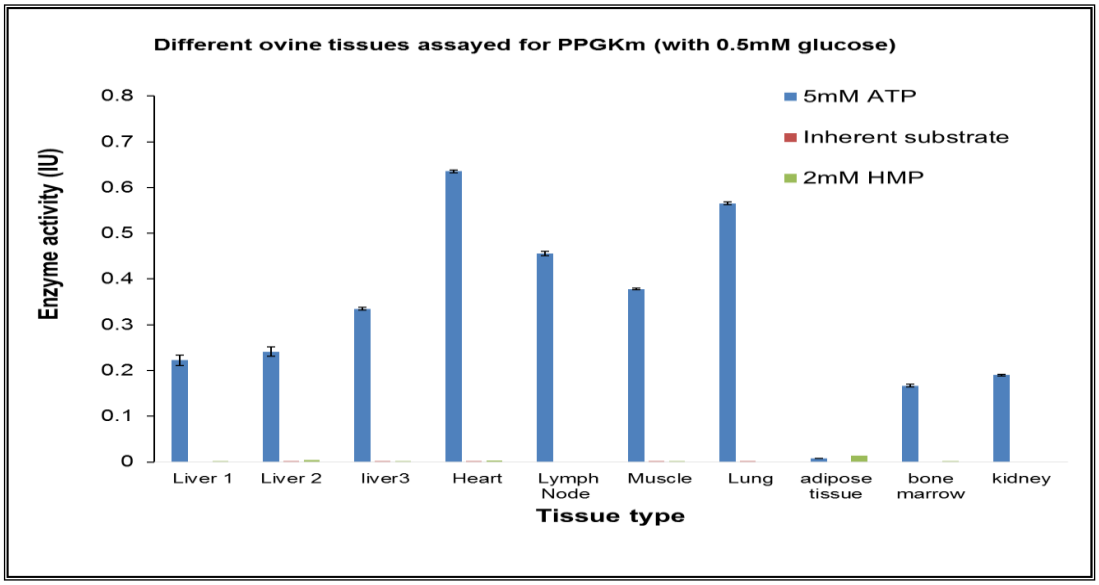
Activity of PPGKm with 0.5mM glucose and different phosphoryl donors											
	inherent substrate	ATP	ADP	AMP	HMP	AP4A	AP5A	UTP	CTP	GTP	P700	Tri-p
	0.0009	0.1120	0.0346	0.0036	0.0219	0.0008	0.0143	0.0024	0.0025	0.0050	0.0010	0.0005
	0.0009	0.1014	0.0269	0.0027	0.0173	0.0008	0.0110	0.0002	0.0003	0.0030	0.0013	0.0004
	0.0007	0.1200	0.0326	0.0034	0.0141	0.0009	0.0127	0.0099	0.0027	0.0037	0.0020	0.0005
AVERAGE	0.0008	0.1111	0.0314	0.0032	0.0178	0.0008	0.0127	0.0041	0.0018	0.0039	0.0014	0.0005
STDEV	0.0001	0.0093	0.0040	0.0005	0.0039	0.0001	0.0017	0.0051	0.0013	0.0010	0.0005	0.0001
SEM	0.0001	0.0054	0.0023	0.0003	0.0023	0.0000	0.0010	0.0029	0.0008	0.0006	0.0003	0.0000

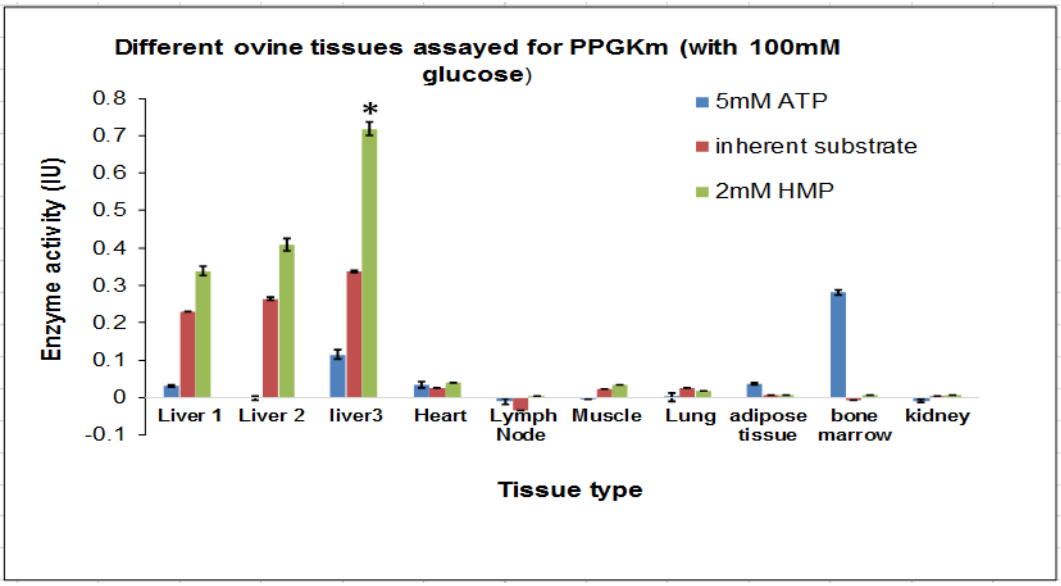


Different ovine tissues

Different ovine tissues assayed for PPGKm activity

	0.5mM Glucose									
	Liver 1	Liver 2	liver3	Heart	Lymph Node	Muscle	Lung	adipose tissue	bone marrow	kidney
ATP	0.2010	0.2250	0.3340	0.6400	0.4630	0.3780	0.5690	0.0078	0.1630	0.1910
	0.2250	0.2610	0.3410	0.6310	0.4460	0.3760	0.5680	0.0076	0.1720	0.1920
	0.2410	0.2370	0.3300	0.6350	0.4570	0.3810	0.5590	0.0070	0.1640	0.1870
Average	0.2223	0.2410	0.3350	0.6353	0.4553	0.3783	0.5653	0.0075	0.1663	0.1900
STDEV	0.0201	0.0183	0.0056	0.0045	0.0086	0.0025	0.0055	0.0004	0.0049	0.0026
SEM	0.0116	0.0106	0.0032	0.0026	0.0050	0.0015	0.0032	0.0002	0.0028	0.0015
Inherent substrate	0.0012	0.0019	0.0021	0.0019	0.0026	0.0001	0.0022	0.0018	0.0012	0.0010
	0.0010	0.0016	0.0018	0.0015	0.0015	0.0013	0.0131	0.0016	0.0013	0.0010
	0.0008	0.0017	0.0018	0.0012	0.0013	0.0011	0.0014	0.0018	0.0011	0.0010
Average	0.0010	0.0017	0.0019	0.0015	0.0018	0.0008	0.0056	0.0017	0.0012	0.0010
STDEV	0.0002	0.0002	0.0002	0.0003	0.0007	0.0006	0.0065	0.0001	0.0001	0.0000
SEM	0.0001	0.0001	0.0001	0.0002	0.0004	0.0004	0.0038	0.0001	0.0001	0.0000
HMP	0.0011	0.0016	0.0014	0.0026	0.0011	0.0020	0.0012	0.0138	0.0038	0.0010
	0.0014	0.0017	0.0012	0.0025	0.0014	0.0020	0.0011	0.0143	0.0034	0.0016
	0.0017	0.0019	0.0014	0.0025	0.0012	0.0019	0.0014	0.0135	0.0029	0.0014
Average	0.0014	0.0017	0.0013	0.0026	0.0012	0.0020	0.0012	0.0139	0.0034	0.0013
STDEV	0.0003	0.0002	0.0001	0.0001	0.0002	0.0001	0.0002	0.0004	0.0005	0.0003
SEM	0.0002	0.0001	0.0001	0.0000	0.0001	0.0001	0.0001	0.0002	0.0003	0.0002

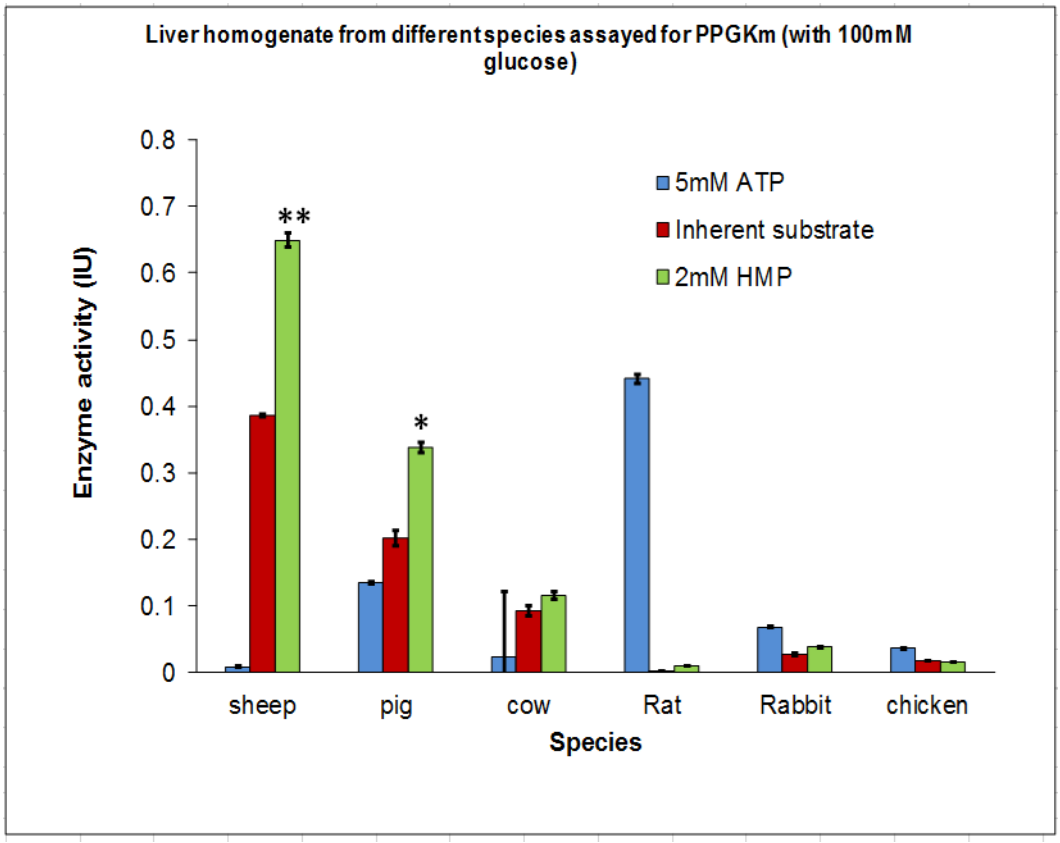




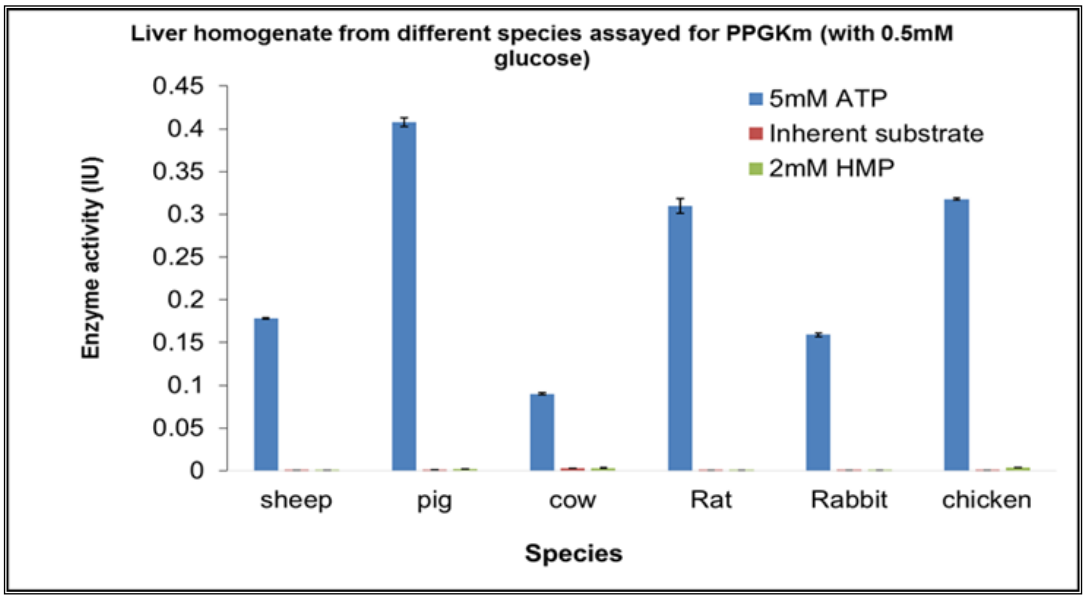
	100mM Glucose									
	Liver 1	Liver 2	liver3	Heart	Lymph Node	Muscle	Lung	adipose tissue	bone marrow	kidney
	0.0400	0.0408	0.0910	0.0310	-0.0630	-0.0080	-0.0820	0.0380	0.2800	-0.0182
ATP	0.0380	0.0590	0.1310	0.0220	-0.0430	-0.0050	-0.0540	0.0380	0.2910	-0.0070
	0.0330	0.0510	0.1210	0.0490	-0.0730	-0.0070	-0.0900	0.0322	0.2710	-0.0050
Average	0.0370	0.0503	0.1143	0.0340	-0.0597	-0.0067	-0.0753	0.0361	0.2807	-0.0101
stdev	0.0036	0.0091	0.0208	0.0137	0.0153	0.0015	0.0189	0.0033	0.0100	0.0071
SEM	0.0021	0.0053	0.0120	0.0079	0.0088	0.0009	0.0109	0.0019	0.0058	0.0041
inherent substrate	0.2270	0.2560	0.3310	0.0260	-0.0340	0.0220	0.0250	0.0064	-0.0073	0.0031
	0.2310	0.2710	0.3420	0.0241	-0.0330	0.0220	0.0240	0.0059	-0.0071	0.0028
	0.2290	0.2630	0.3400	0.0243	-0.0340	0.0220	0.0240	0.0063	-0.0068	0.0026
Average	0.2290	0.2633	0.3377	0.0248	-0.0337	0.0220	0.0243	0.0062	-0.0071	0.0028
stdev	0.0020	0.0075	0.0059	0.0010	0.0006	0.0000	0.0006	0.0003	0.0003	0.0003
SEM	0.0012	0.0043	0.0034	0.0006	0.0003	0.0000	0.0003	0.0002	0.0001	0.0001
HMP	0.3126	0.3752	0.7531	0.0380	0.0043	0.0350	0.0167	0.0059	0.0070	0.0052
	0.3527	0.4261	0.6891	0.0410	0.0039	0.0324	0.0186	0.0068	0.0068	0.0054
	0.3471	0.4223	0.7131	0.0391	0.0040	0.0330	0.0168	0.0071	0.0051	0.0051
Average	0.3375	0.4079	0.7184	0.0394	0.0041	0.0335	0.0174	0.0066	0.0063	0.0052
stdev	0.0217	0.0284	0.0323	0.0015	0.0002	0.0014	0.0011	0.0006	0.0010	0.0002
SEM	0.0125	0.0164	0.0187	0.0009	0.0001	0.0008	0.0006	0.0004	0.0006	0.0001

PPGKm activity in different species

	100mM glucose					
	sheep	pig	cow	Rat	Rabbit	chicken
ATP	0.0002	0.1481	0.0287	0.4345	0.0726	0.0331
	0.0001	0.1521	0.3124	0.4581	0.0763	0.0371
	0.0003	0.1542	0.3341	0.4451	0.0735	0.0321
stdev	0.0001	0.0031	0.1698	0.0118	0.0019	0.0026
SEM	0.0001	0.0018	0.0980	0.0068	0.0011	0.0015
Average	0.0002	0.1515	0.2251	0.4459	0.0741	0.0341
inherent substrate	0.3940	0.2140	0.0950	0.0029	0.0283	0.0203
	0.4010	0.2310	0.1100	0.0032	0.0352	0.0212
	0.3980	0.2530	0.1210	0.0034	0.0299	0.0204
stdev	0.0032	0.0195	0.0130	0.0002	0.0036	0.0005
SEM	0.0019	0.0112	0.0075	0.0001	0.0021	0.0003
Average	0.3977	0.2327	0.1087	0.0032	0.0311	0.0206
HMP	0.6681	0.3460	0.1224	0.0106	0.0391	0.0154
	0.7012	0.3541	0.1421	0.0110	0.0413	0.0162
	0.6981	0.3724	0.1243	0.0112	0.0401	0.0157
Average	0.6891	0.3575	0.1296	0.0109	0.0402	0.0158
stdev	0.0180	0.0135	0.0109	0.0003	0.0011	0.0004
SEM	0.0104	0.0078	0.0063	0.0002	0.0006	0.0002

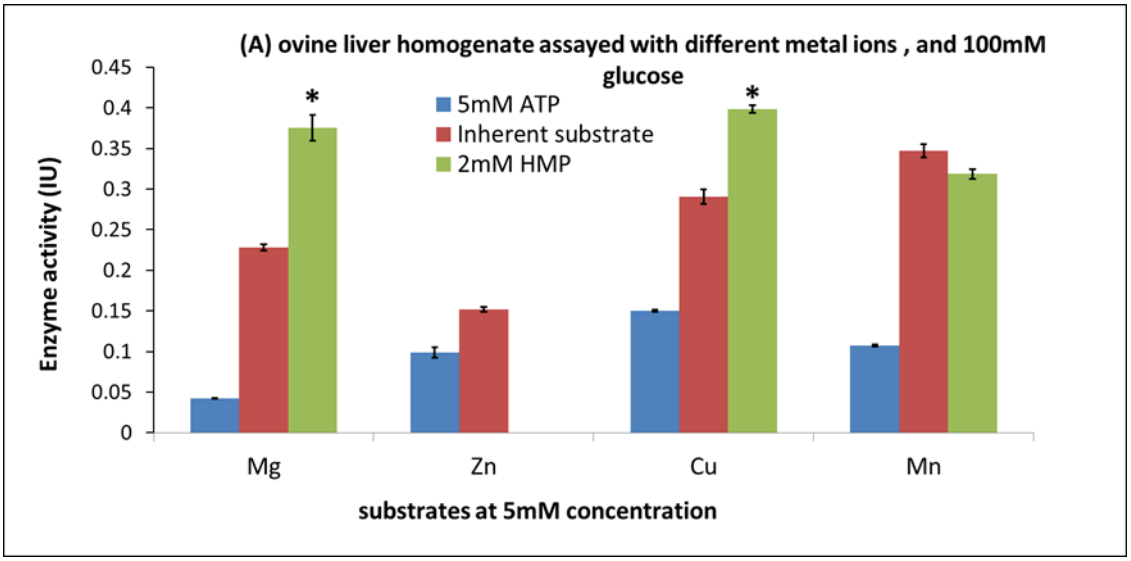


	0.5mM glucose						
		sheep	pig	cow	Rat	Rabbit	chicken
	ATP	0.1772	0.4072	0.0880	0.2943	0.1550	0.3156
		0.1802	0.3985	0.0921	0.3102	0.1632	0.3201
		0.1783	0.4171	0.0901	0.3251	0.1586	0.3172
	Average	0.1786	0.4076	0.0901	0.3099	0.1589	0.3176
	Stdev	0.0015	0.0093	0.0021	0.0154	0.0041	0.0023
	SEM	0.0009	0.0054	0.0012	0.0089	0.0024	0.0013
	inherent substrate	0.0015	0.0015	0.0030	0.0002	0.0002	0.0010
		0.0013	0.0015	0.0028	0.0001	0.0001	0.0011
		0.0012	0.0014	0.0026	0.0001	0.0002	0.0009
	Average	0.0013	0.0015	0.0028	0.0001	0.0002	0.0010
	Stdev	0.0002	0.0001	0.0002	0.0000	0.0000	0.0001
	SEM	0.0001	0.0000	0.0001	0.0000	0.0000	0.0001
	HMP	0.0001	0.0020	0.0039	0.0002	0.0002	0.0039
		0.0002	0.0018	0.0028	0.0002	0.0002	0.0041
		0.0001	0.0019	0.0026	0.0002	0.0003	0.0035
	Average	0.0001	0.0019	0.0031	0.0002	0.0002	0.0038
	Stdev	0.0000	0.0001	0.0007	0.0000	0.0001	0.0003
	SEM	0.0000	0.0001	0.0004	0.0000	0.0000	0.0002

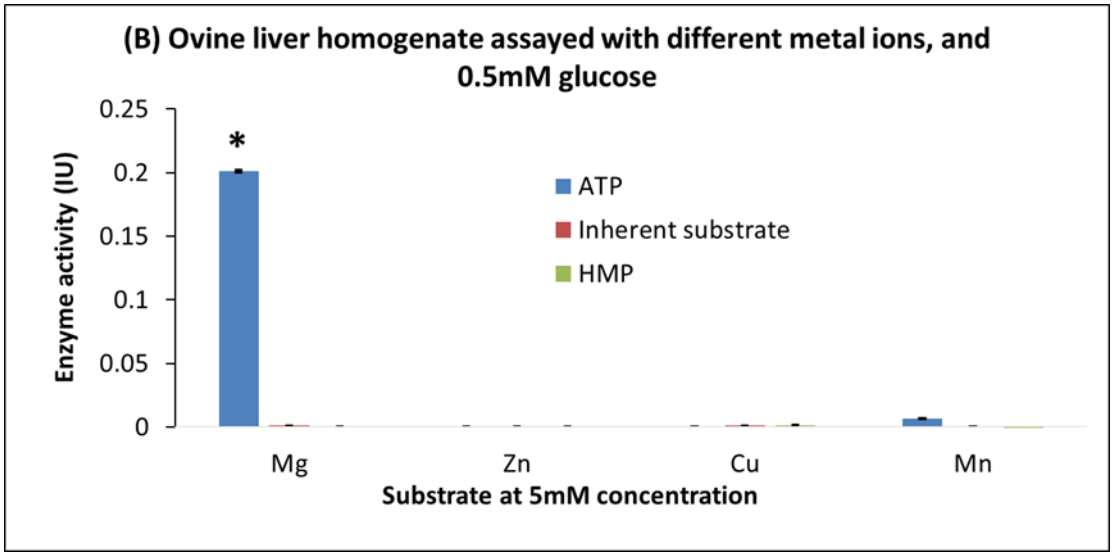


Metal ions dependency assay

		100mM glucose		
		ATP	Inherent substrate	HMP
	Mg	0.0423	0.2279	0.3752
		0.0441	0.2418	0.4310
		0.0436	0.2357	0.3981
	Average	0.0433	0.2351	0.4014
	Stdev	0.0009	0.0070	0.0280
	SEM	0.0005	0.0040	0.0162
	Zn	0.0987	0.1616	0.0000
		0.1103	0.1521	0.0000
		0.1212	0.1571	0.0000
	Average	0.1101	0.1569	0.0000
	Stdev	0.0113	0.0048	0.0000
	SEM	0.0065	0.0027	0.0000
	Cu	0.1498	0.2905	0.3984
		0.1512	0.3011	0.4121
		0.1469	0.3211	0.3991
	Average	0.1493	0.3042	0.4032
	Stdev	0.0022	0.0155	0.0077
	SEM	0.0013	0.0090	0.0045
	Mn	0.1072	0.3463	0.3184
		0.1102	0.3732	0.3210
		0.1089	0.3510	0.3012
	Average	0.1088	0.3568	0.3136
	Stdev	0.0015	0.0144	0.0108
	SEM	0.0009	0.0083	0.0062

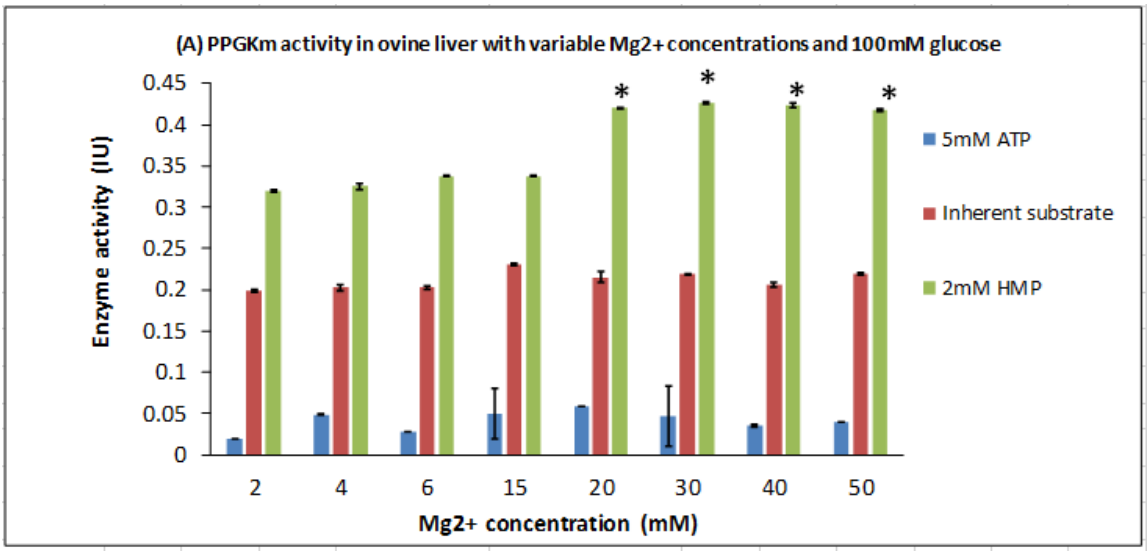


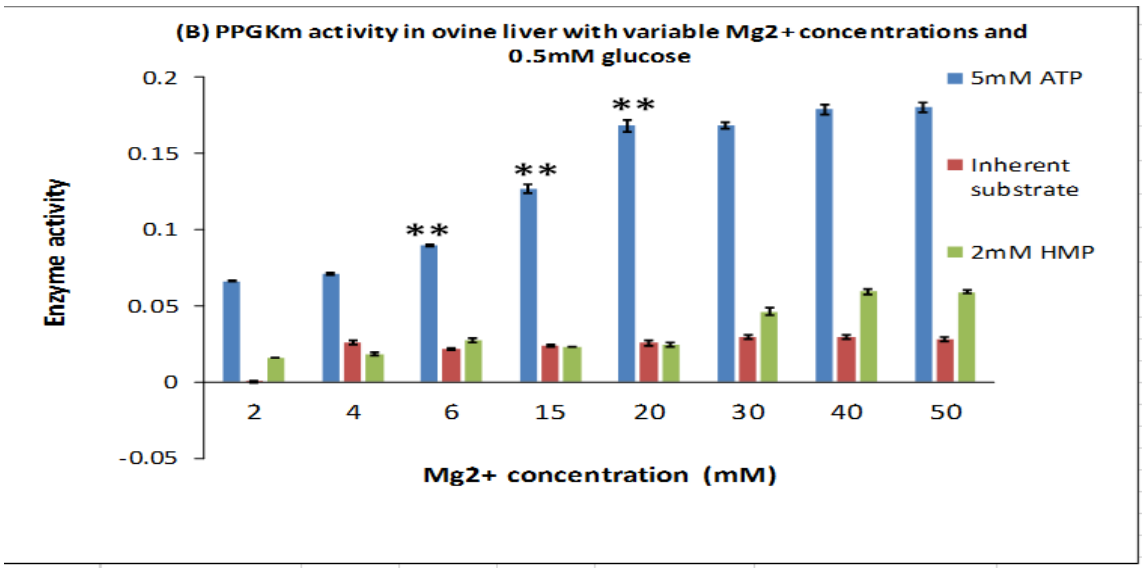
		Assay with 0.5mM glucose		
		ATP	Inherent substrate	HMP
	Mg	0.2011	0.0010	0.0001
		0.1984	0.0010	0.0001
		0.2010	0.0010	0.0001
	Average	0.2002	0.0010	0.0001
	Stdev	0.0015	0.0000	0.0000
	SEM	0.0009	0.0000	0.0000
	Zn	0.0002	0.0002	0.0001
		0.0002	0.0002	0.0001
		0.0002	0.0002	0.0001
	Average	0.0000	0.0002	0.0001
	Stdev	0.0000	0.0000	0.0000
	SEM	0.0000	0.0000	0.0000
	Cu	0.0000	0.0012	0.0010
		0.0000	0.0012	0.0014
		0.0000	0.0011	0.0013
	Average	0.0000	0.0012	0.0010
	Stdev	0.0000	0.0000	0.0005
	SEM	0.0000	0.0000	0.0003
	Mn	0.0067	0.0000	-0.0010
		0.0060	0.0000	-0.0021
		0.0067	0.0000	-0.0020
	Average	0.0065	0.0000	-0.0020
	Stdev	0.0004	0.0000	0.0001
	SEM	0.0002	0.0000	0.0000



PPGKm activity with variable Mg^{2+} concentrations

	with 100 mM		
	5mM ATP	Inherent substrate	2mM HMP
2mM	0.0183	0.1974	0.3194
	0.0191	0.2011	0.3216
	0.0189	0.1982	0.3187
Average	0.0188	0.1989	0.3199
stdev	0.0004	0.0020	0.0015
SEM	0.0002	0.0011	0.0009
4mM	0.0483	0.1989	0.3226
	0.0501	0.2103	0.3318
	0.0485	0.1990	0.3214
Average	0.0490	0.2028	0.3253
stdev	0.0010	0.0065	0.0057
SEM	0.0006	0.0038	0.0033
6mM	0.0172	0.2045	0.3386
	0.0177	0.1984	0.3401
	0.0188	0.2041	0.3354
Average	0.0179	0.2023	0.3380
stdev	0.0008	0.0034	0.0024
SEM	0.0005	0.0020	0.0014
15mM	0.0198	0.2294	0.3363
	0.1102	0.2348	0.3401
	0.0199	0.2288	0.3376
Average	0.0499	0.2310	0.3380
stdev	0.0522	0.0033	0.0019
SEM	0.0301	0.0019	0.0011
20mM	0.0088	0.2210	0.4196
	0.0087	0.2014	0.4217
	0.0090	0.2230	0.4198
Average	0.0089	0.2152	0.4204
stdev	0.0002	0.0119	0.0011
SEM	0.0001	0.0069	0.0007
30mM	0.0113	0.2180	0.4255
	0.1211	0.2201	0.4301
	0.0145	0.2180	0.4246
Average	0.0490	0.2187	0.4267
stdev	0.0625	0.0012	0.0030
SEM	0.0361	0.0007	0.0017
40mM	0.0259	0.2028	0.4213
	0.0261	0.2111	0.4301
	0.0236	0.2035	0.4201
Average	0.0252	0.2058	0.4238
stdev	0.0014	0.0046	0.0055
SEM	0.0008	0.0027	0.0031
50mM	0.0392	0.2171	0.4152
	0.0403	0.2211	0.4216
	0.0398	0.2198	0.4164
Average	0.0398	0.2193	0.4177
stdev	0.0006	0.0020	0.0034
SEM	0.0003	0.0012	0.0020

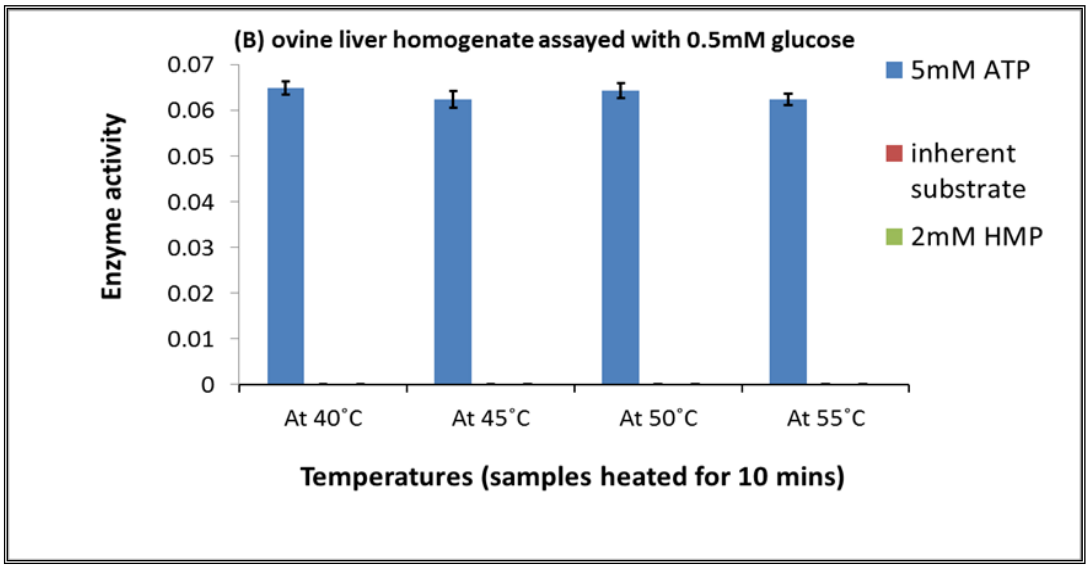

(A) PPGKm activity in ovine liver with variable Mg2+ concentrations and 100mM glucose
Enzyme activity (IU)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
2
4
6
15
20
30
40
50
Mg2+ concentration (mM)
5mM ATP
Inherent substrate
2mM HMP
*
*
*
*


(B) PPGKm activity in ovine liver with variable Mg2+ concentrations and 0.5mM glucose
Enzyme activity
0.2
0.15
0.1
0.05
0
-0.05
2
4
6
15
20
30
40
50
Mg2+ concentration (mM)
5mM ATP
Inherent substrate
2mM HMP
**
**
**

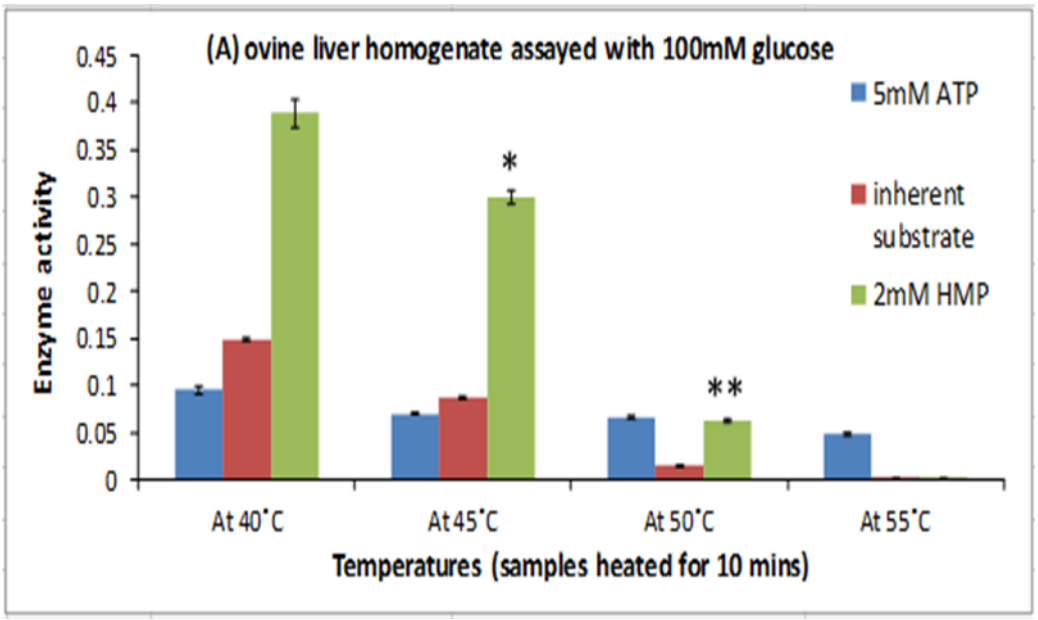
	with 0.5mM glucose			
		5mM ATP	Inherent substrate	2mM HMP
	2mM	0.0664	0.0175	0.0154
		0.0659	0.0201	0.0163
		0.0667	0.0178	0.0158
	Average	0.0663	0.0185	0.0158
	stdev	0.0004	0.0014	0.0004
	SEM	0.0002	0.0008	0.0003
	4mM	0.0727	0.0229	0.0166
		0.0698	0.0287	0.0201
		0.0702	0.0264	0.0178
	Average	0.0709	0.0260	0.0182
	stdev	0.0016	0.0029	0.0018
	SEM	0.0009	0.0017	0.0010
	6mM	0.0901	0.0225	0.0253
		0.0879	0.0201	0.0275
		0.0912	0.0226	0.0301
	Average	0.0897	0.0217	0.0276
	stdev	0.0017	0.0014	0.0024
	SEM	0.0010	0.0008	0.0014
	15mM	0.1223	0.0222	0.0227
		0.1322	0.0254	0.0235
		0.1267	0.0235	0.0228
	Average	0.1271	0.0237	0.0230
	stdev	0.0050	0.0016	0.0004
	SEM	0.0029	0.0009	0.0003
	20mM	0.1614	0.0229	0.0223
		0.1743	0.0253	0.0268
		0.1687	0.0287	0.0247
	Average	0.1681	0.0256	0.0246
	stdev	0.0065	0.0029	0.0023
	SEM	0.0037	0.0017	0.0013
	30mM	0.1647	0.0275	0.0422
		0.1723	0.0321	0.0456
		0.1687	0.0289	0.0510
	Average	0.1686	0.0295	0.0463
	stdev	0.0038	0.0024	0.0044
	SEM	0.0022	0.0014	0.0026
	40mM	0.1735	0.0277	0.0566
		0.1851	0.0321	0.0632
		0.1781	0.0289	0.0583
	Average	0.1789	0.0296	0.0594
	stdev	0.0058	0.0023	0.0034
	SEM	0.0034	0.0013	0.0020
	50mM	0.1746	0.0252	0.0575
		0.1865	0.0301	0.0610
		0.1798	0.0287	0.0583
	Average	0.1803	0.0280	0.0589
	stdev	0.0060	0.0025	0.0018
	SEM	0.0034	0.0015	0.0011

Thermal stability assay for PPGKm activity

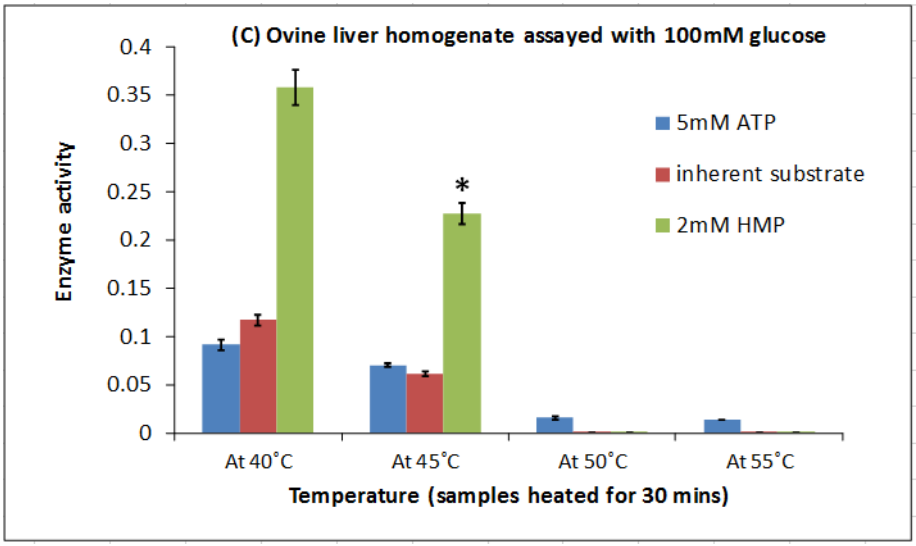
	Heated for 10 minutes with 0.5mM glucose		
	5mM ATP	inherent substrate	2mM HMP
At 40˚C	0.0650	0.0000	0.0000
	0.0685	0.0000	0.0000
	0.0701	0.0000	0.0000
AVERAGE	0.0679	0.0000	0.0000
stdev	0.0026	0.0000	0.0000
SEM	0.0015	0.0000	0.0000
At 45˚C	0.0625	0.0000	0.0000
	0.0575	0.0000	0.0000
	0.0569	0.0000	0.0000
AVERAGE	0.0589	0.0000	0.0000
stdev	0.0031	0.0000	0.0000
SEM	0.0018	0.0000	0.0000
At 50˚C	0.0644	0.0000	0.0000
	0.0588	0.0000	0.0000
	0.0631	0.0000	0.0000
AVERAGE	0.0621	0.0000	0.0000
stdev	0.0029	0.0000	0.0000
SEM	0.0017	0.0000	0.0000
At 55˚C	0.0626	0.0000	0.0000
	0.0636	0.0000	0.0000
	0.0667	0.0000	0.0000
AVERAGE	0.0643	0.0000	0.0000
stdev	0.0022	0.0000	0.0000
SEM	0.0013	0.0000	0.0000



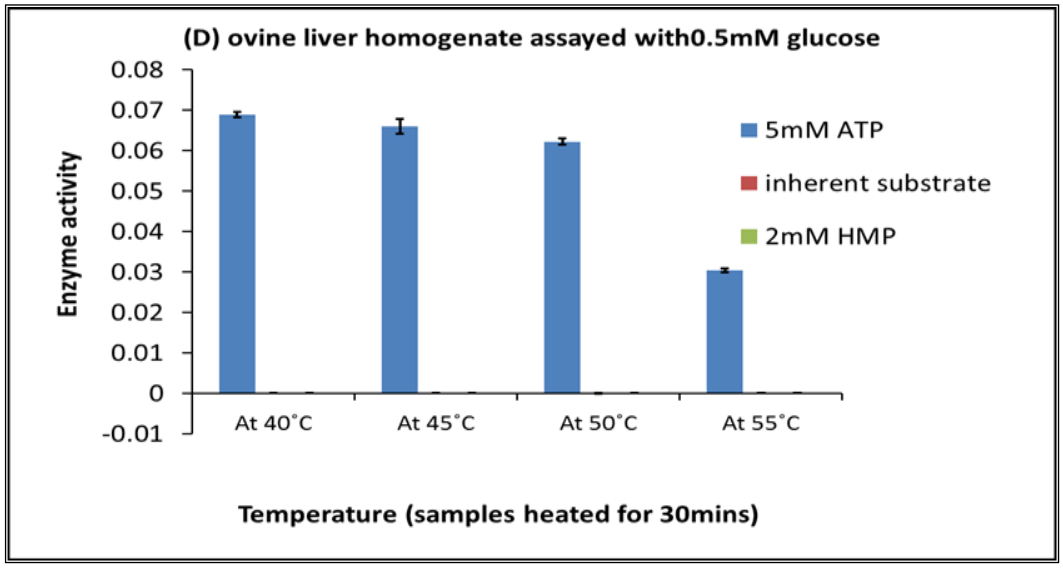
	Heated for 10 minutes with 100 mM glucose			
		5mM ATP	inherent substrate	2mM HMP
	At 40˚C	0.0951	0.1483	0.3883
		0.1102	0.1552	0.4211
		0.0988	0.1503	0.3675
	AVERAGE	0.1014	0.1513	0.3923
	stdev	0.0079	0.0036	0.0270
	SEM	0.0046	0.0021	0.0156
	At 45˚C	0.0701	0.0868	0.3000
		0.0660	0.0932	0.3216
		0.0695	0.0897	0.3011
	AVERAGE	0.0685	0.0899	0.3076
	stdev	0.0022	0.0032	0.0122
	SEM	0.0013	0.0019	0.0070
	At 50˚C	0.0656	0.0141	0.0627
		0.0687	0.0150	0.0675
		0.0701	0.0148	0.0701
	AVERAGE	0.0682	0.0146	0.0668
	stdev	0.0023	0.0005	0.0038
	SEM	0.0013	0.0003	0.0022
	At 55˚C	0.0487	0.0000	0.0000
		0.0532	0.0000	0.0000
		0.0497	0.0000	0.0000
	AVERAGE	0.0505	0.0000	0.0000
	stdev	0.0024	0.0000	0.0000
	SEM	0.0014	0.0000	0.0000



	Heated for 30 minutes with 100 mM glucose			
		5mM ATP	inherent substrate	2mM HMP
	At 40˚C	0.0916	0.1174	0.3574
		0.1102	0.1351	0.4211
		0.0984	0.1198	0.3876
	AVERAGE	0.1001	0.1241	0.3887
	stdev	0.0094	0.0096	0.0318
	SEM	0.0054	0.0055	0.0184
	At 45˚C	0.0701	0.0615	0.2271
		0.0660	0.0685	0.2654
		0.0714	0.0675	0.2451
	AVERAGE	0.0692	0.0658	0.2459
	stdev	0.0028	0.0037	0.0192
	SEM	0.0016	0.0022	0.0111
	At 50˚C	0.0155	0.0000	0.0000
		0.0167	0.0000	0.0000
		0.0201	0.0000	0.0000
	AVERAGE	0.0174	0.0000	0.0000
	stdev	0.0024	0.0000	0.0000
	SEM	0.0014	0.0000	0.0000
	At 55˚C	0.0135	0.0000	0.0000
		0.0143	0.0000	0.0000
		0.0139	0.0000	0.0000
	AVERAGE	0.0139	0.0000	0.0000
	stdev	0.0004	0.0000	0.0000
	SEM	0.0002	0.0000	0.0000

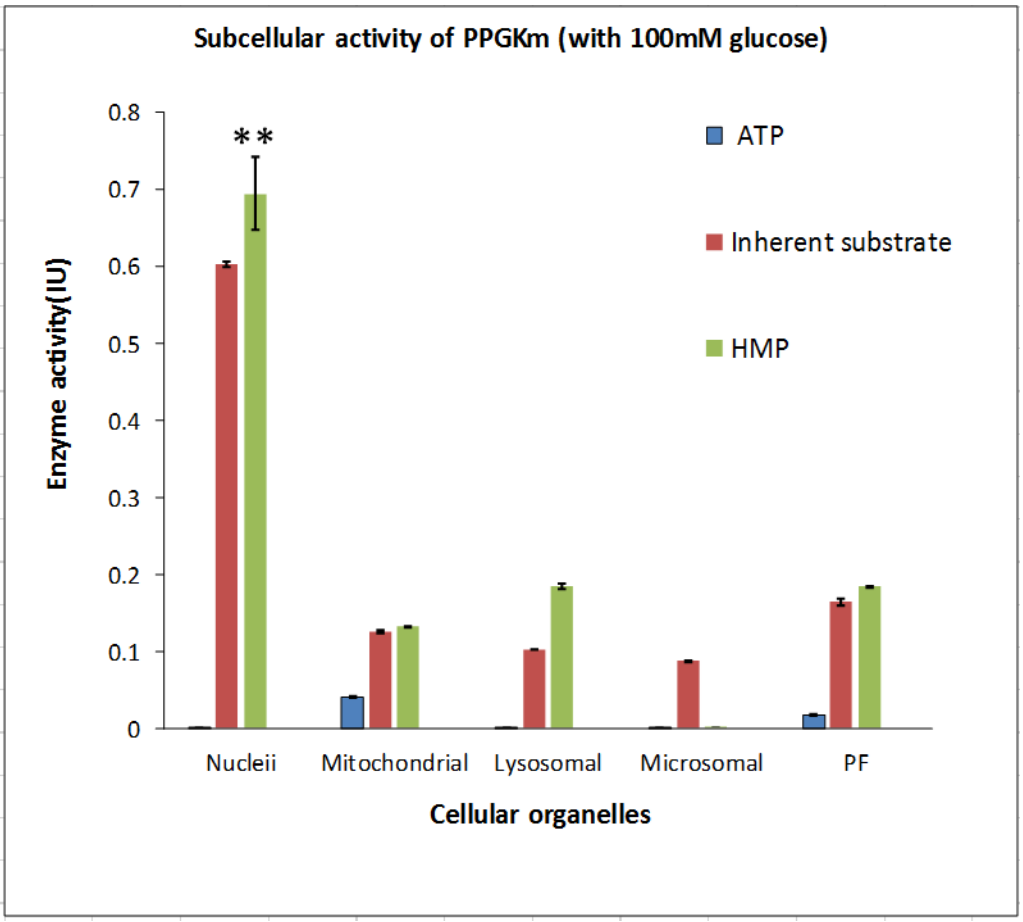


	Heated for 30minutes with 0.5mM glucose			
		5mM ATP	inherent substrate	2mM HMP
	At 40˚C	0.0689	0.0000	0.0000
		0.0712	0.0000	0.0000
		0.0696	0.0000	0.0000
	AVERAGE	0.0699	0.0000	0.0000
	stdev	0.0012	0.0000	0.0000
	SEM	0.0007	0.0000	0.0000
	At 45˚C	0.0660	0.0000	0.0000
		0.0721	0.0000	0.0000
		0.0685	0.0000	0.0000
	AVERAGE	0.0689	0.0000	0.0000
	stdev	0.0031	0.0000	0.0000
	SEM	0.0018	0.0000	0.0000
	At 50˚C	0.0622	0.0001	0.0000
		0.0597	0.0000	0.0000
		0.0615	0.0000	0.0000
	AVERAGE	0.0612	0.0000	0.0000
	stdev	0.0013	0.0000	0.0000
	SEM	0.0008	0.0000	0.0000
	At 55˚C	0.0304	0.0000	0.0000
		0.0290	0.0000	0.0000
		0.0306	0.0000	0.0000
	AVERAGE	0.0300	0.0000	0.0000
	stdev	0.0009	0.0000	0.0000
	SEM	0.0005	0.0000	0.0000

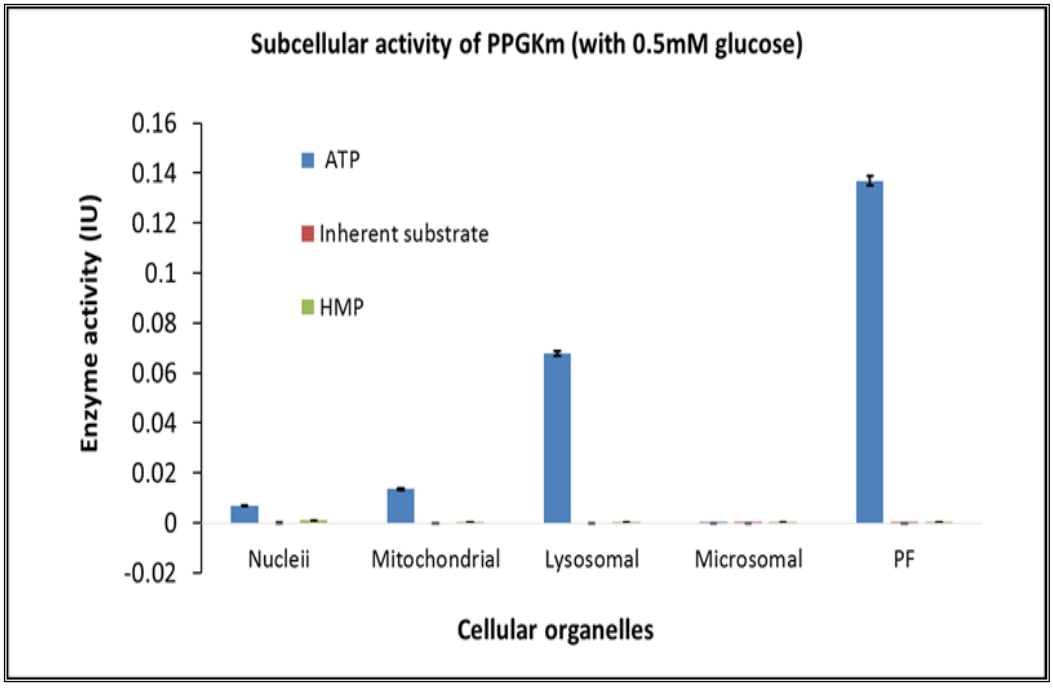


Subcellular fractionation assay

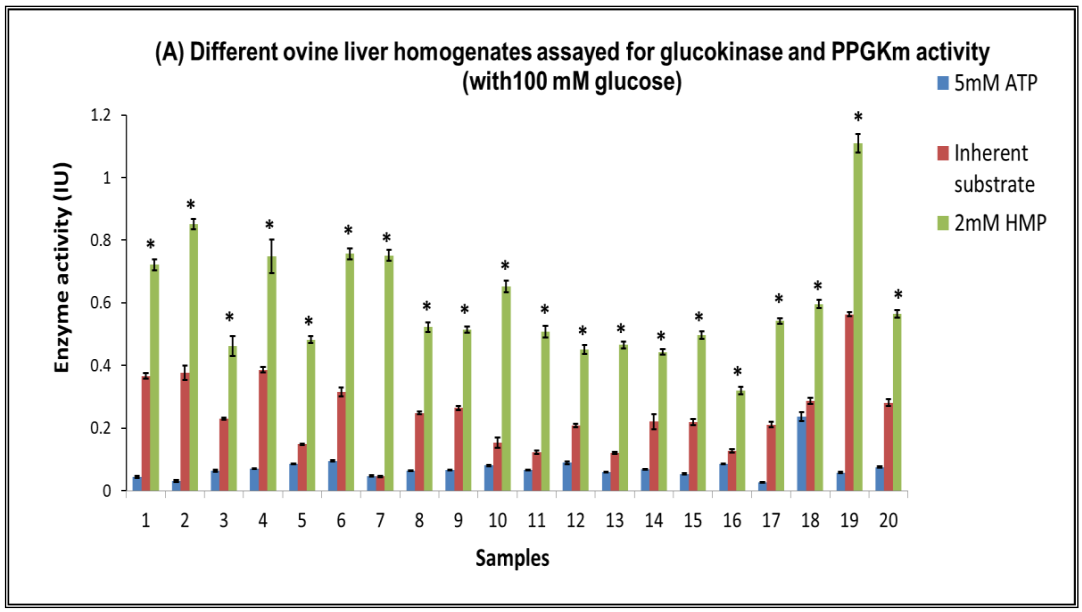
	100mM GLUCOSE				
	Nucleii	Mitochondrial	Lysosomal	Microsomal	PF
ATP	0.0002	0.0423	0.0003	0.0005	0.0180
	0.0002	0.0411	0.0003	0.0004	0.0172
	0.0002	0.0389	0.0003	0.0005	0.0179
Average	0.0002	0.0408	0.0003	0.0005	0.0177
stdev	0.0000	0.0017	0.0000	0.0001	0.0004
SEM	0.0000	0.0010	0.0000	0.0000	0.0003
inherent substrat	0.6005	0.1231	0.1029	0.0864	0.1681
	0.5973	0.1301	0.1031	0.0901	0.1546
	0.6105	0.1254	0.1026	0.0859	0.1701
Average	0.6028	0.1262	0.1029	0.0875	0.1643
stdev	0.0069	0.0036	0.0003	0.0023	0.0084
SEM	0.0040	0.0021	0.0001	0.0013	0.0049
HMP	0.6869	0.1298	0.1883	0.0001	0.1833
	0.8211	0.1321	0.1763	0.0002	0.1841
	0.6754	0.1342	0.1896	0.0001	0.1854
Average	0.7278	0.1321	0.1848	0.0001	0.1843
stdev	0.0810	0.0022	0.0073	0.0001	0.0011
SEM	0.0468	0.0013	0.0042	0.0000	0.0006

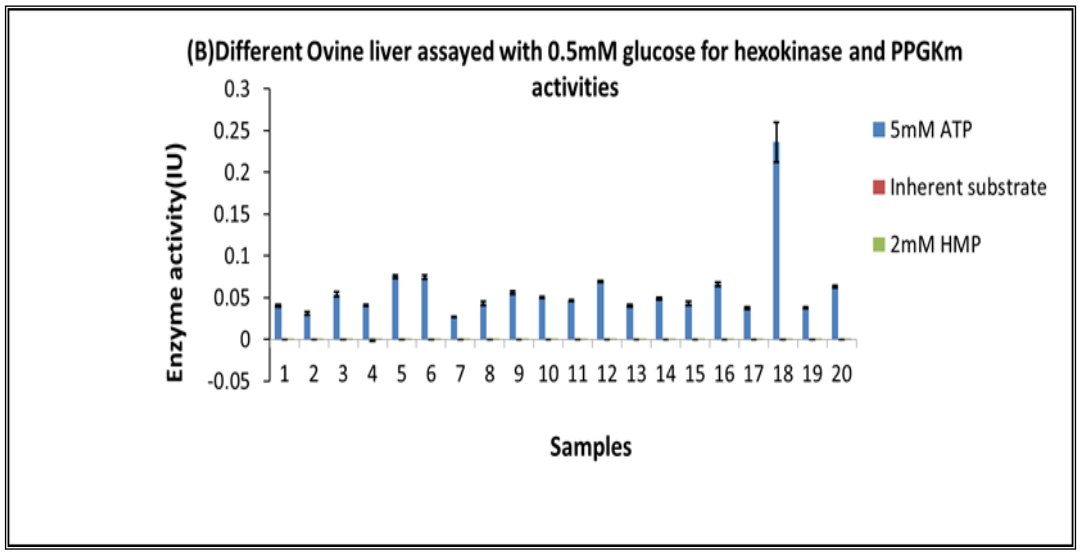


	Assay with 0.5mM glucose				
	Nucleii	Mitochondrial	Lysosomal	Microsomal	PF
ATP	0.0070	0.0131	0.0651	0.0000	0.1334
	0.0062	0.0143	0.0682	0.0000	0.1372
	0.0072	0.0136	0.0675	0.0000	0.1401
Average	0.0068	0.0137	0.0669	0.0000	0.1369
stdev	0.0005	0.0006	0.0016	0.0000	0.0034
SEM	0.0003	0.0003	0.0009	0.0000	0.0019
inherent substrat	0.0014	0.0002	0.0002	0.0002	0.0002
	0.0011	0.0002	0.0001	0.0002	0.0002
	0.0013	0.0002	0.0002	0.0003	0.0002
Average	0.0013	0.0002	0.0002	0.0002	0.0002
stdev	0.0002	0.0000	0.0000	0.0000	0.0000
sem	0.0001	0.0000	0.0000	0.0000	0.0000
HMP	0.0000	0.0000	0.0000	0.0000	0.0000
	0.0000	0.0000	0.0000	0.0000	0.0000
	0.0000	0.0000	0.0000	0.0000	0.0000
Average	0.0000	0.0000	0.0000	0.0000	0.0000
stdev	0.0000	0.0000	0.0000	0.0000	0.0000
SEM	0.0000	0.0000	0.0000	0.0000	0.0000



PPGKm activity assay in 20 sheep livers





PPGKm activity in 20 different ovine liver samples assayed with 100 mM glucose			
Sample	ATP	Inherent substrate	HMP
1	0.04440	0.36600	0.72100
	0.04530	0.38400	0.75300
	0.05430	0.35400	0.68900
Average	0.04800	0.36800	0.72100
stdev	0.00547	0.01510	0.03200
SEM	0.00316	0.00872	0.01848
2	0.03100	0.37620	0.85200
	0.03400	0.42130	0.90100
	0.04200	0.34210	0.85100
Average	0.03567	0.37987	0.86800
stdev	0.00569	0.03973	0.02858
SEM	0.00328	0.02294	0.01650
3	0.06390	0.23100	0.46100
	0.06850	0.24100	0.50100
	0.05780	0.23100	0.57100
Average	0.06340	0.23433	0.51100
stdev	0.00537	0.00577	0.05568
SEM	0.00310	0.00333	0.03215
4	0.07040	0.38600	0.74900
	0.07410	0.41030	0.91030
	0.06870	0.37950	0.75100
Average	0.07107	0.39193	0.80343
stdev	0.00276	0.01623	0.09255
SEM	0.00159	0.00937	0.05344
5	0.08500	0.14800	0.48200
	0.09120	0.15512	0.51213
	0.08861	0.14693	0.47612
Average	0.08827	0.15002	0.49008
stdev	0.00312	0.00445	0.01932
SEM	0.00180	0.00257	0.01115
6	0.09450	0.31520	0.75610
	0.10110	0.35100	0.76150
	0.09650	0.30140	0.81240
Average	0.09737	0.32253	0.77667
stdev	0.00338	0.02560	0.03106
SEM	0.00195	0.01478	0.01793
7	0.04700	0.04500	0.75110
	0.05200	0.05400	0.81250
	0.04900	0.04800	0.78910
Average	0.04933	0.04900	0.78423
stdev	0.00252	0.00458	0.03099
SEM	0.00145	0.00265	0.01789
8	0.06300	0.24800	0.52200
	0.06610	0.26130	0.55110
	0.06340	0.25620	0.50120
Average	0.06417	0.25517	0.52477
stdev	0.00169	0.00671	0.02506
SEM	0.00097	0.00387	0.01447
9	0.06600	0.26410	0.51410
	0.06830	0.28400	0.55100
	0.07130	0.26610	0.53150
Average	0.06853	0.27140	0.53220
stdev	0.00266	0.01096	0.01846
SEM	0.00153	0.00633	0.01066
10	0.08000	0.15410	0.65100
	0.07600	0.21030	0.71200
	0.07900	0.17620	0.66400
Average	0.07833	0.18020	0.67567
stdev	0.00208	0.02831	0.03213
SEM	0.00120	0.01635	0.01855

11	0.06600	0.12300	0.50700
	0.07100	0.14150	0.55120
	0.06800	0.12620	0.48900
Average	0.06833	0.13023	0.51573
stdev	0.00252	0.00989	0.03201
SEM	0.00145	0.00571	0.01848
12	0.08890	0.20800	0.45110
	0.09120	0.22100	0.47100
	0.07890	0.22500	0.50160
Average	0.08633	0.21800	0.47457
stdev	0.00654	0.00889	0.02544
SEM	0.00378	0.00513	0.01469
13	0.06000	0.12230	0.46500
	0.05891	0.13216	0.50132
	0.06210	0.12613	0.47751
Average	0.06034	0.12686	0.48128
stdev	0.00162	0.00497	0.01845
SEM	0.00094	0.00287	0.01065
14	0.06850	0.22300	0.44310
	0.07120	0.24200	0.47100
	0.06830	0.30100	0.45120
Average	0.06933	0.25533	0.45510
stdev	0.00162	0.04067	0.01435
SEM	0.00094	0.02348	0.00829
15	0.05290	0.21900	0.49700
	0.05510	0.22400	0.52150
	0.04860	0.25100	0.54110
Average	0.05220	0.23133	0.51987
stdev	0.00331	0.01721	0.02210
SEM	0.00191	0.00994	0.01276
16	0.08600	0.12700	0.32100
	0.09110	0.14200	0.36310
	0.08800	0.12810	0.33410
Average	0.08837	0.13237	0.33940
stdev	0.00257	0.00836	0.02154
SEM	0.00148	0.00483	0.01244
17	0.02700	0.21100	0.54210
	0.03230	0.24100	0.55810
	0.02910	0.21810	0.57410
Average	0.02947	0.22337	0.55810
stdev	0.00267	0.01568	0.01600
SEM	0.00154	0.00905	0.00924
18	0.23600	0.28720	0.59600
	0.22000	0.32100	0.63130
	0.18960	0.29860	0.58700
Average	0.21520	0.30227	0.60477
stdev	0.02357	0.01720	0.02342
SEM	0.01361	0.00993	0.01352
19	0.05790	0.56300	1.11000
	0.06210	0.58100	1.21000
	0.05890	0.55700	1.18000
Average	0.05963	0.56700	1.16667
stdev	0.00219	0.01249	0.05132
SEM	0.00127	0.00721	0.02963
20	0.07500	0.28100	0.56400
	0.08300	0.31650	0.60400
	0.07700	0.28810	0.58120
Average	0.07833	0.29520	0.58307
stdev	0.00416	0.01878	0.02007
SEM	0.00240	0.01085	0.01158

	With 0.5mM glucose			
	Sample	5mM ATP	Inherent substrate	2mM HMP
	1	0.04010	0.00002	0.00011
		0.03720	0.00002	0.00023
		0.04120	0.00001	0.00012
	Average	0.03950	0.00002	0.00015
	Stdev	0.00207	0.00000	0.00007
	SEM	0.00119	0.00000	0.00004
	2	0.03110	0.00001	0.00003
		0.02800	0.00002	0.00002
		0.03500	0.00001	0.00002
	Average	0.03137	0.00001	0.00003
	Stdev	0.00351	0.00001	0.00000
	SEM	0.00203	0.00000	0.00000
	3	0.05390	0.00001	0.00002
		0.04720	0.00001	0.00001
		0.05510	0.00001	0.00001
	Average	0.05207	0.00001	0.00001
	Stdev	0.00426	0.00000	0.00000
	SEM	0.00246	0.00000	0.00000
	4	0.04040	-0.00100	0.00001
		0.04410	-0.00110	0.00001
		0.04250	-0.00200	0.00001
	Average	0.04233	-0.00137	0.00001
	Stdev	0.00186	0.00055	0.00000
	SEM	0.00107	0.00032	0.00000
	5	0.07512	0.00004	0.00013
		0.08102	0.00003	0.00016
		0.07814	0.00004	0.00016
	Average	0.07809	0.00004	0.00015
	Stdev	0.00295	0.00001	0.00002
	SEM	0.00170	0.00000	0.00001
	6	0.07450	-0.00001	0.00001
		0.08131	-0.00001	0.00002
		0.07842	-0.00002	0.00002
	Average	0.07808	-0.00001	0.00002
	Stdev	0.00342	0.00000	0.00001
	SEM	0.00197	0.00000	0.00000
	7	0.02700	-0.00001	0.00000
		0.02400	-0.00001	0.00001
		0.03100	-0.00001	0.00001
	Average	0.02733	-0.00001	0.00001
	Stdev	0.00351	0.00000	0.00000
	SEM	0.00064	0.00000	0.00000
	8	0.04324	-0.00002	0.00001
		0.04700	-0.00001	0.00002
		0.03813	-0.00002	0.00002
	Average	0.04279	-0.00002	0.00002
	Stdev	0.00445	0.00000	0.00000
	SEM	0.00257	0.00000	0.00000
	9	0.05600	-0.00002	0.00003
		0.06200	-0.00001	0.00002
		0.05800	-0.00002	0.00003
	Average	0.05867	-0.00002	0.00003
	Stdev	0.00306	0.00000	0.00001
	SEM	0.00176	0.00000	0.00000
	10	0.05000	-0.00002	0.00001
		0.04912	-0.00003	0.00001
		0.04631	-0.00002	0.00002
	Average	0.04848	-0.00002	0.00001
	Stdev	0.00193	0.00000	0.00001
	SEM	0.00111	0.00000	0.00000

11	0.04600	0.00003	0.00002
	0.04841	0.00002	0.00003
	0.04510	0.00003	0.00002
Average	0.04650	0.00003	0.00002
Stdev	0.00171	0.00000	0.00000
SEM	0.00099	0.00000	0.00000
12	0.06890	0.00001	0.00004
	0.07210	0.00002	0.00003
	0.06680	0.00001	0.00004
	0.06927	0.00001	0.00004
Average	0.00267	0.00001	0.00001
Stdev	0.00154	0.00000	0.00000
SEM	0.00089	0.00000	0.00000
13	0.04321	-0.00001	0.00003
	0.03900	-0.00002	0.00004
	0.03611	-0.00002	0.00004
Average	0.03944	-0.00002	0.00004
Stdev	0.00357	0.00000	0.00000
SEM	0.00206	0.00000	0.00000
14	0.04850	-0.00001	0.00003
	0.05120	-0.00002	0.00003
	0.04621	-0.00001	0.00003
Average	0.04864	-0.00001	0.00003
Stdev	0.00250	0.00000	0.00000
SEM	0.00144	0.00000	0.00000
15	0.04290	-0.00002	0.00001
	0.04410	-0.00002	0.00002
	0.05131	-0.00002	0.00001
Average	0.04610	-0.00002	0.00001
Stdev	0.00455	0.00000	0.00001
SEM	0.00263	0.00000	0.00000
16	0.06600	-0.00002	0.00002
	0.06821	-0.00003	0.00003
	0.07311	-0.00002	0.00003
Average	0.06911	-0.00002	0.00003
Stdev	0.00364	0.00000	0.00001
SEM	0.00210	0.00000	0.00000
17	0.03700	-0.00002	0.00004
	0.04430	-0.00003	0.00004
	0.03521	-0.00003	0.00005
Average	0.03884	-0.00003	0.00004
Stdev	0.00481	0.00000	0.00000
SEM	0.00278	0.00000	0.00000
18	0.23600	0.00003	0.00003
	0.25311	0.00002	0.00004
	0.31523	0.00003	0.00003
Average	0.26811	0.00003	0.00003
Stdev	0.04169	0.00000	0.00000
SEM	0.02407	0.00000	0.00000
19	0.03790	0.00001	0.00001
	0.04112	0.00002	0.00001
	0.03855	0.00001	0.00001
Average	0.03919	0.00001	0.00001
Stdev	0.00170	0.00001	0.00000
SEM	0.00098	0.00000	0.00000
20	0.06300	-0.00001	0.00003
	0.06816	-0.00002	0.00002
	0.06641	-0.00002	0.00003
Average	0.06586	-0.00002	0.00003
Stdev	0.00262	0.00000	0.00000
SEM	0.00152	0.00000	0.00000