

**UNIVERSITY OF KWAZULU-NATAL**

**Localization and Partial  
Purification of Phosphate  
Dependent Glutaminase from  
Rat Small Intestine**

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# **Localization and Partial Purification of Phosphate Dependent Glutaminase from Rat Small Intestine**

**BY**

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**Submitted in fulfilment of the requirements for the degree of Master of  
Science in the Discipline of Biochemistry, School of Life Sciences,  
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Small Intestine”**

is the result of my own investigation and research and that it has not been submitted in part or in full for any other degree or to any other university. Where use of the work of others was made, it is duly acknowledged in the text.

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**“UJEHOVA UNGUMALUSI OMUHLE, ANGIYI KWESHWELA...”** Amahubo 23

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## LIST OF ABBREVIATIONS

A	Alpha
B	Beta
$\Delta$	Gamma
$\mu$	Micro
ADP	Adenosine 5'-diphosphate
AMP	Adenosine 5'- monophosphate sodium salt monohydrate
ATP	Adenosine 5'-triphosphate
BRU	Biomedical Resource Unit
BSA	Bovine serum albumin
CMC	Critical micelle concentration
CPSI	Carbamylphosphate synthetase I
DEAE-Sepharose	Diethylaminoethyl–Sepharose
GAC	Glutaminase C
GDH	Glutamate dehydrogenase
GI	Gastrointestinal tract
H <sub>2</sub> O	Water
HCl	Hydrochloric acid
$\alpha$ -KG	$\alpha$ -Ketoglutarate
kDa	KiloDalton
KGA	Kidney-type glutaminase
K <sub>m</sub>	Michaelis-Menten constant
LGA	Liver-type glutaminase
MCF-7	Breast cancer cells
Mers	Mersalyl
MPP	Matrix processing peptidase
mRNA	Messenger ribonucleic acid
NAD <sup>+</sup>	Beta-Nicotinamide adenine dinucleotide
NADH	Beta-Nicotinamide adenine dinucleotide, reduced form
NADP <sup>+</sup>	Beta-Nicotinamide adenine dinucleotide phosphate
NEM	N-Ethylmaleimide

NH <sub>3</sub>	Ammonia
NH <sub>4</sub> <sup>+</sup>	Ammonium ion
PDG	Phosphate dependent glutaminase
P <sub>i</sub>	Phosphate
SDS-PAGE	Sodium-dodecyl sulphate-polyacrylamide gel electrophoresis
spp.	Species
TCA	Tricarboxylic acid cycle

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## ABSTRACT

Phosphate dependent glutaminase (PDG) is a key enzyme in intestinal energy metabolism and hence has an impact on nutrient absorption and nutritional status of the whole animal. PDG is known to be a mitochondrial enzyme but its sub-mitochondrial location is still controversial. Due to its instability, PDG has never been purified from the small intestine. The sub-mitochondrial localization of PDG was investigated by tracking the release of PDG and that of marker enzymes for sub-mitochondrial compartments following fractionation of mitochondria using digitonin. The dependence of PDG activity on the membrane phospholipids was investigated using phospholipase A<sub>2</sub> treatment while the orientation of the enzyme in mitochondria was probed using sulphhydryl inhibitors Mersalyl (Mers) and N-ethylmaleimide (NEM). PDG was partially purified after solubilizing PDG using different methods including lyophilization combined with digitonin fractionation and sonication in the presence of a stabilizing buffer. Solubilized proteins were separated by gel filtration chromatography. SDS-PAGE and Western blotting were conducted on fractions collected to show protein profiles and location of PDG bands. Release of PDG was intermediate between that of cytochrome c oxidase and matrix enzymes. Phospholipase A<sub>2</sub> treatment exhibited a time dependent loss of PDG activity. In intact mitochondria Mers inhibited 97% and NEM inhibited 64% of PDG activity at 1 mM concentration; with a more pronounced effect when combined with sonication. Pre-incubation of mitochondria in a stabilizing buffer before solubilization activated PDG 37-fold. Partial purification was achieved after using Sephacryl S-300 HR. Coomassie stained SDS-PAGE confirmed the partial purification of PDG with bands on Western blot observed to be 63-65 kDa, 50 kDa and 42 kDa. In conclusion, the results suggest that intestinal PDG is localized in two sub-mitochondrial fractions with each displaying a different form: PDG with a molecular weight of 50 kDa being localized in the mitochondria matrix and a 63-65 kDa PDG being bound to the mitochondria inner membrane. The membrane bound PDG requires the presence of phospholipids to retain its activity.

# CHAPTER 1

## 1. Literature review

### 1.1 Introduction

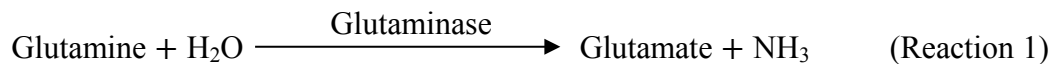
Phosphate dependent glutaminase (PDG) is an enzyme which catalyses the hydrolysis of glutamine. Glutamine is the most abundant amino acid, found in almost all organs within the body. The body uses by-products generated through glutamine catabolism as precursors for the synthesis of biomolecules and to generate energy. The major site for glutamine degradation in the body is the small intestine which uses glutamine as the primary source of energy generation. Any malfunction that occurs in the small intestine that affects energy production also directly impacts the capability of the intestine to absorb nutrients. Even though PDG plays such a crucial role in the small intestine little is known about its localization within the mitochondria and its chemical structure; a characteristic which is crucial in studying the regulation of PDG under metabolic conditions that affect the functioning of this enzyme. It has been shown that under different metabolic disorders the activity of glutaminase may be suppressed or increased. In this study we aim to localize PDG within mitochondrial compartments and purify intestinal PDG in an active form.

### 1.2 Physiological role of glutamine

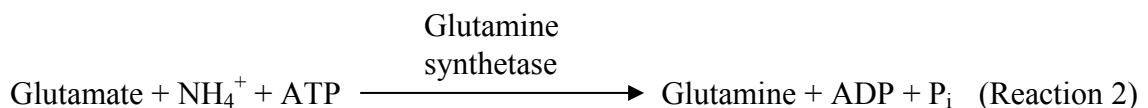
Glutamine is the most abundant amino acid in the blood stream and is actively transported and metabolized in nearly all tissues. It is found in plasma at a concentration of 0.5-0.8 mM (20%-25% of circulating free amino acid) while its concentration in skeletal muscle is greater than 20 mM (Souba *et al.*, 1990b). Glutamine is part of the amino acid pool in muscle, adipose, lungs, brain, intestine, kidneys, liver and rat lenses (Rudermann and Lund, 1972; Curthoys and Watford, 1995; Labow and Souba, 2000). The amino acid is essential for the growth of rapidly dividing cells that include intestinal mucosa, cells of the immune system, cancer cells and foetal skin fibroblast (Calder and Yaqoob, 1999; Reeds and Burrin, 2001; Vermeulena *et al.*, 2008; Carr *et al.*, 2010; Hensley *et al.*, 2013). However, the major glutamine utilizing cells and organs are enterocytes, macrophages, lymphocytes (Newsholme

*et al.*, 1985; Grohmann and Bronte, 2010), the kidney during metabolic acidosis (Poll *et al.*, 2004) and the mammary gland during lactation (Manso *et al.*, 2012).

Within cells glutamine is utilized in maintaining the cellular redox state by regulating glutathione synthesis (an antioxidant that prevents cellular damage by binding reactive oxygen species) (Labow and Souba, 2000). Glutamine is also used to supply nitrogen and carbon for synthesis of macromolecules and metabolic intermediates (Watford, 2000) (Figure 1) and is involved in the maintenance of gut integrity, metabolism and structure (Lacey and Wilmore, 1990; Souba *et al.*, 1990a; Elia, 1992) (Figure 1). The amino acid also serves as an energy source through the conversion of glutamine to glutamate to  $\alpha$ -ketoglutarate ( $\alpha$ -KG) which is oxidized in the TCA (tricarboxylic acid cycle) (Windmueller and Spaeth, 1974). Glutamine is categorized as a non-essential amino acid which may be termed conditionally essential when the body is under stress (Elia, 1992; Lacey and Wilmore, 1990). The first enzyme involved in the catabolism of glutamine is glutaminase. Phosphate dependent glutaminase (L-glutamine amidohydrolase; E.C number 3.5.1.2) is an enzyme localized in mitochondria that catalyzes the hydrolytic deamidation of glutamine to yield ammonia and glutamate as shown in Reaction 1 (Watford, 2000).



Under normal conditions in the body glutamine is not acquired from dietary intake but rather synthesized by glutamine synthetase (E.C 6.3.1.2) which converts ammonia and glutamate in the presence of ATP to glutamine (Reaction 2).



Glutamine synthetase is a cytosolic enzyme that is expressed in most cells. It is most abundant in tissues that contain low levels of glutaminase these being muscle, lungs and adipose tissue which release glutamine into the blood. During the post-absorptive state in humans, glutamine has a turnover rate of 350  $\mu\text{mol/kg/h}$  which is almost like that of glucose

(Gerich *et al.*, 2000). The main site for glutamine synthesis is the muscle, this is not due to a large amount of activity which can be recovered in this organ but it is due to its size (Watford, 1993). The liver is the second main site where glutamine is synthesized (Watford, 1993). Plasma glutamine homeostasis is maintained by balancing the release of glutamine from the muscle, adipose tissue and lungs and uptake by the splanchnic bed which supplies the small intestine and liver (Stumvoll *et al.*, 1999).

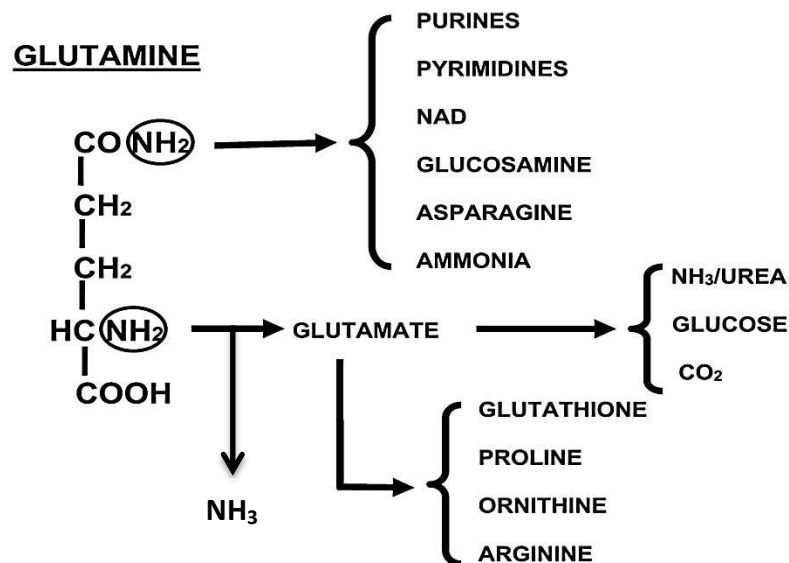


Figure 1: The fate of glutamine in the body (Watford, 2000).

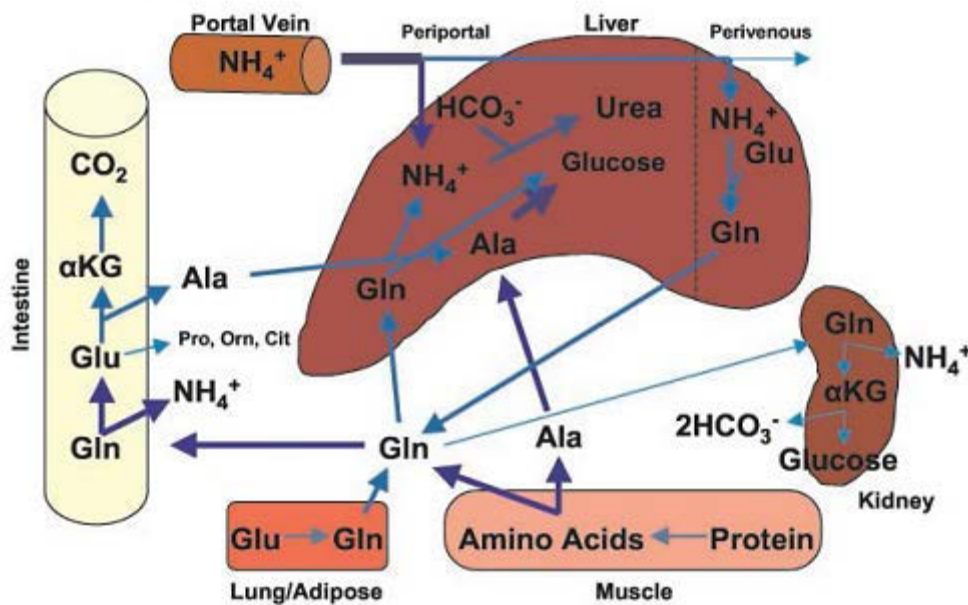
The only organ that does not release the by-product ammonia to the plasma after catabolism of glutamine is the liver, it utilizes ammonia in the ureagenesis (Watford, 2000). Other organ systems like the muscle release ammonia to the plasma; this ammonia is taken up by the liver and synthesized into urea. Watford (1993) proposed that the fate of the ammonia released after catabolism of glutamine is dependent on the type of glutaminase protein that is present in that tissue. In the presence of kidney-type glutaminase (KGA) most of the amide ammonia is released from the cells. The pathway that glutamine end products will follow is tissue-dependent, for example, in the kidney both nitrogen groups are released as ammonia and the carbon skeleton is directed to gluconeogenesis (Watford, 1993).

## **1.3 Glutamine metabolism in tissues**

### **1.3.1. Glutamine metabolism in the liver**

A major role of the liver is to detoxify the ammonium ions that are generated by the catabolism of amino acids coming through the portal vein. A large fraction of glutamine turnover occurs in the epithelial cells of the small intestine producing a significant amount of ammonium ions (Watford, 2000). These ions are drained into the portal vein and directed to the liver as shown in Figure 2 below. The liver glutaminase (LGA) and enzymes of urea synthesis are most abundant in the periportal hepatocytes which are located near the portal venule (Watford, 2000), while glutamine synthetase is located in the perivenous hepatocytes; the outer most layer that surrounds the hepatic venule. As a result of this distribution of enzymes along the portal venule, ammonium ions are first converted into urea in the periportal hepatocytes and the remainder channelled to the perivenous hepatocytes to be used for glutamine synthesis (Taylor and Curthoys, 2004b).

This continuous synthesis of urea and glutamine is an efficient system which ensures that systemic levels of ammonium ions are maintained at non-toxic levels (Häussinger, 1998). The ammonium ions produced during intestinal glutamine catabolism act in a feed forward activation of LGA to ensure the appropriate mitochondrial synthesis of glutamate and N-acetylglutamate in the liver (Brosnan and Brosnan, 2002). This apparent contradiction that ammonia would stimulate the production of more ammonia was resolved by Brosnan and Brosnan (2002) who explained that glutamate is the required product which is further used in the synthesis of N-acetylglutamate; an obligatory activator of carbamylphosphate synthetase I (CPSI). Activation of CPSI increases urea synthesis thus removing the excess ammonia from the system. Hence this pathway is important in maintaining non-toxic levels of ammonium ions and urea cycle activity (Taylor and Curthoys, 2004b). The usage and synthesis of glutamine is also affected by an acidosis, no alterations to glutamine synthetase activities are acquired but a change in release and uptake of glutamine is observed (Watford, 2000). During acidosis the liver decreases the rate of glutamine degradation and urea synthesis, such an alteration results in the liver releasing, rather than taking glutamine. These changes are followed by an increase in removal of glutamine by the kidneys (Taylor and Curthoys, 2004a).



**Figure 2: Inter-organ metabolism of glutamine under physiologic circumstances. The thickness of the arrow represents the relative influx of the metabolite (Taylor and Curthoys, 2004b). Ala - alanine, Gln - glutamine, Glu - glutamate,  $\text{HCO}_3^-$  - bicarbonate ion,  $\alpha\text{KG}$  -  $\alpha$ -ketoglutarate, Pro - proline, Orn - ornithine, Cit - citrulline and  $\text{NH}_4^+$  - ammonium ions.**

### 1.3.2. Glutamine metabolism in the kidney

Glutamine is by far the most important donor of ammonia in the kidney (Newsholme *et al.*, 2003a). The glutamine in the kidney is transported across the apical brush border membrane and most of it is later returned to the blood across the basolateral membrane (Curthoys, 2001). During normal acid-base balance the kidney utilizes a small portion of plasma glutamine. The small fraction of glutamine extracted by the kidney is transported into the mitochondria for hydrolysis by glutaminase. Under normal physiological conditions the ammonia is exported to the lumen of the collecting tubules where it combines with hydrogen to form ammonium which is excreted in urine (Newsholme *et al.*, 2003a). The hydrogen that combines with ammonia is derived from the dissociation of carbonic acid into hydrogen and bicarbonate which is essential in maintaining physiological pH in blood. Hence the kidney is amongst other organs that are important in maintaining plasma homeostasis (Curthoys, 2001). The glutamate formed is converted to  $\alpha$ -Ketoglutarate ( $\alpha$ -KG) *via* transamination. This  $\alpha$ -KG is then oxidized by the TCA cycle to malate which is either converted to pyruvate *via*

NADP<sup>+</sup>-dependent malic enzyme or converted to oxaloacetate *via* malate dehydrogenase. These by-products are then directed into gluconeogenesis and the glucose produced provides up to 25% of circulating plasma glucose *in vivo* (Newsholme *et al.*, 2003a).

Changes in the inter-organ flux of glutamine are triggered at the onset of metabolic acidosis by inducing an increase in renal uptake and breakdown of glutamine. Within 1-3 hours of metabolic acidosis onset, the arterial plasma glutamine concentrations in rats is increased two-fold (Hughey *et al.*, 1980) due to release of glutamine by the skeletal muscle. The kidney therefore plays an important role of ammonia disposal in this situation. The total amount of nitrogen excreted in the urine as urea and ammonia does not change significantly during acidosis. In this situation urinary ammonia excretion is enhanced while urea excretion diminishes (Poll *et al.*, 2004). Nussbaum and Berry (1996) suggested that nitrogen disposal remains unchanged probably due to diminished urea synthesis in the liver.

### **1.3.3 Glutamine metabolism in the brain**

The key role of glutaminase in the central nervous system is to produce glutamate, a principal excitatory neurotransmitter. Glutamate is responsible for promoting the synthesis of GABA (gamma-Aminobutyric acid) which is an important brain neurotransmitter. It is also involved in normal brain functions including memory and learning (Hawkins, 2009). Glutamate does not cross the blood-brain barrier; therefore the pool of glutamate found in neurons is derived from glutamine that is synthesized within the brain by astrocytes (Hawkins, 2009). Astrocytes *via* glutamine synthetase convert glutamate into glutamine. Glutamine is released into the extracellular region where it can be used by neurons as a precursor for glutamate. Glutamate is formed through the action of glutaminase; this glutamate is later captured by the astrocytes and converted back to glutamine. Thus a glutamate - glutamine shuttle existing between the astrocytes and neurones is responsible for recycling this neurotransmitter which is continuously released and removed from the extracellular fluid (Márquez *et al.*, 2009). A great pool of glutamate is found in the brain, with a small fraction on the outside or in between brain cells while the highest concentrations are found inside nerve terminals (Danbolt, 2001). An equilibrium between glutamine and glutamate distribution must always exist since the latter, at high extracellularly concentration, is toxic and results in complications in the brain (Bak *et al.*, 2006). Rat brains have been shown to express liver-type glutaminase (LGA), glutaminase B (GAB) and kidney-type glutaminase (KGA), the

latter being predominant at greater than 93% in both mouse and rat brain (Márquez *et al.*, 2013). In the brain the LGA is reported to be localized in the nuclei of neurons while KGA is dominant in the mitochondria. It has been suggested that LGA in the nuclei might be serving as a transcriptional co-regulator (Szeliga *et al.*, 2008)

#### **1.3.4 Glutamine metabolism by cancer cells**

The metabolism of glutamine by cancer cells has received much attention over the years. Numerous studies have reported glutaminase as a potential target that may be suppressed to control cancer proliferation. It has been widely reported that cancer cells utilize large quantities of glucose and glutamine compared to normal cells. The carbon skeleton produced after metabolism of glucose and glutamine is used mainly in the production of lactate and the remainder is used for the synthesis of ATP and other biomolecules (such as those highlighted in Figure 1) (Macintyre and Rathmell, 2013). Cancer cells were described by Warburg *et al.* (1927) as cells that utilized more glucose compared to normal cells exhibiting a high rate of glycolysis, producing lactate even in the presence of oxygen. Different methods which have been investigated as potential suppressors of cancer cell proliferation are presented below. The prominent involvement of glutamine in cancer cell metabolism presents the possibility to develop anti-cancer treatment by targeting oncogenes and tumour suppressors which are involved in metabolism of glutamine (Rajagopalan and DeBerardinis, 2011). Zhao *et al.* (2013) reported that the drug resistance observed in cancer cells may be due to abnormal metabolism, hence the need to target metabolic enzymes for therapeutic potential so as to increase the effectiveness of currently existing cancer treatments.

KGA and LGA glutaminase isoforms share 81% similarity of their core amino acid sequence. This characteristic is being exploited in development of specific allosteric inhibitors that will selectively target the heterogeneous regions of glutaminase in order to regulate cancer cell proliferation (Lee *et al.*, 2014). Some exploited allosteric inhibitors of glutaminase include BPTES [Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulphide], 6-Diazo-5-oxo-L-norleucine (DON) and dibenzophenanthridine-968. Cassagoa *et al.* (2012) reported that BPTES inhibited glutaminase C (GAC) by binding an allosteric site therefore securing GAC in a non-functional tetramer conformation. He also showed that BPTES at this allosteric site specifically interacts with residues Phe 322 and Tyr 394 since point mutations to these site caused BPTES to be ineffective as an inhibitor. Lee *et al.* (2014) reported another allosteric

inhibitor dibenzophenanthridine-968 which decreased the activity of GAC but upon conducting a two point mutation of residues Arg 539 and Phe 532 exhibited less inhibition effects on GAC activity. Lee *et al.* (2014) also reported AV-1 allosteric inhibitors which were natural alkyl benzoquinones which bonded at different KGA and GAB allosteric sites compared to GAC allosteric sites bonded by dibenzophenanthridine-968 and BPTES. Other inhibitors of glutaminase include 6-Diazo-5-oxo-L-norleucine (DON) and acivicin their mechanism of action is to covalently modify residues which are located in the active site but these have been shown to be toxic (Ahluwalia *et al.*, 1990).

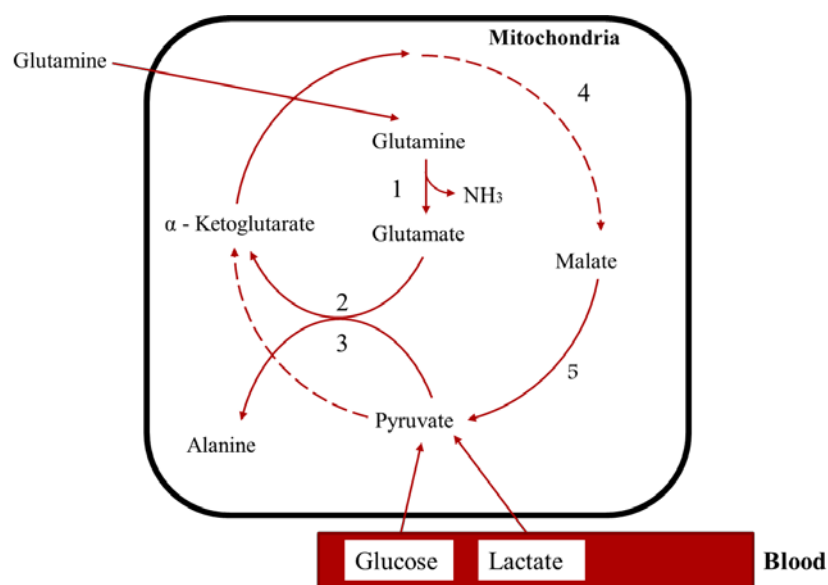
The rate of GAC in some cancer cells are dependent on the abnormal oncogene MYC. This oncogene in cancer cells induces a network of transcriptional responses associated with cell growth and proliferation which are altered when compared with normal cells (Dang, 2013). Glutamine metabolism was shown to correlate to an increase in the rate of glutaminase activity in cancer cells (Perez-Gomez *et al.*, 2005). Durán *et al.* (2012) reported the survival of cancer cell is through activating mTORC1 (mammalian target of rapamycin complex 1) signalling pathway which is involved in stimulating cell growth while at the same time inhibiting degradation of damaged or unwanted cellular components by lysosomes. Hu *et al.* (2010) concluded that glutamine in combination with leucine has a dual role of activating the mTORC1 and providing the TCA cycle with precursors. Some other evidence suggests that glutamine is used by the cell to adapt to acidic environments. Huang *et al.* (2013) have suggested that cancer cells use glutamine for acid resistance rather than providing energy. Such results showcase the dynamic role of glutaminase in cancer cells metabolism and how it has a potential as a target in cancer therapeutic drug design.

### **1.3.5 Glutamine metabolism in the small intestine**

Glutamine is a major respiratory fuel in the small intestine, approximately 35-77% CO<sub>2</sub> released by the small intestine is derived from this amino acid, depending on the metabolic state of the animal (Windmueller and Spaeth, 1974; James *et al.*, 1998; Langhans, 2010; Davila *et al.*, 2013). The small intestine plays a critical role in the absorption of nutrients from food which provides essential amino acids for synthesis of biomolecules while also providing substrates for the liver and other organs (Salloum *et al.*, 1991; Newsholme *et al.*, 2003b). Inadequate absorption of nutrients may lead to nitrogen imbalances, loss of weight and slow biosynthesis of biomolecules. Arterio-venous difference measurements in fasted

rats revealed that most circulatory glutamine is taken up by organs that are drained by the hepatic-portal vein. These organs are the large and small intestine, spleen, caecum, stomach and pancreas but mainly the small intestine (Windmueller and Spaeth, 1974).

The mucosal epithelium in the small intestine acts as a protective barrier against harmful substances. When the barrier integrity is compromised either due to starvation, infection, chemotherapy or other conditions harmful substances cross the mucosal barrier and stimulate immune response and inflammation. Glutamine supplements may be used as therapy to improve the health state in animals this is achieved by increasing splanchnic circulation, reducing atrophy of the intestine and reducing infections through repairing the leaky mucosal epithelium (Houdijk *et al.*, 1994; De-Souza and Greene, 2005; Rao and Samak, 2012). Under normal circumstances the intestine utilizes endogenous glutamine that is synthesized in the muscle since the intestine has low glutamine synthesizing capabilities (Rao and Samak, 2012). In the small intestine, as is the case in other organs, glutamine is hydrolysed to glutamate and ammonia. The resulting glutamate is used to transaminate pyruvate by alanine aminotransferase catalysis to produce  $\alpha$ -KG and alanine. The  $\alpha$ -KG is channelled to the TCA cycle while the alanine is transported *via* the hepatic portal vein to the periportal hepatocytes. The  $\alpha$ -KG also results in the production of malate, which *via* NADP<sup>+</sup>-dependent malic enzyme, produces pyruvate. Along the span of TCA cycle (Figure 3) NADH and FADH<sub>2</sub> are produced, these produce ATP through oxidative phosphorylation.



**Figure 3: Metabolism of glutamine in the intestine mucosa cell. 1: Glutaminase, 2: Glutamate dehydrogenase, 3: Alanine aminotransferase, 4: Span of TCA cycle reactions and 5: NADP<sup>+</sup> dependent malic enzyme (Newsholme *et al.*, 2003a) with modifications.**

Masola and colleagues (1985) have suggested that glucose and lactate in the blood are the main sources of pyruvate used for alanine synthesis. While Newsholme *et al.* (2003b) reported that the major end product after glutamine metabolism in the intestine is alanine. Within the gastro-intestinal tract (GI) approximately 90% glutamine synthesis occurs in the stomach but not much glutamine is recovered within this organ. James and colleagues (1998b) suggested that since glucose is the preferred energy source in the stomach glutamine is either channelled out to other parts of the gastrointestinal tract (GI) or is either rapidly used within the stomach.

#### **1.4 Glutaminase**

In 1935 Krebs first described glutaminase-type proteins (isoforms) based on their kinetic differences and tissue distribution as KGA and liver-type glutaminase (LGA). KGA is expressed mostly in the brain, kidney, lymphocytes, foetal liver cells and intestinal epithelial cells (Elgadi *et al.*, 1999) whereas LGA is expressed in the brain, pancreas and the periportal hepatocytes of the post-natal liver (Gomez-Fabre *et al.*, 2000). Expression of the enzyme is from distinct but structurally related genes (Curthoys and Watford, 1995). The KGA gene is located on chromosome 2, while the LGA gene is located on chromosome 12. In mammals KGA is expressed in two isoforms. KGA, which was first described in the human kidney and a splice variant of KGA glutaminase C (GAC) which was first described in human kidney and cancerous colon cells. These isoforms are composed of 19 and 15 exons respectively (Elgadi *et al.*, 1999; Porter *et al.*, 2002). LGA is also expressed in two isoforms, glutaminase B (GAB) and LGA which are composed of 18 and 17 exons, respectively (Martín-Rufián *et al.*, 2012).

The lengths of exons 3 to 17 of the human KGA gene are identical to the corresponding exons for the human LGA gene (Porter *et al.*, 2002). The conserved exons on the KGA and LGA genes give rise to nucleotide sequences which share a 69% similarity and encode amino acid sequences that are 77% identical. A significant difference is observed in their C-terminal regions where KGA and LGA share only 33% similarity (Taylor and Curthoys, 2004b). The first 16 amino acids encoded by these genes form an amphipathic  $\alpha$ -helix that functions as a mitochondrial targeting sequence. A truncated form of rat recombinant KGA glutaminase that lacks a C-terminal was found to retain full activity (Kenny *et al.*, 2003). Taylor and Curthoys (2004) hypothesised that the role of the distinct C-terminal domains of KGA is to act as a

protein-binding domain that leads to association with an anchoring protein of the inner mitochondrial membrane or with different enzymes of glutamate metabolism. This is supported by the findings of Ollala *et al.* (2001) who found that the C-terminus of the human LGA binds to a unique glutaminase-interacting protein.

Porter *et al.* (2002) also showed that the mature 66 kDa form lacks a targeting sequence that would initiate the translocation of the mature subunit to the intermembrane space. The human GAC mRNA contains a unique C-terminus coding sequence and a 3'-untranslated region (Porter *et al.*, 2002). GAC also contains the same sequence transcribed from exons 1 to 14. The sequence which is coded by exon 15 distinguishes KGA from GAC (Porter *et al.*, 2002). KGA contains a unique 133 amino acid sequence on the N-terminal (Shapiro *et al.*, 1991). A 74 kDa renal glutaminase mRNA is processed to 72 kDa from which mature glutaminase proteins of 63 kDa and 66 kDa are synthesized in 1:3 ratio, respectively (Svinivasan *et al.*, 1995). It was speculated that the different glutaminase proteins may arise due to alternative proteolytic processing or initial synthesis of the 66 kDa subunit followed by its covalent modification to generate the 63 kDa subunit.

Svinivasan and colleagues (1995) suggested that the 63 kDa subunit is not produced by covalent modification of a 66 kDa subunit, but rather the mature subunits of glutaminase are produced by alternative processing reactions catalysed by a matrix processing peptidase (MPP). During synthesis of proteins destined for the mitochondria the protease MPP is responsible for cleaving the N-terminal from cytosolic precursors which allows them to bind to the outer mitochondrial membrane. The precursors are then translocated into the mitochondria (Svinivasan *et al.*, 1995). Shenoy *et al.* (1996) reported that in the small intestine of rats the expression of glutaminase is dependent on the maturity of the rat. They showed (using protein immunoblots) that in the proximal region of the small intestine during the foetal stage, bands of 65 and 63 kDa were detected. After 10 days and 19 days only a 65 kDa band was expressed; and after 32 days bands of 65, 68 and 72 kDa were detected. While in the distal region of the small intestine during the foetal stage only bands of 65 and 63 kDa were detected and after 10, 19 and 32 days only the 72 kDa band was detected as a faint band, and its intensity increased with time (Shenoy *et al.*, 1996).

### 1.4.1 Regulation of glutaminase by ions and metabolites

Calcium has been found to be an indirect activator of phosphate dependent glutaminase (PDG) in renal mitochondria (Kvamme *et al.*, 1982) and the brain cortex (Bradford *et al.*, 1984) but exhibits no activation profile for purified PDG. This activation was thought to be through calcium binding to an unidentified compound which in turn enhances the binding of phosphate (Kvamme *et al.*, 2001). Kvamme and colleagues (2001) suggested that this compound could be an acyl CoA derivative or a long chain fatty acid since they demonstrated that certain fatty acyl CoA derivatives activate PDG at low concentrations but inhibit the enzyme at higher concentrations. Brain and kidney glutaminases have been shown to be allosteric enzymes with different effectors. In the presence of high phosphate concentrations and optimal assay conditions (pH and glutamine concentration) the inhibition of PDG by sulphhydryl inhibitors, N-Ethylmaleimide (NEM) and Mersalyl (Mers), is terminated in intact pig renal mitochondria (Kvamme *et al.*, 1991), which is the opposite to what was found with purified PDG. The termination was thought to be due to the high phosphate concentrations altering the conformation of glutaminase to a higher molecular weight form (activated form) that is not sensitive to the sulphhydryl reagents (Kvamme *et al.*, 2001).

The phosphate activated KGA of mammals is stimulated by inorganic phosphate and alkaline pH and inhibited by the product glutamate (Pinkus and Windmueller, 1977). The most potent physiological inhibitors of mammalian PDG are the products glutamate and ammonia (Kvamme *et al.*, 1988) which are thought to act by promoting depolymerisation of the active tetrameric form of the enzyme (Curthoys and Watford, 1995). Márquez *et al.* (2013) showed that phosphate activated KGA is inhibited by glutamate unlike the LGA. Cassagoa *et al.* (2012) reported that phosphate competes with the by-product glutamate to bind active site to avoid GAC activity inhibition. PDG in intact mitochondria and membrane bound fraction is less sensitive to inhibition by glutamate than the solubilized PDG from rat kidney (Roberg *et al.*, 2000). Ions that also influence the activity of intestinal PDG include bicarbonate, sulphate and phosphate ions (Pinkus and Windmueller, 1977; Masola and Zvinavashe, 2003) but none has been found to be as good an activator in intact intestinal mitochondria as ADP (Masola and Ngubane, 2010). The exact mechanism of how ADP regulates the PDG activity in intact intestinal mitochondria is not known but is thought to be a response to low energy levels in the intestinal mucosa (Masola and Ngubane, 2010). The activation of PDG by ATP (adenosine triphosphate) is through the lowering of phosphate required for half-maximal

activity of the enzyme; this is evident of glutaminases isolated from the liver, heart (Curthoys *et al.*, 1976b; Nelson *et al.*, 1994) and the islet of Langerhans (Michalik *et al.*, 1992).

Phosphate, sulphate and bicarbonate ions are speculated to cause activation of the intestinal glutaminase by inducing dimer formation (Masola and Zvinavashe, 2003) since dimeric KGA is known to have a high  $K_m$  for glutamate and low  $K_m$  for glutamine. Phosphate is the most common activator of PDG producing half-maximal activity at 20-30 mM concentration, however; the activation process has shown a broad specificity (Curthoys *et al.*, 1976b). Masola and Ngubane (2010) reported that the enzyme from intact intestinal mitochondria is activated differently at different phosphate concentrations but all phosphate concentrations showed maximal activation at pH 8.6. PDG requires the presence of a polyvalent anion for enhanced activity. In the absence of a polyvalent anion, phosphate-activated brain KGA is a partially active protomer but addition of a polyvalent anion leads to a more active dimer (Godfrey *et al.*, 1977). Cassagoa *et al.* (2012) reported that GAC in a solution which either contained or did not contain phosphate existed as a dimer and tetramer contrary to what has been reported in other studies.

They concluded that tetramerization of GAC is phosphate concentration dependent and that phosphate increases the affinity for glutamine to bind into the cationic active site by opening the gating loop (Cassagoa *et al.*, 2012). The activity of PDG is also highly dependent on the stability of the enzyme and it has been shown that borate stabilizes PDG (Durá *et al.*, 2002). Klingman and Handler (1958) showed that upon addition of borate, phosphate-activated KGA exhibited properties of being stable and in a purified form had a 3-fold higher activity than the labile low molecular weight enzyme formed in the absence of borate. The optimum pH of PDG is dependent on the origin of mitochondria from which it has been isolated. PDG from Ehrlich ascites tumour cells have an optimum pH of 9 (Quesada *et al.*, 1988), that from intestinal mitochondria had an optimum pH of 8.6-9 (Masola and Ngubane, 2010) while those isolated from liver had an optimum pH at 7.8-8.2 (Smith and Watford, 1988). Besides the origin of the mitochondria, buffers also influence the activity of glutaminase. Glutaminase from pig renal cortex in a Tris-HCl buffer has been reported to have optimum activity at pH 9 while that in a phosphate-borate buffer had optimal activity at pH 8-9 (Kvamme *et al.*, 1970).

PDG isolated from freeze-thawed and Triton X-100 treated mitochondria revealed that about two thirds of glutaminase activity is associated with the pellet (membrane bound PDG) and one third with the supernatant (soluble PDG) (Roberg *et al.*, 2000). This is unlike the LGA

that has been reported to be a matrix enzyme (Heini *et al.*, 1987). The activity of glutaminase is also influenced by the form of the enzyme, whether it is the membrane bound form or it is in a soluble form. Soluble (solubilized glutaminase) and membrane bound forms of PDG isolated from the pig and rat kidney mitochondria possess distinct enzymatic properties (Roberg *et al.*, 1997). The activity of membrane PDG is less dependent on pH than the soluble PDG which is also affected by low temperatures (Roberg *et al.*, 1997). The phosphate activation curve for membrane-bound glutaminase and intact mitochondria originating from pig brain, pig and rat kidney mitochondria exhibits an almost hyperbolic curve (Roberg *et al.*, 2000).

For soluble glutaminase from pig and rat brain, the phosphate activation curve is sigmoid (Roberg *et al.*, 2000). Soluble glutaminase from the brain and kidney have a higher association rate constant value  $K_a$  for phosphate than membrane-bound glutaminase from intact mitochondria (Roberg *et al.*, 2000). The soluble glutaminase activity is extremely sensitive to pH levels in order maintain enzyme activity compared to membrane-bound glutaminase and glutaminase in intact mitochondria. Membrane-bound glutaminase and glutaminase in intact mitochondria are less sensitive to inhibition by glutamate (Kvamme *et al.*, 2001). Glutamate and ammonia (by-products) that result from the glutaminase reaction restrain the activation of PDG by phosphate in rat brain synaptosomes and kidney cortex homogenates; showing competitive inhibition in rat brain synaptosomes (Bradford *et al.*, 1984). These by-products also increase the sigmoidicity of the phosphate activation curve in the human brain cortex (Svenneby *et al.*, 1986).

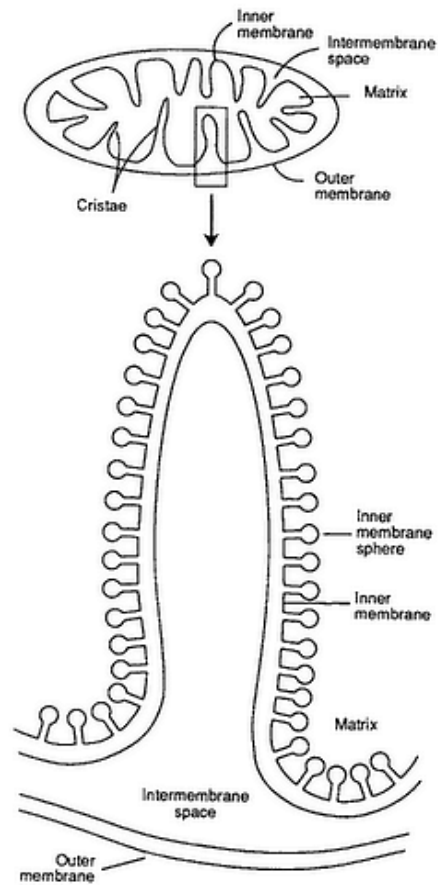
#### **1.4.2 Regulation of intestinal glutaminase by different metabolic states**

The role of glutamine in the small intestine is more important when the gut is under stress. Glutaminase activity/expression is regulated according to the health condition of an animal. It is decreased by starvation, systemic inflammatory response syndrome (Ardawi *et al.*, 1991), hypothyroidism (Ardawi *et al.*, 1991), reperfusion injury (Mueller *et al.*, 1993), cytokines interleukin-1 and interferon- $\alpha$  (Zhao *et al.*, 2012). The enzyme's activity/expression is increased during late pregnancy and lactation (Ardawi, 1987), weaning (Cabrera *et al.*, 2013), glucocorticoid administration (Salleh *et al.*, 1988), glutamine enriched enteral nutrition (Klimberg *et al.*, 1990), glutamine enriched parental nutrition (Haque *et al.*, 1996), by enteral branched chain amino acids in nutrient enriched solutions (McCauley *et al.*, 1997), during

diabetes and high protein diet (McCauley *et al.*, 1999). During starvation glutaminase mRNA levels increase while glutaminase specific activity is decreased (Kong *et al.*, 2000). Glucocorticoids are thought to increase glutamine utilization by increasing the expression of glutaminase; an adaptive response which may provide energy for intestinal cells during stress (Sarantos *et al.*, 1992).

### **1.5 Localization of glutaminase**

Information acquired during protein localization studies is vital in characterizing the cellular functions of an enzyme and elucidating its molecular interacting patterns. When an enzyme is localized within sub-mitochondrial compartments, it impacts how it interacts with other biomolecules within a network of metabolic processes. Discovering where an enzyme is localized is of great importance in drug design (Mintz-Oron *et al.*, 2009). The compartment where an enzyme is localized influences how the substrate will reach the enzyme and how the by-products will be translocated to the correct intra- or extracellular compartments for further metabolism and whether the process requires transporter proteins, chemical energy or it is passively transported across membranes (Mintz-Oron *et al.*, 2009). Mitochondria are the power house of the cell. They generate energy in the form of ATP, a biomolecule which is needed by every cell to meet their energy requirements and sustain life. Mitochondria obtain energy by oxidizing the substrates of the Citric acid cycle (Rizzo, 2009). There exist networks of enzymes localized in the mitochondria which serve a common goal of synthesizing ATP. Mitochondria are rod shaped organelles that are composed of two membranes; the outer and inner membrane. In between the outer and inner membrane membranes lies an inter-membrane space. The inner membrane folds into cristae, within the cristae lies the matrix (Bhagavan, 2002) (Figure 4). There are a variety of methods which can be used to localize PDG. Up to date methods which have been used to localize PDG in the small intestine include the use of immunohistology, immunofluorescence (Shenoy *et al.*, 1996) and studying the net glutamine uptake by the intestine (Pinkus and Windmueller, 1977). Other localization methods that may be used to study the localization of PDG from small intestine included fractionating mitochondria into different compartments and using marker enzymes to determine where the enzyme of interest is located. Mitochondrial compartment marker enzymes are enzymes whose localization within mitochondria has been well established.

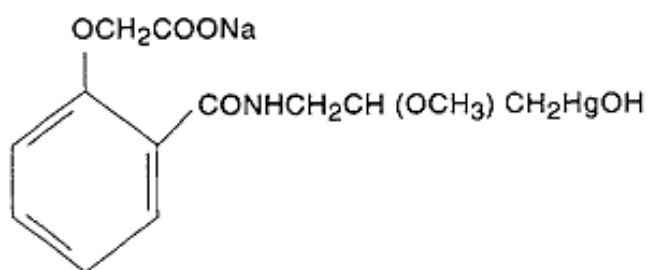


**Figure 4: The anatomy of a mitochondrion showing sub-mitochondrial compartments (Bhagavan, 2002).**

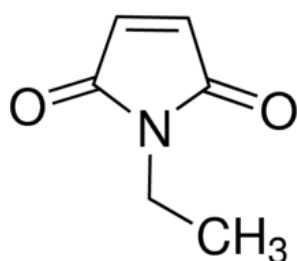
These enzymes include outer membrane marker monoamine oxidase, intermembrane space adenylate kinase (Rompay *et al.*, 2000), inner mitochondria membrane (cytochrome c oxidase and  $\beta$ -hydroxybutyrate dehydrogenase) and matrix markers malate and glutamate dehydrogenase (Brown and Chattopadhyay, 2012). The marker for the outer face of inner mitochondrial membrane is cytochrome c oxidase (Dumont *et al.*, 1991; Schneider, 2013) and that of the inner face of the inner mitochondrial membrane is  $\beta$ -hydroxybutyrate dehydrogenase (Mihara *et al.*, 1982; Chelius *et al.*, 2000).

If an enzyme of interest is found associated with the inner mitochondrial membrane after mitochondria had been fractionated into sub-mitochondrial fractions, these two marker enzymes can be used to determine the orientation of the enzyme of interest by comparing its activity to cytochrome c oxidase and  $\beta$ -hydroxybutyrate dehydrogenase. Another method which may be used to investigate the orientation of an enzyme includes utilizing membrane permeable and membrane non-permeable sulphhydryl alkylating reagents which bind

covalently and modify free cysteine residues. Mersalyl and N-ethylmaleimide are sulfhydryl alkylating reagent which bind covalently and modify –SH residues in proteins. Cysteine residues in proteins exist mainly as disulphide bonds and to a lesser degree they occur as free cysteine groups. The disulphide bonds are important for maintaining protein structure rigidity while the free cysteine groups in some cases are located within an enzyme catalytic site and are involved in the enzyme’s catalytic potential (Trivedi *et al.*, 2009). Both these reagents were used to study whether PDG from the rat small intestine possesses any cysteine groups which are essential for PDG activity and whether they are located on the outer or inner side of the inner mitochondrial membrane.



**Figure 5: Chemical structure of mersalyl (Zeidel, 1997).**



**Figure 6: Chemical structure of N-Ethylmaleimide (Sigma-Aldrich).**

PDG of small intestinal origin has long been known to be a mitochondrial enzyme (Pinkus and Windmueller, 1977), but its exact sub-mitochondrial location is still not clear. Over the years studies have shown different, sometimes contradictory sub-mitochondrial locations of

PDG. In the intestine PDG has been reported to be localized in the mucosal layer (Hahn *et al.*, 1988) of the villus and crypt cell with another small portion located in the lamina propria using immunohistochemistry (Nagy *et al.*, 1988; Shenoy *et al.*, 1996). Studies on rat kidney indicated PDG to be localized in the matrix, matrix side of the inner mitochondria membrane (Shapiro *et al.*, 1985) or localized concurrently in the two sub-mitochondrial locations (Shapiro *et al.*, 1985). Brain PDG was suggested by Kvamme *et al.* (2001) to be localized on the outer side of the inner mitochondrial membrane, while Bak *et al.* (2008) reported matrix localization. Kvamme *et al.* (1991) reported the localization of pig renal mitochondria glutaminase to be the outer surface of the inner mitochondrial membrane. By conducting inhibition studies using sulphhydryl inhibitors which are impermeable to the inner mitochondria membrane [Mers and PMB (p-hydroxymercuribenzoate)] they showed that in intact pig renal mitochondria the activity of  $\beta$ -hydroxybutyrate dehydrogenase and glutaminase were less affected by presence of Mers and PMB when compared to that in vesicles which were formed after sonication. According to these authors this implied that glutaminase is localized on the outer side of the inner mitochondria membrane.

The localization of PDG can be investigated by comparing its distribution to mitochondrial compartment markers following differential centrifugation of ruptured mitochondria. Rat LGA was recovered in a fraction that had succinate-cytochrome c reductase, an enzyme found in the inner mitochondria membrane. Mitochondria were ruptured using different methods and the soluble and membrane bound fractions were collected by centrifugation. After comparison of glutaminase distribution to glutamate dehydrogenase and succinate cytochrome c reductase it was concluded that LGA is localized in the matrix as its distribution resembled that of glutamate dehydrogenase (Kalra and Brosnan, 1973). Intramitochondrial studies (digitonin fractionation) conducted by Masola and Devlin (1995) also indicated that LGA from rat liver is associated with the inner membrane-bound fraction (mitoplast). After fractionating mitoplast they recovered 90% LGA in the supernatant compared to ruptured mitoplast indicating that LGA is a matrix localized enzyme. Immunohistochemical and immunofluorescence analysis showed that KGA in prostate, lung and breast cancer cell lines was localized in the cytosol while GAC was localized inside the mitochondria (Cassagoa *et al.*, 2012).

## **1.6 Solubilization of glutaminase**

A critical step in purifying membrane-bound enzymes is to solubilize the enzyme in a stable form in which it retains its activity. The procedures used must also be compatible with subsequent purification steps and further analyses to be conducted. This process is delicate as disrupting the proteins and lipids held together in membranes may render the enzyme completely inactive (Scopes, 1993). Different methods that may be used to solubilize membrane bound enzymes are available but detergents are highly recommended (Linke, 2009). Detergents tend to conserve the native structure while solubilizing membrane bound enzymes. The principles of the methods used in this project to solubilize PDG of rat small intestine origin are outlined below.

### **1.6.1 Lyophilization**

Lyophilization is based on the principle that the sample material is frozen under vacuum then heated slowly to enhance sublimation where ice crystals that have formed on the sample material will slowly evaporate. Before lyophilization occurs the sample must first be dialysed in water to remove buffer components since the buffer pH may drastically decrease the sample temperature during freezing which may result in denaturation of proteins (Rosenberg, 2006). The sample is shell frozen before it is freeze dried. PDG of renal mitochondrial origin were reported to have solubilized after being lyophilized in the presence of hypertonic borate and phosphate buffer and then resuspended in hypotonic buffer (Curthoys *et al.*, 1976a).

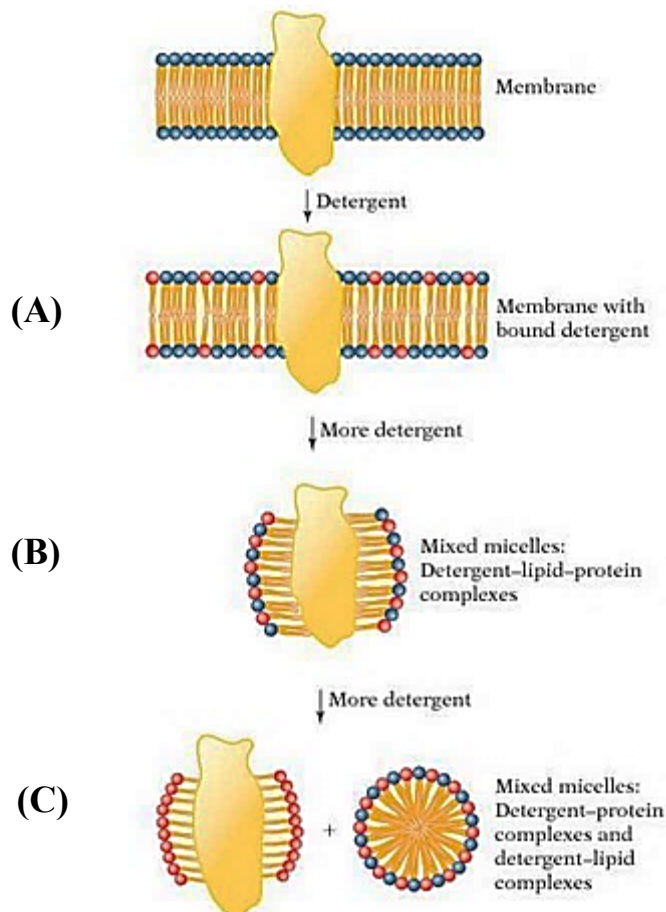
### **1.6.2 Sonication**

Sonication is a physical disruption method used to lyse cells and cell organelles by suspending them in high frequency sound waves that can shear the cell membranes. The ultrasonic power supply converts 50/60 Hz voltage into high electrical energy which is transmitted to the piezoelectric transducer and converted to mechanical vibrations which are intensified by a probe immersed in the liquid cell suspension creating microscopic vapour bubbles that form briefly to shear cell membranes (Walker, 2010). The microscopic bubbles create shock waves and release high levels of energy into the suspension. To prevent excessive heating ultrasonic treatment is applied in multiple short bursts to a sample

immersed in an ice bath (Walker, 2010). Sonication causes the mitochondria to turn inside out exposing any enzymes contained in the matrix. The enzymes which have been exposed therefore exhibit increased activity due to greater interaction with substrates. The activity of glutaminase in mitochondria isolated from *Escherichia coli* was increased after sonication due to effective disruption of the mitochondria membranes resulting in more assayable glutaminase (Kenny *et al.*, 2003). However, it has also been reported that disrupting intact intestinal mitochondria with sonication leads to loss of enzyme activity (Masola and Ngubane, 2010).

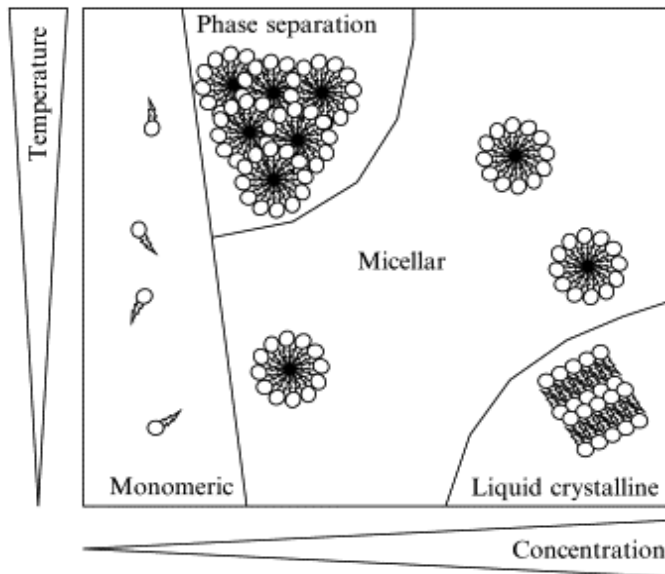
### **1.6.3 Detergents**

Detergents are organic compounds which are used widely for solubilization of membrane bound enzymes and keeping them in solution during subsequent purification procedures (Roos, 2000b). Most membrane proteins are stable within a limited number of different detergent-buffer systems. Different membrane proteins have different preferences for detergent which may be used to solubilize them. Detergents have physical and chemical properties which can be exploited for protein purification. Generally detergents are amphiphilic in nature; with their hydrophobic domains made of hydrocarbons and the hydrophilic domains being either a polar or charged head group (Linke, 2009) (Figure 7). The hydrocarbon moiety is an unbranched saturated alkane whose hydrophobicity increases as its length is increased. Alternatively branched-chain or unsaturated alkanes, steroids or aromatic hydrocarbon moieties are found in the lipophilic group of detergents, sometimes in combination (Luckey, 2014). Detergents are classified based on their head groups, namely non-ionic, anionic or cationic; with a tendency of migrating to the interface in solution assuming the most energetically favoured orientation (Roos, 2000b). Membrane proteins have hydrophobic regions buried in the membrane and a hydrophilic side with polar residues. In aqueous solutions detergents form spherical aggregates known as micelles. The hydrophobic tails form the core while the hydrophilic heads form the surface of the micelles (Seddon *et al.*, 2004). It must be noted that other factors like temperature, detergent concentration, ionic strength, pH and the presence of other variable compounds in the buffer affect the formation of micelles.



**Figure 7: An illustration of solubilization of integral proteins from membrane bi-layer using an increasing detergent concentration. (A) At low detergent concentrations the detergent integrates into the bilayer. (B) A higher detergent concentration causes integral proteins to be solubilized. (C) Increasing the concentration further strips all the phospholipids and the integral protein is only surrounded by detergent (Luckey, 2014).**

The extent of solubilization of membrane proteins is dependent on the formation of micelles where the hydrophobic membrane protein part is infused to the detergent, while hydrophilic head groups protrude (Linke, 2009; Luckey, 2014). An adequate concentration of the detergent must be present in the solution for the membrane protein to be solubilized, denoted as the critical micelle concentration (CMC). If there is too little detergent in the solution the detergent will exist as monomer structures but at higher concentrations detergent aggregates form resulting in phase separation. This phenomenon is best demonstrated using a phase diagram (Figure 8).

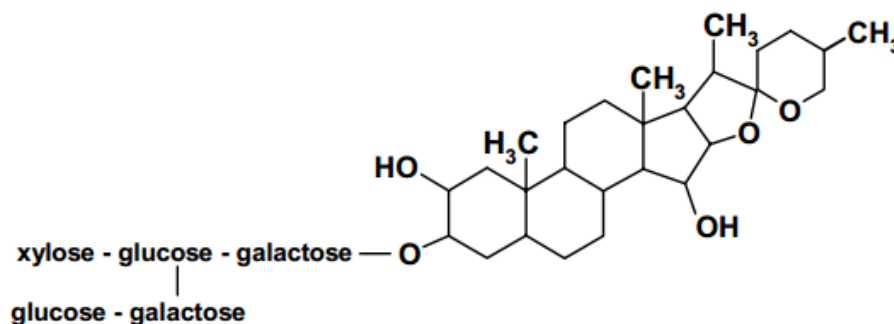


**Figure 8: Illustration of a phase separation of a detergent (Linke, 2009).**

### 1.6.3.1 Digitonin and lubrol

Digitonin is a steroid glycoside obtained from a plant *Digitalis purpurea* (Figure 9). It belongs to a class of non-ionic detergents which are commonly used for solubilizing membrane proteins, making cell membranes permeable and precipitating cholesterol. It is composed of complex polysaccharide head groups and a cholesterol derivative tail with an aggregation number of 60-70 and a CMC of < 0.05 mM. Schaitman and Greenawalt (1968) showed that digitonin (at certain concentrations) does not affect the integrity of the inner membrane and maintains the inner membrane matrix. They became the first scientists to isolate a morphologically and biochemically intact preparation of the inner membrane-matrix fraction (mitoplast) from rat liver mitochondria which showed respiratory control using digitonin fractionation.

Lubrol is a mild/non-ionic detergent that has the potential of solubilizing membrane bound proteins while preserving their biological structure and activity. Schaitman and Greenawalt (1968) also reported that lubrol (polyoxyethylene) may be used instead of sonication to activate mitochondrial enzymes. This activation by lubrol is through the separation of the inner membrane from matrix thereby exposing enzymes which are located in these regions. They also observed that at concentrations of 0.1 mg lubrol/mg protein cytochrome oxidase and other mitochondrial enzymes were activated.



**Figure 9: Chemical structure of digitonin (Paila *et al.*, 2005).**

When using this non-ionic detergent, solubilization of membrane protein is not clear cut since the protein may then become encapsulated by the detergent (move into vesicles) (Roos, 2000b). Experiments by Schaitman and Greenawalt (1968) using their digitonin-lubrol fractionation procedure showed that renal PDG was quantitatively recovered in the inner membrane and matrix fractions following digitonin fractionation. The subsequent treatment of the mitoplasts with lubrol resulted in a 70% loss of activity but when borate was added PDG regained activity, exhibiting an increase of 50-100%. The use of mild treatments like the swell and shrink procedure, freezing and thawing and sonication in the absence of borate did not result in more than 10% PDG activity recovery (Curthoys and Weiss, 1974). Curthoys *et al.* (1976) showed that using 1% digitonin or 1% lubrol more than 50% and 65% rat kidney glutaminase was solubilized, respectively. On the other hand research has shown that use of aggressive detergents such as Triton X-100 greatly solubilized glutaminase enzyme from the mitochondria but it had a negative impact on the enzyme's activity (Curthoys *et al.*, 1976a; Curthoys and Watford, 1995). Roos (2000b) showed that using 0.003% Triton X-100 produced high glutaminase activity compared to untreated mitochondria.



**Figure 10: Chemical structure of lubrol (GuideChem).**

## 1.7 Purification of glutaminase from various organisms and cell types

Glutaminase has been purified from different animal organs including the brain from rats, pigs, cows and humans (Chiu and Boeker, 1979; Nimmo and Tipton, 1980; Svenneby *et al.*, 1986; Kaneko *et al.*, 1987; Holcomb *et al.*, 2000), and the kidney from pigs and rats (Kvamme *et al.*, 1970; Curthoys *et al.*, 1976a). The enzyme has also been purified from rat liver (Patel and McGivan, 1984; Smith and Watford, 1988), Ehrlich ascites tumour cells (Quesada *et al.*, 1988) and microorganisms [*Debaryomyces* spp. (Durá *et al.*, 2002) and *Lactobacillus reuteri* (*L. reuteri*) KCTC3594 (Jeong-Min *et al.*, 2010)]. PDG isolated from Ehrlich ascites tumour cells and purified by DEAE-cellulose (anion-exchanger) and gel filtration chromatography using Sephacryl S-300 was shown to have a molecular weight of 135 kDa (Quesada *et al.*, 1988). Sodium-dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of purified glutaminase from Ehrlich cells showed two protein bands of molecular weight 64 and 56 kDa (Quesada *et al.*, 1988). Glutaminase isolated from *L. reuteri* cells was purified using HiTrap-DEAE-FF (DEAD column) and Superose 12HR. Its molecular weight using SDS-PAGE was determined to be 70 and 50 kDa (Jeong-Min *et al.*, 2010). Svenneby *et al.* (1973) purified PDG from pig brain by fractionating acetone precipitated proteins using sodium sulphate and further precipitating PDG using phosphate and borate after dialysing the sodium sulphate precipitate in a buffer containing mercaptoethanol, Tris-HCl and EDTA.

Sedimentation equilibrium centrifugation was used to determine the molecular weight of purified pig brain PDG and it was determined to be 187 kDa. Using SDS-PAGE a molecular weight of 64 kDa was observed (Svenneby *et al.*, 1973). Rat brain PDG fractionated using ammonium sulphate precipitation followed by purification on Sepharose 4B was shown to have a molecular weight of 63 kDa and 65 kDa using SDS-PAGE (Haser *et al.*, 1985). Kaneko *et al.* (1987) reported only one band with a molecular weight of 62 kDa on SDS-PAGE after purifying glutaminase from rat brain on a different gel filtration chromatography column (Sephacryl S- 400 HR followed by lysine- Sepharose and then a Sepharose 4B). A purified native Tris-form KGA (KGA in a Tris buffer) lacking a polyvalent ion exists as an inactive protomer with a molecular weight range of 90-137 kDa, which upon addition of phosphate forms a dimer and experiences reversible polymerization into a high molecular mass aggregate in borate buffer (Curthoys *et al.*, 1976b). Partially purified LGA had a molecular weight of 290 kDa when purified on a DEAE column followed by Sephacryl S-300 HR gel filtration chromatography and displayed molecular mass subunits of 73.5 kDa on

SDS-PAGE (Patel and McGivan, 1984). Smith and Watford (1987) successfully purified LGA from the rat liver after initially fractionating liver proteins using ammonium sulphate and polyethylene glycol fractionation methods, followed by purifying LGA using different chromatography columns. Their pure LGA proteins after being analysed using SDS-PAGE yielded a subunit of approximately 58 kDa.

Despite the importance of PDG in the small intestine (during in energy provision for absorptive processes) (Newsholme *et al.*, 2003a), there exists no work which reports a purified PDG protein which has further been characterized to understand its molecular structure. Since the intestinal enzyme is the KGA-type, it is likely that it also polymerizes in hypertonic buffers, a property that may enhance its purification using gel filtration chromatography as has been the case with kidney and Ehrlich ascites tumour cell enzymes (Curthoys *et al.*, 1976a; Quesada *et al.*, 1988). However, if substantial activity is only maintained in such buffers during the purification process, ion exchange chromatography as used for the Ehrlich ascites tumour cell enzyme may not be suitable.

## **1.8 Potential purification techniques for intestinal PDG**

### **1.8.1 Ammonium sulphate precipitation**

Ammonium sulphate protein precipitation is a technique which is used to separate proteins by changing their phase from being soluble to being insoluble. This is achieved by altering the ionic strength of the surrounding buffer or altering the protein charge characteristics (Burgess, 2009). Proteins possess outward facing free amino acid residues which are hydrophilic, these amino acid are responsible for interacting with water rendering the protein soluble (Nigam and Ayyagari, 2007). Slowly saturating the solution with ammonium sulphate disturbs the amino acid-water interactions. This favours proteins to interact with each other rather than with water therefore forming precipitates. Different proteins suspended in an aqueous solution have different solubility potential, adding ammonium sulphate initially allows proteins which are least hydrophilic to precipitate first and the hydrophilic proteins to precipitate at higher ammonium sulphate concentrations (Duong-Ly and Gabelli, 2014). Proteins precipitate at different concentrations of ammonium sulphate added into solution, allowing proteins to separate at different points. Protein precipitation is useful in

concentrating samples and is ideal as an initial purification step which eliminates components that may interfere with downstream purification procedures.

Different salts are available which may be used for precipitation of proteins but ammonium sulphate possesses properties which makes it the most preferred choice. These properties include protecting proteins from denaturation, allowing heat generated to be easily released from the solution, having a low density (1.235 g/ml) upon saturation which does not affect sedimentation of precipitated proteins during centrifugation, dissolving at high concentrations and causing most proteins to precipitate (Bonner, 2007). When used as a method for purification, proteins are differentially precipitated using low salt concentration collecting the precipitate and increasing the salt concentration in the collected supernatant (Scopes, 1993). This allows proteins to be separated depending at which concentration it is precipitated. The ammonium sulphate salt is dialysed against a buffer which is compatible with downstream process.

### **1.8.2 Ion-exchange chromatography**

The ion exchange chromatography method reversibly separates biomolecules according to differences in their net surface charge. It takes advantage of the electrostatic force that exists between ions on the protein and matrix according to Coulomb's Law. The ions on the surface of the protein are influenced by the pH of a buffer on which the protein is suspended (Fritz and Gjerd, 2009). This technique requires that the isoelectric point (pI) of the protein must be known. The pI is the pH at which the protein has a net charge of zero. A protein at its pI will not interact with a charged medium; however at a pH above its pI a protein will bind to a positively charged medium or anion exchanger and at a pH below its pI a protein will bind to a negatively charged medium or cation exchanger (Simpson, 2004). Molecules exhibit different degrees of interaction with charged chromatography media according to differences in their overall charge, charge density and surface charge distribution (Roos, 2000a). To elute the proteins from an ionic chromatography column the bond between the proteins and matrix must be weakened by lowering the pH which will reduce the charge on the protein or by increasing the ionic strength of the eluting buffer; this should be done in time before the protein changes conformation assuming a more strong interactive bond with the matrix (Simpson, 2004).

### **1.8.3 Gel filtration chromatography**

Gel filtration chromatography also known as size exclusion chromatography is a method that separates proteins based on shape and size as they pass through a matrix packed in a column. The matrix is a bed of porous beads with internal channels with a defined fractionation range allowing smaller proteins to enter but excluding proteins larger than the pore diameter (Seddon *et al.*, 2004). Larger proteins elute out of the column first, the log of a molecule's molecular weight is proportional to the distance that molecule migrates. Also proteins with spherical shape migrate easier than those with an elongated shape (Gupta, 2008). The protein size in a given sample can be determined by first calibrating the gel in the column with proteins that have known sizes. In this method molecules do not bind to the chromatography matrix so the buffer composition does not directly affect the resolution.

This method is suitable for proteins that may be sensitive to pH, concentration of metal ions, co-factors or harsh environmental conditions (Gupta, 2008). Samples are separated isocratically therefore there is no need to use different buffers. Different matrices are available and the selection of a matrix to be utilized is dependent on the molecular weight of the protein to be purified (Walsh, 2007). Factors that affect resolution include flow rate (slower flow rate is better), column size (usually tall with 1-2 cm diameter) and type of matrix. In this project Sephacryl S-100 HR, Sephacryl S-300 HR and Sephacryl S- 400 HR matrices will be used. The Sephacryl matrix is a cross-linked allyl dextran/N, N'-methylenebisacrylamide copolymer which is hydrophilic in nature. It's hydrophilic nature lowers unspecific adsorption of proteins onto the surface of the matrix while increasing recovery (Janson and Jonsson, 2011). Sephacryl S-100 HR, Sephacryl S-300 HR, Sephacryl S- 400 HR have different fractionation molecular weight range 1 - 100 kDa, 10 - 1500 kDa and 20 - 8000 kDa, respectively.

### **1.9 Rationale of the study**

PDG is responsible for hydrolyzing glutamine, a major and obligatory source of energy in the small intestine. Proper functioning of PDG is vital as glutamine, besides being the main energy source in the small intestine, also supplies precursors for other pathways essential for proliferating cells and maintaining gut structure. The functioning of PDG therefore has an impact on the efficiency of nutrient absorption in the gut and implications on the nutritional

status of the whole animal. Over the year studies in an effort to understand cancer metabolism and derive ways to control the high proliferation rate of cancer cells metabolic enzymes have been identified as targets. Glutaminase is amongst other metabolic enzymes which are being exploited to suppress and control cancer proliferation. Localization of intestinal PDG and acquiring a stable partially purified enzyme will aid in studies which aim at developing therapeutic drugs that target PDG (Zhao *et al.*, 2013), particularly those that may target glutamine metabolism in colon and intestinal cancers. To optimize the design of therapeutic drugs that target intestinal PDG it is essential to know the molecular structure and characteristics of the enzyme. Hence the aim of this study is to localize and partially purify glutaminase of small intestinal origin as the first step in this endeavour.

## **1.10 Aim and Objectives**

### **Aim**

To localize and purify phosphate dependent glutaminase from rat small intestine

### **Objectives:**

1. To determine the sub-mitochondrial location of PDG using mitochondrial compartment marker enzymes.
2. To determine the lipid-dependence of PDG by phospholipase A<sub>2</sub> treatment.
3. To determine the sub-mitochondrial orientation of PDG using sulphydryl inhibitors.
4. To solubilize PDG in an active form.
5. To purify PDG by ammonium sulphate fractionation, ion exchange chromatography and gel filtration chromatography.
6. To assess the purity and integrity of PDG by using SDS-PAGE and Western blot analysis.

## CHAPTER 2

### 2. Materials and Methods

#### 2.1 Materials

##### 2.1.1 Animals

Male Sprague-Dawley rats weighing (230-250 g) were obtained from the Biomedical Research Unit at the University of KwaZulu-Natal, Westville campus (Ethical clearance reference number 089/13/Animal and 096/14/Animal). The animals had free access to water and were fed standard rat chow *ad libitum* (Meadows, Pietermaritzburg, South Africa).

##### 2.1.2 Chemicals

The sodium chloride (NaCl), sodium hydroxide (NaOH), sodium bicarbonate (NaHCO<sub>3</sub>), sodium phosphate monobasic dihydrate (NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O), sodium phosphate dibasic (Na<sub>2</sub>HPO<sub>4</sub>), potassium chloride (KCl), potassium phosphate monobasic anhydrous (KH<sub>2</sub>PO<sub>4</sub>), potassium pyrophosphate (K<sub>4</sub>P<sub>2</sub>O<sub>7</sub>), magnesium sulphate heptahydrate (MgSO<sub>4</sub>·7H<sub>2</sub>O), ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N' tetra acetic acid (EGTA), bovine serum albumin (BSA), sucrose (C<sub>12</sub>H<sub>22</sub>O<sub>11</sub>), borax anhydrous (B<sub>4</sub>Na<sub>2</sub>O<sub>7</sub>), ammonium sulphate [(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>], Hepes (N-[2-hydroxyethyl] piperazine-N'-ethanesulfonic acid) (C<sub>8</sub>H<sub>18</sub>N<sub>2</sub>O<sub>4</sub>S), N-ethylmaleimide (NEM) (C<sub>6</sub>H<sub>7</sub>NO<sub>2</sub>), mersalyl acid (C<sub>13</sub>H<sub>17</sub>HgNO<sub>6</sub>), digitonin (C<sub>56</sub>H<sub>92</sub>O<sub>29</sub>), lubrol [HO(CH<sub>2</sub>CH<sub>2</sub>O)<sub>n</sub>C<sub>16</sub>H<sub>33</sub> n≈23], calcium chloride dehydrate (CaCl<sub>2</sub>·2H<sub>2</sub>O), TRIZMA-Base [NH<sub>2</sub>C(CH<sub>2</sub>OH)<sub>3</sub>], triethanolamine (TEA) [(HOCH<sub>2</sub>CH<sub>2</sub>)<sub>3</sub>N], DL-dithiothreitol (DTT) [HSCH<sub>2</sub>CH(OH)CH(OH)CH<sub>2</sub>SH], phosphoenolpyruvate (PEP) tricyclohexylammonium salt, β-nicotinamide adenine dinucleotide (NAD) (C<sub>21</sub>H<sub>27</sub>N<sub>7</sub>O<sub>14</sub>P<sub>2</sub>), benzamidinium hydrochloride hydrate (C<sub>6</sub>H<sub>5</sub>C(=NH)NH<sub>2</sub>·HCl·xH<sub>2</sub>O), DL-β-hydroxybutyric acid sodium salt (C<sub>4</sub>H<sub>7</sub>O<sub>3</sub>Na), BCIP/NBT alkaline phosphatase substrate, benzylamine hydrochloride (C<sub>6</sub>H<sub>5</sub>CH<sub>2</sub>NH<sub>2</sub>·HCl), phenylmethanesulfonyl fluoride (PMSF), 2-mercaptoethanol (HSCH<sub>2</sub>CH<sub>2</sub>OH), sodium dodecyl sulfate (SDS) [CH<sub>3</sub>(CH<sub>2</sub>)<sub>11</sub>OSO<sub>3</sub>Na], bis (N,N'-Methylene-bis-acrylamide, acrylamide, β-nicotinamide adenine dinucleotide, reduced disodium salt hydrate (NADH) (C<sub>21</sub>H<sub>27</sub>N<sub>7</sub>Na<sub>2</sub>O<sub>14</sub>P<sub>2</sub>·xH<sub>2</sub>O), adenosine 5'-diphosphate (ADP) (C<sub>10</sub>H<sub>15</sub>N<sub>5</sub>O<sub>10</sub>P<sub>2</sub>), glycine (C<sub>2</sub>H<sub>5</sub>NO<sub>2</sub>), L-glutamine [H<sub>2</sub>NCOCH<sub>2</sub>CH<sub>2</sub>CH(NH<sub>2</sub>)CO<sub>2</sub>H], cytochrome c, oxaloacetic acid

(HOOCCH<sub>2</sub>COCOOH), lactate dehydrogenase and L-glutamic dehydrogenase solutions in 50% phosphate buffered glycerol from bovine liver were purchased from SIGMA-ALDRICH, South Africa. Potassium hydroxide (KOH), hydrazinium-hydroxide (N<sub>2</sub>H<sub>5</sub>OH), TWEEN 20, perchloric acid, acetic acid, butanol, methanol, sodium carbonate anhydrous (Na<sub>2</sub>CO<sub>3</sub>), cupric sulphate (CuSO<sub>4</sub>.5H<sub>2</sub>O), Folin & Ciocalteu's phenol reagent, and potassium sodium tartate (KNaC<sub>4</sub>H<sub>4</sub>O<sub>4</sub>.H<sub>2</sub>O) were bought from Merck, Halfway House, South Africa. Tris [NH<sub>2</sub>C (CH<sub>2</sub>OH)<sub>3</sub>], ammonium persulfate, 10× Tris/Glycine/SDS buffer, TEMED and precision plus protein standards were bought from BIORAD. Kidney-type glutaminase antibody (KGA) H-45: sc-366447 was bought from Santa Cruz Biotechnology, INC. The kidney-type glutaminase antibody used when we investigated different PDG solubilizing method was a kind gift from Professor Norman Curthoys, Colorado State University, USA. The secondary antibody was goat anti-rabbit IgG alkaline phosphatase conjugate purchased from BIORAD, South Africa. 95% Oxygen: 5% Carbon dioxide gas was bought from AFROX (South Africa). All solutions were prepared in deionized water unless stated otherwise.

## **2.2 Methods**

### **2.2.1 Preparation of enterocytes**

#### **2.2.1.1 Isolating media**

Medium 1, 2 and 3 were used in the process of isolating enterocytes. Their components are described below. These media are used used to maintain the physiological pH.

**Medium 1** contained 118 mM NaCl, 25 mM NaHCO<sub>3</sub>, 4.7 mM KCl, and 1.18 mM KH<sub>2</sub>PO<sub>4</sub> and 1.9 mM MgSO<sub>4</sub>.7H<sub>2</sub>O in deionized water, pH 7.4

**Medium 2** was prepared by dissolving 5 mM EGTA and 0.25% BSA in of medium 1, pH 7.4. Medium 2 contains EGTA which functions to loosen the intestinal mucosa from the underlying muscularis mucosae.

**Medium 3** was prepared by dissolving 0.25% BSA in of medium 1, pH 7.4.

### **2.2.1.2 Procedure for isolation of enterocytes**

Enterocytes were prepared using a method by Watford *et al.* (1979) modified by Masola and Evered (1984). During each preparation two rats were euthanized using an overdose of halothane. The small intestines were harvested and the mesenteric fat was removed. Intestines were then washed to remove digester residues using a syringe containing medium 1 which was saturated with 95% O<sub>2</sub>:5% CO<sub>2</sub>. Each intestine was ligated on one end, filled with medium 2 and then the other end was also ligated. The intestines were placed in a conical flask containing 250-300 ml of medium 1 (pre-incubated at 37°C), incubated in a water bath at 37°C and continuously gassed with 95% O<sub>2</sub>/5% CO<sub>2</sub> for 12 minutes. Medium 2 was then removed from each intestine, which were then rinsed with medium 1 and half filled with ice cold medium 3. Enterocytes were detached by gently rubbing the intestine along its length using the forefinger and thumb. One end of the intestine was then unligated and detached enterocytes were released into a beaker.

### **2.2.1.3 Preparation of mitochondria from enterocytes**

Mitochondria were prepared from enterocytes using a method by Masola and Evered (1984). The enterocyte suspension was divided into four 15 ml centrifuge tubes and washed three times using medium 1 by centrifugation at 3000 rpm for 5 minutes using a Hettich Zentrifugen D-7200 bench top centrifuge, Germany. After the third wash, the pellets were washed twice with ice cold mitochondria preparation medium (250 mM sucrose, 5 mM Hepes, 1 mM EGTA, pH 7.4) using the previous centrifugation conditions. The four pellets were each then re-suspended in ice cold mitochondrial preparation medium and homogenized using a Dounce homogenizer (7 passes) while chilled on ice.

Two homogenates from above were combined and each of the two combined homogenates was re-homogenized using a motor driven Glas-Col homogeniser, Terre Haute (USA) by another 7 passes at 4000 rpm. All homogenates were combined and diluted to a total volume of 190 ml, divided between eight 50 ml polycarbonate centrifuge tubes and centrifuged at  $1482 \times g$  for 10 minutes at 4°C using a Beckman Coulter J26-XPI centrifuge (USA). Supernatants were recovered and centrifuged at  $9\ 798 \times g$  for 10 minutes at 4°C using the above mentioned centrifuge. The resulting pellets were combined into one 50 ml polycarbonate tube and washed three times with 25 ml of mitochondria preparation medium.

After the first wash the fluffy layer on top of the mitochondria was removed. The final pellet was re-suspended in 3 ml of mitochondrial preparation medium and assayed for PDG fresh, depending on the experiment, or stored in eppendorf tubes at  $-20^{\circ}\text{C}$ . For solubilization and purification studies mitochondrial preparations were assayed for PDG activity and then divided into three groups based on their specific activity:

**Group 1** (low PDG specific activity)

**Group 2** (intermediate PDG specific activity)

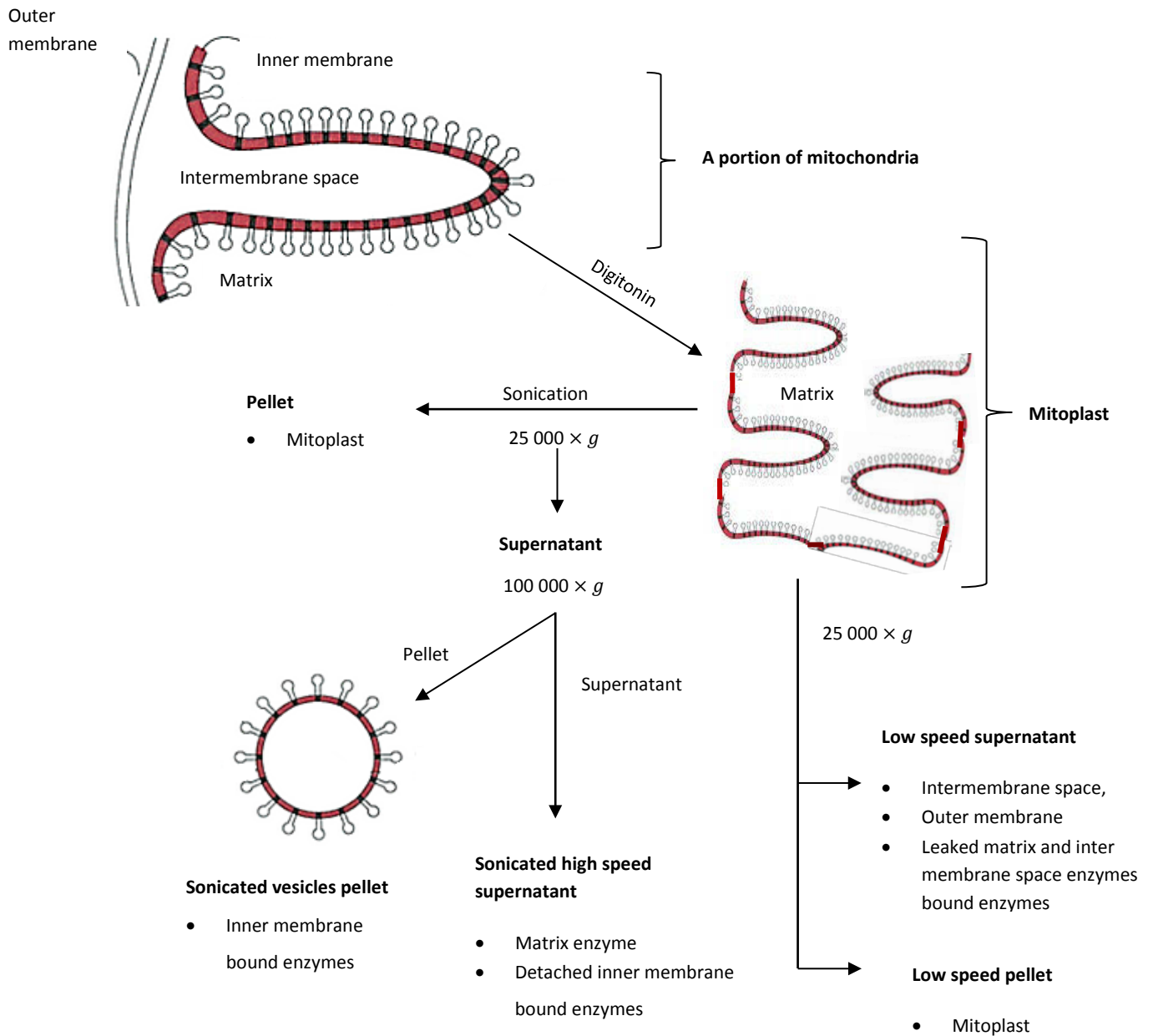
**Group 3** (high PDG specific activity)

Group 1 and group 2 mitochondria were used to optimize the solubilization procedure.

## **2.3 Sub-mitochondrial localization of PDG from rat intestinal mitochondria**

### **2.3.1 Location of PDG in sub-fractions of mitochondria**

Mitochondria were fractionated using a method by Masola and Devlin (1995) with modifications (Figure 11). The outer membrane of rat intestinal mitochondria was stripped out to yield mitoplasts using 2 mg digitonin/mg of protein. This concentration will completely strip liver mitochondria of the outer membrane but will also cause substantial leakage of matrix enzymes (Schaitman and Greenawalt, 1968; Masola and Devlin 1995). Enzymes strongly bound to the inner membrane should still retain a substantial association with the low speed pellet following centrifugation. After adding digitonin to mitochondria this was mixed thoroughly by inversion, incubated for 20 minutes on ice and centrifuged at  $25\,000 \times g$  for 15 minutes using a Beckman L-80 ultracentrifuge, USA. The resulting supernatant was denoted “low speed supernatant” while the pellet contained mitoplasts. The latter was sonicated three times using an 80 W digital ultrasonic cleaner UD80SH-2L (Hong Kong), 10 seconds each time with cooling on ice in between intervals. Sonicated mitoplasts were centrifuged at  $25\,000 \times g$  for 15 minutes and the resulting pellet was denoted “sonicated mitoplast”, whilst the supernatant was centrifuged further at  $100\,000 \times g$  for 1 hour. The supernatant and pellet were collected and the pellet was denoted sonicated vesicles (inside-out mitoplast).



**Figure 11: Illustration of the fractionation of mitochondria into mitochondrial sub-fractions using digitonin and sonication.**

Enzymatic assays were then conducted in these collected fractions to investigate whether the distribution of PDG could be correlated to marker enzymes whose localization is known from studies in liver mitochondria (Schaitman and Greenawalt, 1968; Masola and Devlin, 1995). The marker enzymes assayed were adenylate kinase, a marker of the inter-membrane space; cytochrome oxidase, a marker of the inner membrane and also a marker for the outer surface of the inner membrane;  $\beta$ -hydroxybutyrate dehydrogenase, a marker of the inner membrane

and also a marker for the inner surface of the inner membrane; glutamate dehydrogenase and malate dehydrogenase, markers for the matrix.

### **2.3.2 Investigating membrane association and solubilization of PDG using lyophilization, detergents and sonication**

To investigate the solubility of PDG from rat intestinal mitochondria the method of Schaintman and Greenwalt (1968) as modified by Masola and Devlin (1995) was used. Mitochondria were lyophilized overnight using a VirTis SP Scientific Sentry 2.0 bench top lyophilizer (USA). After lyophilization mitochondria were resuspended in 15 ml hypotonic phosphate-borate buffer (10 mM  $B_4Na_2O_7$ , 100 mM  $K_4P_2O_7$  and 100 mM  $KH_2PO_4$ , pH 8.6), incubated for 30 minutes on ice then homogenized using a motor driven GLAS-COL homogenizer, Terre Haute (USA) at 3000 rpm (7 passes). The sample was then centrifuged at  $20\,000 \times g$  for 30 minutes using a Beckman L-80 ultracentrifuge (USA). The supernatant (LYO supernatant) was collected whilst the pellet (LYO pellet) was suspended in 15 ml hypotonic phosphate-borate buffer and homogenized using a motor driven GLAS-COL homogenizer, Terre Haute (USA) at 3000 rpm (7 passes). Since most of PDG activity could still be recovered in the pellet after this step, PDG was further solubilized from lyophilized mitochondria using further methods.

Digitonin and lubrol were prepared in a hypotonic phosphate-borate buffer at concentrations of 4 mg digitonin/10 mg protein and 1 mg lubrol/mg protein, respectively. At these concentrations digitonin solubilizes substantial amounts of membrane bound mitochondrial enzymes while lubrol causes their substantial activation, presumably through solubilization (Masola and Devlin, 1995). Equal amounts of lyophilized mitochondria protein were divided into six tubes. Into two of the tubes hypotonic phosphate-borate buffer was added followed by sonication of one tube and these mitochondrial samples were designated US - unsonicated and S - sonicated. Digitonin was added to two of the remaining tubes while lubrol was added to the other two. This was followed by sonication of one tube from each of the detergent treated samples. These tubes/samples were designated according to their treatment as D, DS, L and LS for digitonin, digitonin + sonication, lubrol and lubrol + sonication, respectively. Sonication was carried out immediately following addition of detergent once for 10 seconds using a 120 W Virsonic ultrasonic disintegrator at 50% power (Gardiner, NY). All samples were then incubated for 30 minutes on ice. After the incubation period samples were

homogenized using a Dounce homogenizer (7 passes) then centrifuged at  $20\,000 \times g$  using a Beckman L-80 ultracentrifuge, USA. The supernatants were collected into different tubes and the treated pellets were re-suspended into hypotonic phosphate-borate buffer plus digitonin/lubrol solution as per treatment, while the untreated pellets were re-suspended into hypotonic phosphate-borate buffer. All re-suspended pellets were homogenized using a Dounce homogenizer as above. Samples were then assayed for PDG activity.

### **2.3.3 Determination of the lipid dependence of PDG**

The determination of the lipid dependence of PDG was investigated using the method of McGivan and Bradford (1983), with minor modifications. This method uses phospholipase A<sub>2</sub> to digest membrane phospholipids and monitors changes in enzyme activity. Fresh mitochondria were prepared as outlined above and the final pellet was suspended in a buffer containing 250 mM sucrose, 10 mM borax and 5 mM Tris, pH 8.0. All other solutions were also prepared in the same buffer. A 2 ml mitochondria sample containing 2.5 mg/ml protein was frozen and thawed three times using ethanol cooled with a Cryocool immersion cooler model cc-80, Thermo NESLAB (USA). The frozen and thawed mitochondria along with 1 ml phospholipase A<sub>2</sub> solution (25 U/ml) were pre-incubated at 30°C. To 2 ml of the frozen and thawed mitochondria sample 10 µl of 1 mM CaCl<sub>2</sub> solution and 1 ml phospholipase A<sub>2</sub> solution were added. At 0, 15, 30, 45 and 60 minutes, 0.5 ml of the mixture was withdrawn and placed in a clean eppendorf tube containing 100 µl of 10 mM EGTA solution to terminate the reaction. The samples were immediately assayed for PDG activity. The same procedure was adopted for solubilized PDG but the samples were not subjected to freeze-thaw cycles.

### **2.3.4 Investigation of PDG orientation with sulphhydryl group targeting inhibitors**

The orientation of PDG was investigated using the method of Aledo *et al.* (1997) with minor modifications. The method uses membrane permeate and impermeate sulphhydryl inhibitors to probe mitochondria. Fresh mitochondria were frozen and thawed using a Cryocool immersion cooler model cc-80, Thermo NESLAB (USA). Equal amounts of mitochondria protein were divided into ten tubes. Into two of the tubes an equal volume of buffer (250 mM sucrose, 10 mM borate and 5 mM Tris pH 8.0) was added followed by sonication of one tube and these

mitochondrial samples were designated as unsonicated mitochondria and sonicated mitochondria. Sulphydryl reagents were added into the remaining tubes: 1 mM Mers into the first two tubes, 1 mM NEM into another set and the final two had 0.1 mM Mers. This was followed by sonication of one tube from each of the detergent treated samples. These were incubated for 12 minutes at 25°C. The reaction was terminated by adding 200-fold concentration of mercaptoethanol (i.e. with respect to the concentration of inhibitor). The samples were assayed for PDG activity.

## **2.4 Purification of phosphate dependent glutaminase from rat small intestine**

### **2.4.1 Solubilizing PDG using osmotic imbalance and lubrol detergent of Group 1 mitochondria**

A method by Curthoys *et al.* (1976) with minor modifications was used to solubilize PDG of small intestinal origin for purification purposes. To a suspension of mitochondria, four volumes of hypertonic phosphate-borax buffer (0.1 M  $B_4Na_2O_7$ , 1 M  $K_4P_2O_7$  and 1 M  $KH_2PO_4$ , pH 8.6 with 2 mg lubrol/mg protein) was added. This was stirred for 30 minutes at room temperature, homogenized using a Dounce homogenizer (7 passes), frozen using liquid nitrogen and lyophilized overnight using a VirTis SP Scientific Sentry 2.0 bench top lyophilizer (USA). After lyophilization the mitochondria were diluted 10× with deionized water and incubated on ice for 30 minutes. The sample was then homogenized (as per previous conditions) and centrifuged at  $20\,000 \times g$  for 30 minutes at 4°C using a Beckman Coulter J26-XPI centrifuge (USA). The supernatant (**1<sup>st</sup> supernatant**) was collected and the pellet was resuspended in a hypotonic phosphate-borax buffer which was half the volume of the supernatant. The pellet was re-homogenized and centrifuged (as per previous conditions). The supernatant (**2<sup>nd</sup> supernatant**) was collected, combined with the previously collected supernatant while the pellet was resuspended in 15 ml of hypotonic phosphate-borate buffer.

#### **2.4.1.1 Ammonium sulphate precipitation**

Proteins in the 1<sup>st</sup> and 2<sup>nd</sup> supernatant were precipitated sequentially using 20%, 40%, 70% and 100% ammonium sulphate. The precipitated proteins were collected by centrifugation at  $9\,798 \times g$  for 5 minutes at 4°C. The pellets of precipitated proteins were resuspended in 5 ml

of hypotonic phosphate-borate buffer and dialyzed against hypotonic phosphate-borate buffer for 24 hours at 4°C (with buffer changed every 8 hours) using a membrane with a 12 000 Da cut-off. The collected fractions were assayed for PDG activity.

#### **2.4.2 Solubilization of PDG in Group 2 mitochondria using osmotic imbalance and digitonin combined with sonication**

Group 2 mitochondria were lyophilized as described previously. Digitonin solution (4 mg/10 mg protein) was prepared in the hypotonic phosphate-borax buffer and added to the lyophilized mitochondria to a final volume of 23 ml. Immediately after adding the digitonin solution the sample was sonicated three times for 10 seconds each time (with chilling on ice in between) using a 120 W Virsonic ultrasonic disintegrator, 50% power, VirTis (USA). The sample was incubated on ice for 30 minutes, then homogenized using a Dounce homogeniser (7 passes) followed by centrifugation at 20 000 x *g* for 30 minutes at 4°C using a Beckman Coulter J26-XPI centrifuge (USA). The supernatant was collected, designated **1<sup>st</sup> supernatant** and stored at -20°C. The pellet designated 1<sup>st</sup> pellet was resuspended in 11.5 ml hypotonic phosphate-borate buffer containing 4 mg digitonin/10 mg protein, sonicated, incubated, homogenized and centrifuged as per previous conditions. The supernatant was collected (**2<sup>nd</sup> supernatant**) and stored. The pellet (2<sup>nd</sup> pellet) was resuspended in 8 ml of hypotonic phosphate-borate buffer.

##### **2.4.2.1 Ammonium sulphate precipitation**

The 1<sup>st</sup> and 2<sup>nd</sup> supernatants from group 2 mitochondria were combined and proteins were precipitated using 70% ammonium sulphate. Most proteins were precipitated at 70% ammonium sulphate. The precipitate (AS) was collected using previously mentioned conditions, and resuspended in 7.5 ml hypotonic phosphate-borate buffer. This sample was divided between two separate dialysing tubes, the first tube was dialysed in low phosphate buffer (10 mM  $B_4Na_2O_7$ , 20 mM  $K_4P_2O_7$  and 20 mM  $KH_2PO_4$ , pH 8.6) and the other was dialysed in a hypotonic phosphate-borax buffer for 24 hours with buffer changes every 8 hours. The samples were collected and concentrated using Millipore Ultrafree-15 with Biomax-30K membrane centrifuge filter units. The collected fractions were assayed for PDG activity.

### **2.4.3 Solubilization of PDG in Group 3 mitochondria using lyophilization and digitonin combined with sonication**

Group 3 mitochondria were treated the same as group 2 with the following modifications. The lyophilized mitochondria were first resuspended in 6 ml of hypotonic phosphate-borax buffer and incubated on ice for 3 hours before adding the digitonin detergent solution. After the incubation period 3 ml digitonin solution (4 mg digitonin/mg protein) was added to the lyophilized mitochondria. This sample was homogenized as per previous conditions, the **1<sup>st</sup> supernatant** was then collected. After the collection of **1<sup>st</sup> supernatant** the resulting pellet was resuspended in 10.5 ml of hypotonic phosphate-borate buffer. The 1<sup>st</sup> pellet was re-homogenized and re-centrifuged to collect the **2<sup>nd</sup> supernatant**. The 2<sup>nd</sup> pellet was resuspended in 2 ml of hypotonic phosphate-borate buffer. The 1<sup>st</sup> and 2<sup>nd</sup> supernatants were concentrated separately using Millipore Ultrafree-15 with Biomax-10K membrane centrifuge filter units. The collected fractions were assayed for PDG activity.

## **2.5 Gel filtration chromatography**

### **2.5.1 Sephacryl S-100 HR column**

A 1.5 cm × 30 cm column was packed with Sephacryl S-100 HR under gravity. Gel filtration chromatography was conducted using an automated AKTA purifier system, GE Healthcare Life Sciences (USA). The column was equilibrated with 3 column volumes (CV) of deionized water and 2 CV of hypotonic phosphate-borate buffer at a flow rate of 0.3 ml/minute. The volume of sample injected into the column was 0.5 ml, a flow rate of 0.5 ml/minute was used and 2 ml fractions were collected. The maximum pressure was set at 0.15 MPa to avoid high pressure which would collapse the matrix bed. After the run was complete the column was washed with 2 CV of deionized water and 2 CV of 20% ethanol. All solutions used in this system were pre-filtered with a 0.22µm Millipore membrane filter. Collected fractions were assayed for glutaminase activity.

### **2.5.2 Sephacryl S-300 HR column**

A 1.0 cm × 60 cm Pharmacia column was packed with Sephacryl S-300 HR matrix under gravity. The matrix was degassed for three hours prior to packing the column. The column

was equilibrated with 3 CV of deionized water and 2 CV of hypotonic phosphate-borate buffer at as flow rate of 0.3 ml/minute. Gel filtration chromatography was conducted using the automated AKTA purifier system with the Sephacryl S-300 HR column connected. The chromatography conditions were set up as follows: 1 ml sample injection volume, 0.3 ml/minute flow rate and 2.5 CV column elution. For the initial run 3 ml fractions were collected but for the second run 1 ml fractions were collected starting immediately after the injection of sample. The maximum pressure was set at 0.15 MPa to avoid high pressure which would collapse the matrix bed. After the run the column was washed with 2 CV of deionized water and 2 CV of 20% ethanol. All solutions used in this system were pre-filtered with a 0.22  $\mu$ m Millipore membrane filter. The void volume of this column was determined using the same buffer and variables mentioned above, 0.5 ml of blue dextran 2000 was loaded into the column and the void volume was determined using UNICORN software.

### **2.5.3 Sephacryl S- 400 HR column**

A 1.0 cm  $\times$  60 cm Pharmacia column was packed with Sephacryl S-400 HR matrix under gravity. The matrix was degassed for three hours prior to packing the column. The column was equilibrated with 3 CV of deionized water and 2 CV of hypotonic phosphate-borate buffer at as flow rate of 0.3 ml/minute. Gel filtration chromatography was conducted using an automated AKTA purifier system, GE Healthcare Life Sciences (USA) with the Sephacryl S-400 HR column connected. The chromatography conditions were set up as follows: 0.47 ml sample injection volume, 0.3 ml/minute flow rate, 2.5 CV column elution and 1 ml fractions were collected starting immediately after sample injection. The maximum pressure was set at 0.15 MPa to avoid high pressure which would collapse the matrix bed. After the run the column was washed with 2 CV of deionized water and 2 CV of 20% ethanol. All solutions used in this system were pre-filtered with a 0.22  $\mu$ m Millipore membrane filter.

### **2.6 SDS-PAGE and Western blot analysis**

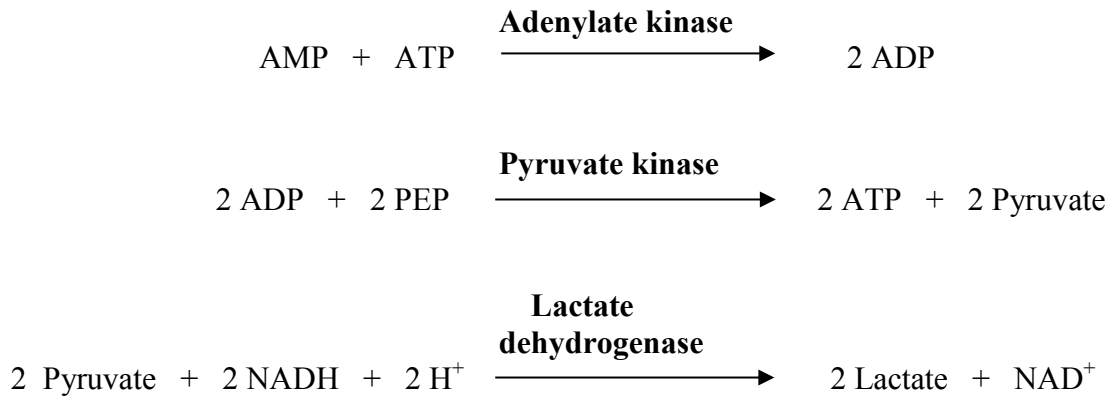
The proteins were resolved by SDS-PAGE (10% acrylamide), using the method by Laemmli (1970). Proteins were electrophoresed in Tris/Glycine/SDS buffer, pH 8.3 at 110 V using a Bio-Rad Mini-PROTEAN 3 Electrophoresis cell, BIORAD (South Africa). The proteins resolved in the gel were then transferred onto a nitrocellulose membrane using a Bio-Rad

Mini Trans-Blot Electrophoretic Transfer Cell, BIORAD (South Africa) run at 100 V for 2 hours. The membrane was blocked in Tris-buffered saline (20 mM Tris, 500 mM NaCl) plus 0.05% Tween 20 (v/v), pH 7.5 (TBST) containing 5% non-fat dry milk at 4°C overnight. The membrane was probed with rabbit anti-rat kidney glutaminase IgG antibody (anti-KGA) diluted (1:500) in TBST containing non-fat dry milk at room temperature for 1 hour at room temperature. The membrane was washed 2 times in TBST for 5 minutes each time. The membrane was then probed with goat anti-rabbit IgG alkaline phosphatase conjugate, diluted 1:1500 in TBST containing 5% non-fat dry milk for 1 hour at room temperature. The membrane was washed 3 times with TBST for 5 minutes each time. BCIP/NBT substrate was then added incubated at room temperature until bands developed followed by washing with deionized water to terminate the reaction. The antibody-bound proteins were visualized using a Syngene G-BOX Chem XR5, Vacutec (South Africa). Images were captured using the GeneSys software and the bands were quantified using GeneTools analysis software.

## **2.7 Enzyme assays**

### **2.7.1 Adenylate kinase**

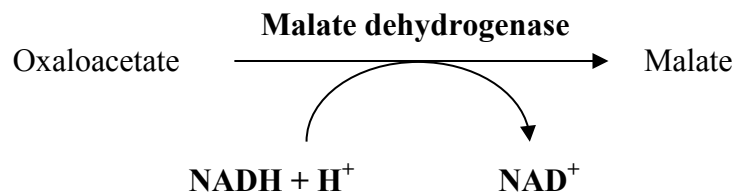
The activity of adenylate kinase was assayed spectrophotometrically using the method of Bergmeyer (1974). A buffer containing 50 mM triethanolamine, 13 mM MgCl<sub>2</sub>, 43 mM potassium chloride, 1 mM dithiothreitol, 0.3 mM phosphoenolpyruvate, 5.4 U/ml pyruvate kinase and 7 U/ml lactate dehydrogenase pH 7.6 was prepared. A 200 µl aliquot of sample was mixed with 2.3 ml of buffer followed by the addition of 100 µl 0.23 mM NADH and incubation for 1 minute at 24°C before the addition 200 µl 1.3 mM ATP. The change in absorbance was monitored at 340 nm using a Varian Cary 50 Conc. UV-Visible Spectrophotometer, USA. The change in absorbance after addition of ATP was due to removal of contaminating ADP. After 5 minutes 1.3 mM AMP was added to initiate adenylate kinase reaction and the reaction monitored for 10 minutes. Adenylate kinase activity was calculated as NADH oxidized using a molar extinction coefficient  $6.22 \times 10^3$  Mole<sup>-1</sup> cm<sup>-1</sup> (Figure 12).



**Figure 12: A coupled reaction used to assay the activity of adenylate kinase.**

### 2.7.2 Malate dehydrogenase assay

Malate dehydrogenase activity was determined as described by McGivan *et al.* (1980). Malate dehydrogenase activity was determined spectrophotometrically by measuring the rate of oxidation of NADH when oxaloacetate was converted to malate as shown in Figure 13. A 2.7 ml aliquot of 20 mM Tris and 100 mM KCl buffer, pH 7.4 was added to a cuvette followed by 1 mM NADH (100  $\mu$ l) and 0.5 ml sample which were then equilibrated at 25°C for 3 minutes. The reaction was started by adding 1 mM oxaloacetate (100  $\mu$ l). The change in absorbance was monitored for 5 minutes at 340 nm using a Varian Cary 50 Conc. UV-Visible Spectrophotometer, USA. Malate dehydrogenase activity was calculated as NADH oxidized using a molar extinction coefficient  $6.22 \times 10^3 \text{ Mole}^{-1} \text{ cm}^{-1}$ .



**Figure 13: Chemical reaction used to assay the activity of malate dehydrogenase.**

### 2.7.3 Glutamate dehydrogenase assay

A method by Schmidt (1974) was used to spectrophotometrically assay glutamate dehydrogenase activity based on the conversion of 2-oxoglutarate to glutamate in the presence of NADH and  $\text{NH}_4^+$  to  $\text{H}_2\text{O}$  and  $\text{NAD}^+$  as shown in Figure 14. The decrease in absorbance of NADH was monitored at 340 nm in a Varian Cary 50 Conc. UV-Visible spectrophotometer at 25°C after the sample was initially incubated with lactate dehydrogenase to remove pyruvate contaminating the sample. The oxidation of NADH to  $\text{NAD}^+$  is directly proportional to the activity of glutamate dehydrogenase. To 2.65 ml of 50 mM Triethanolamine buffer (pH 8.0) containing 0.2 mM NADH, 100 mM  $\text{NH}_4^+$  acetate, 1 mM ADP and 2.5 mM EDTA, 0.1 ml of the sample and 2 units of lactate dehydrogenase were added, mixed and incubated for 3 minutes while monitoring absorbance change. Following the incubation period, 7 mM 2-oxoglutarate prepared in the buffer was added and the change in absorbance was monitored for 5 minutes. Glutamate dehydrogenase activity was calculated as NADH oxidized using a molar extinction coefficient  $6.22 \times 10^3 \text{ Mole}^{-1} \text{ cm}^{-1}$ .

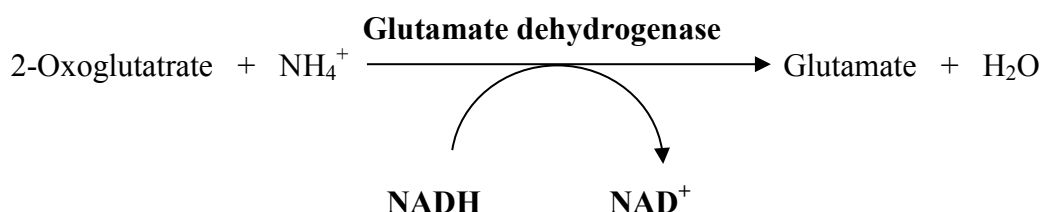


Figure 14: Chemical reaction used to monitor the activity of glutamate dehydrogenase.

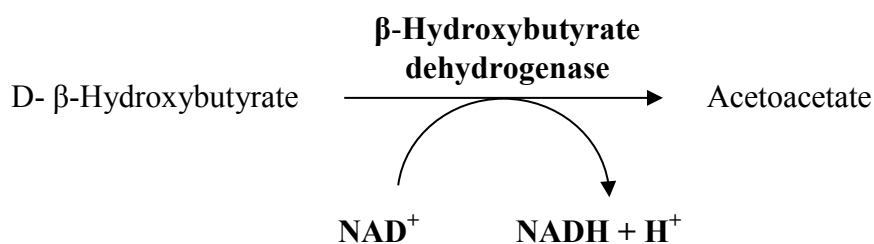
### 2.7.4 Cytochrome c oxidase assay

The method of Sottocasa *et al.* (1967) was used to assay the activity of cytochrome c oxidase. Cytochrome c oxidase activity was determined spectrophotometrically by measuring the rate of oxidation of reduced cytochrome c at 550 nm in a Varian Cary 50 Conc. UV-Visible Spectrophotometer thermostated at 30°C. Cytochrome c was first reduced using sodium borohydride crystals and excess borohydride was neutralized using 100 mM HCl. A cuvette containing 2.8 ml phosphate buffer (75 mM) and 0.150 ml reduced cytochrome c (0.08 mM) was placed in a spectrophotometer and equilibrated for 2 minutes while monitoring

absorbance change before adding 0.05 ml of sample. The absorbance was then monitored for 5 minutes. The molar extinction difference of  $18.7 \times 10^3 \text{ Mole}^{-1} \text{ cm}^{-1}$  was used to calculate the activity of cytochrome c oxidase. This molar extinction difference was attained by subtracting the molar extinction coefficient of oxidized cytochrome c ( $9.0 \times 10^3 \text{ cm}^{-1} \text{ Mole}^{-1}$ ) from molar extinction coefficient of reduced cytochrome c ( $27.7 \times 10^3 \text{ Mole}^{-1} \text{ cm}^{-1}$ ).

### 2.7.5 $\beta$ -Hydroxybutyrate dehydrogenase activity assay

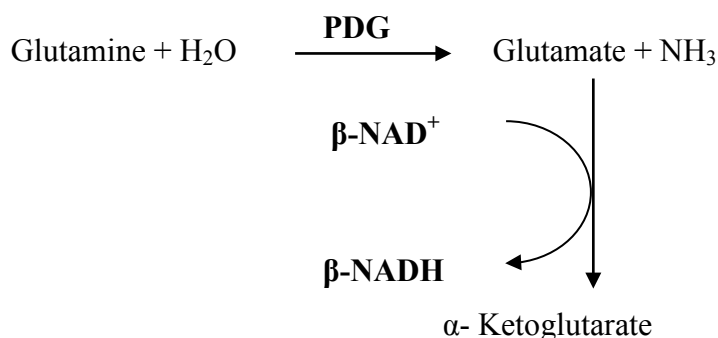
A procedure as described by Gotterer (1967) was used to spectrophotometrically assay the activity of  $\beta$ -hydroxybutyrate dehydrogenase by monitoring the conversion of D- $\beta$ -hydroxybutyrate to acetoacetate in the presence of  $\text{NAD}^+$  as shown in Figure 15. To 2.7 ml 75 mM buffer containing 1 mM  $\text{NAD}^+$ , 210 mM thioglycerol, 0.4  $\mu\text{mole}$  lipid phosphorus/mg protein and 0.1 ml sample was added mixed by inversion and incubated 45 minutes at  $22^\circ\text{C}$ . Following the incubation period 11 mM D- $\beta$ -hydroxybutyrate was added the solution was mixed by inversion and inserted into the spectrophotometer. The change in absorbance as  $\text{NAD}^+$  was reduced to  $\text{NADH}$  was monitored at 340 nm in a thermostated Varian Cary 50 Conc. UV-Visible spectrophotometer at  $22^\circ\text{C}$  for 10 minutes. The change in absorbance was directly proportional to the activity of  $\beta$ -hydroxybutyrate dehydrogenase.  $\beta$ -Hydroxybutyrate dehydrogenase activity was calculated as  $\text{NADH}$  oxidized using a molar extinction coefficient  $6.22 \times 10^3 \text{ Mole}^{-1} \text{ cm}^{-1}$ .



**Figure 15: Chemical reaction used to monitor the activity of  $\beta$ -hydroxybutyrate dehydrogenase.**

### 2.7.6 Phosphate dependent glutaminase activity assay

PDG activity was assayed at 37°C using a two-step procedure described by Curthoys and Weiss (1974) and modified by Masola and Zvinavashe (2003). The PDG activity was determined by measuring the amount of glutamate produced in the PDG catalysed reaction. The glutamate was determined from the amount of NADH produced when glutamate is oxidized by glutamate dehydrogenase at pH 9, as shown in Figure 16. The amount NADH was measured at 340 nm and is directly proportional to PDG activity.



**Figure 16: Chemical reaction used to monitor the activity of phosphate dependent glutaminase.**

Two buffers were used during the assay of PDG activity. Solution 1 had 10 mM potassium phosphate, 50 mM Tris and 120 mM KCl, pH 8.6. On the day of the experiment 20 mM glutamine was made up in solution 1. Solution 2 was constituted of 50 mM Tris and 0.31 M hydrazine, pH 9. On the day on which the experiment was conducted 2 mM NAD<sup>+</sup> and 0.5 mM ADP were added to solution 2 and the pH was adjusted to 9. A 50 µl aliquot of the sample was added into 200 µl solution 1 and the mixture was then incubated at 37°C for 15 minutes. The reaction was stopped by adding 120 µl of ice cold 30% perchloric acid, after which the test tubes were immersed in ice. After 5 minutes the mixture was neutralized with 225 µl ice cold 3 N KOH and a white precipitate was formed. To the mixture, 2 ml of solution 2 containing ADP and NAD<sup>+</sup>, was added followed by centrifugation at 3000 rpm for 5 minutes using a Hettich Zentrifugen D-7200 bench top centrifuge (Germany), to remove the precipitate. An aliquot of 10 µl of glutamate dehydrogenase in 50% glycerol (13 U) was added to the supernatant and incubated for 45 minutes at room temperature. The absorbance of samples was read at 340 nm using a Varian CARY 50 Conc. UV-Visible

Spectrophotometer, USA. Glutaminase activity was calculated from the amount of NADH formed using a molar extinction coefficient  $6.22 \times 10^3 \text{ Mole}^{-1} \text{ cm}^{-1}$ .

## **2.8 Protein determination**

Protein concentration was determined using a method by Lowry *et al.* (1951). Solutions were prepared as follows: Alkaline sodium carbonate reagent (20 mM  $\text{Na}_2\text{CO}_3$  in 0.1 M NaOH) and copper sulphate-sodium potassium tartrate solution (5 mM  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  in 10 mM Na, K tartrate). Alkaline reagent was prepared on the day of experiment by mixing alkaline sodium carbonate solution and copper sulphate-sodium potassium tartrate solution 50:1 whereas the Folin-Ciocalteu's reagent was prepared by diluting stock 1:2 with deionized water. To 0.5 ml of sample, 5 ml of alkaline reagent was added and incubated at  $40^\circ\text{C}$  for 15 minute. Samples were cooled followed by the addition of 0.5 ml Folin-Ciocalteu's reagent, vortexing and incubation at room temperature for 30 minutes. Absorbance was read at 600 nm using a Varian CARY 50 Conc. UV-Visible Spectrophotometer, USA. A range of BSA standards containing 20 - 100  $\mu\text{g}$  protein were used to construct a standard graph. The amount of protein in each sample and hence the concentration, was extrapolated from the standard curve graph.

## **2.9 Data analysis**

All data were expressed as mean  $\pm$  standard error of mean (SEM). Statistical analysis were performed using Graph Pad InStat Software (version5, GraphPad Software, San Diego, California, USA). Statistical comparisons of data between groups were done using one way one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test. Values of  $p < 0.05$  were taken as statistically significant.

## CHAPTER 3

### 3. Results

#### 3.1 Sub-mitochondrial localization of PDG in rat intestinal mitochondria

##### 3.1.1 Localization of PDG in sub-mitochondrial compartments

Mitochondria were successfully prepared from the rat small intestine and utilized for different experiments as presented throughout the results section. As shown in Table 1 below, the specific activities of PDG and cellular compartment marker enzymes in sub-mitochondrial fractions. Adenylate kinase was only found in the low speed supernatant and its specific activity in this fraction was approximately two-fold in intact mitochondria. The specific activity of malate dehydrogenase was significantly decreased in mitoplasts and significantly increased in the low speed supernatant compared to intact mitochondria. The specific activity of malate dehydrogenase in the sonicated mitoplasts pellet and sonicated vesicles was very low compared to that in the sonicated high speed supernatant. Glutamate dehydrogenase specific activity in mitoplasts and low speed supernatant was significantly decreased and increased, respectively, compared to that in intact mitochondria. Sonication of mitoplasts resulted in the highest specific activity of glutamate dehydrogenase being found in the sonicated high speed supernatant compared to that in the sonicated vesicles ( $p < 0.05$ ) and sonicated mitoplasts pellet. The specific activity of cytochrome c oxidase was highest in the mitoplasts and sonicated mitoplasts pellet with extremely low values ( $p < 0.05$ ) in the low and sonicated high speed supernatants compared to other fractions. Notably cytochrome c oxidase specific activity in the sonicated vesicles was much lower compared to that in the sonicated mitoplasts pellet but this was not accompanied by a high specific activity in the sonicated high speed supernatant. There was higher PDG activity in the mitoplast and low speed supernatant compared to mitochondria but the differences were not significant. The sonicated mitoplast pellet had decreased PDG activity compared to mitoplast but the decrease was not significant. A significant increase in PDG activity was observed in the sonicated high speed supernatant compared to sonicated mitoplast pellet and sonicated vesicles. No  $\beta$ -hydroxybutyrate dehydrogenase activity was detected in any of the rat intestinal mitochondria sub-mitochondrial fractions. However, rat liver mitochondria prepared as a positive control

showed  $\beta$ -hydroxybutyrate dehydrogenase activity (results not shown) using the same method used for rat intestinal mitochondria.

**Table 1: Localization of PDG and compartment marker enzymes in sub-mitochondrial fractions of intestinal mitochondria**

Enzymes	Specific activity (nmol substrate/min/mg protein)					
	Intact mitochondria	Mitoplasts	Low speed supernatant	Sonicated mitoplasts pellet	Sonicated vesicles	Sonicated high speed supernatant
Adenylate kinase	12.15 $\pm$ 1.48	0	27.39 $\pm$ 1.07	0	0	0
Malate dehydrogenase	429.3 $\pm$ 3.39	13.10 $\pm$ 3.45*	734.8 $\pm$ 5.93*	4.87 $\pm$ 1.75	5.26 $\pm$ 0.44	43.92 $\pm$ 5.51
Glutamate dehydrogenase	212.7 $\pm$ 2.04	81.69 $\pm$ 5.46*	389.4 $\pm$ 2.36*	39.47 $\pm$ 6.12 <sup>#<math>\delta</math></sup>	10.95 $\pm$ 2.92	126.4 $\pm$ 6.43 <sup><math>\delta</math><math>\gamma</math></sup>
Cytochrome c oxidase	123.0 $\pm$ 2.25	220.3 $\pm$ 5.98*	21.19 $\pm$ 2.46*	275.7 $\pm$ 6.21 <sup>#<math>\delta</math></sup>	148.1 $\pm$ 2.87	6.09 $\pm$ 0.68 <sup><math>\delta</math><math>\gamma</math></sup>
Phosphate dependent glutaminase	55.11 $\pm$ 0.08	72.02 $\pm$ 5.34	60.69 $\pm$ 4.53	44.01 $\pm$ 5.05	39.9 $\pm$ 4.21	99.36 $\pm$ 5.27 <sup><math>\delta</math><math>\gamma</math></sup>

Mitoplasts and low speed supernatant refers to the pellet and supernatant, respectively recovered after centrifugation at 25 000  $\times g$  of mitochondria treated with 2 mg digitonin/10 mg protein. The pellet that resulted after mitoplasts were sonicated and centrifuged at 25000  $\times g$  was denoted as sonicated mitoplasts pellet. The resultant supernatant was centrifuged at 100 000  $\times g$  to collect pellet (sonicated vesicles) and a sonicated high speed supernatant. The specific activity of PDG is presented as mean  $\pm$  SEM (n = 4). \*  $p < 0.05$  compared to intact mitochondria; #  $p < 0.05$  by comparison with mitoplasts, by  $\gamma$   $p < 0.05$  compared to sonicated mitoplast pellet and  $\delta$   $p < 0.05$  compared to sonicated vesicles.

As shown in Table 2 below shows the percentage of total enzyme activity recovered in the mitochondria, mitoplast and in low speed supernatant fractions. The total activity of

adenylate kinase recovered after fractionating mitochondria was greater than 100%, with all of the activity located in the low speed supernatant. Malate dehydrogenase had 74% of total activity recovered after mitochondria were fractionated and 95% of this activity was recovered in the low speed supernatant and only 5% in the mitoplast. The GDH activity recovered after fractionating mitochondria was 98%. Of this recovered activity, 76% was found in the low speed supernatant and 24% in the mitoplast. Cytochrome c oxidase recovery was 149% following fractionation of mitochondria showing activation of the enzyme due to digitonin treatment rendering the enzyme more accessible to interactions with the substrate. The majority (94%) of this activity was recovered in the mitoplast and only 6% was recovered in the low speed supernatant. The total recovered activity for PDG after fractionation of mitochondria was 62% showing loss of enzyme activity due to digitonin treatment. Of this remaining enzyme, 65% was recovered in the mitoplast and 35% in the low speed supernatant.

**Table 2: Recoveries of PDG and compartment marker enzymes in sub-mitochondrial fractions of intestinal mitochondria**

Enzymes	% of Total recovered activity			
	Mitochondria	Mitoplast + Low speed supernatant	Distribution of recovered activity in sub-mitochondrial fractions	
			Mitoplast	Low speed supernatant
Adenylate kinase	100	103	0	100
Malate dehydrogenase	100	74	5	95
Glutamate dehydrogenase	100	98	24	76
Cytochrome c oxidase	100	149	94	6
Phosphate dependent glutaminase	100	62	65	35

The total recovered activities in the mitochondria were expressed as 100%. The recoveries in the mitoplast and low speed supernatant show the amount recovered in each fraction as a percentage of the total recovered from both fractions.

As shown in Table 3 below shows the total enzyme activity recovered in the sonicated mitoplast pellet, sonicated vesicles and sonicated high speed supernatant fractions. No adenylate kinase activity was recovered in any of the above mentioned fractions. The activity of malate dehydrogenase recovered in the fractions was 31% in the sonicated mitoplast, 5% in sonicated vesicles and pellet and 64% in the sonicated high speed supernatant. The distribution of glutamate dehydrogenase for the respective fractions was 34%, 1% and 65% whereas that of cytochrome c oxidase 89%, 10% and 1%. PDG had 34% recovered in the sonicated mitoplast fraction, 7% in sonicated vesicles and 59% in the sonicated high speed supernatant.

**Table 3: Recoveries of PDG and compartment marker enzymes in sub-mitoplast fractions of intestinal mitochondria**

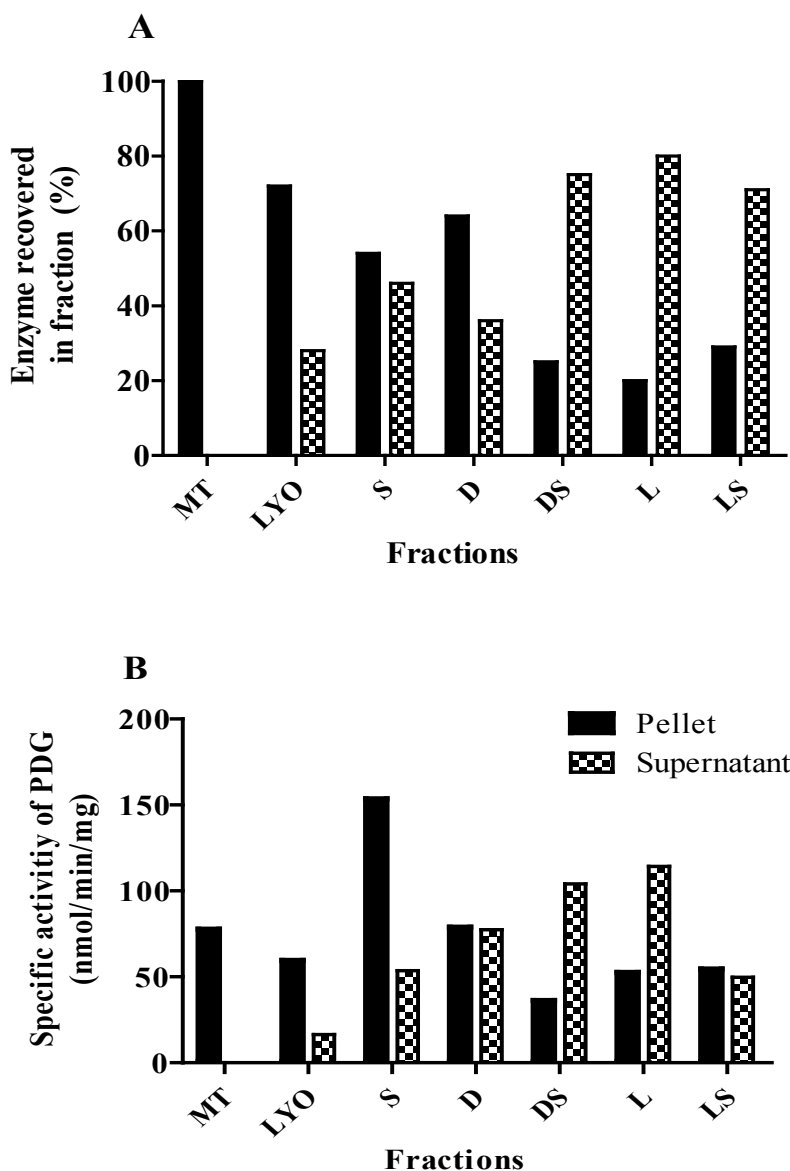
Enzymes	Total enzyme activity of sonicated mitoplast pellet + sonicated vesicles + sonicated high speed supernatant (nmol/min) (%)	% of Total recovered activity		
		Sonicated mitoplast pellet	Sonicated vesicles	Sonicated high speed supernatant
Adenylate kinase	0 (0)	0	0	0
Malate dehydrogenase	100 (5)	31	5	64
Glutamate dehydrogenase	100 (24)	34	1	65
Cytochrome c oxidase	100 (94)	89	10	1
Phosphate dependent glutaminase	100 (65)	34	7	59

The total activity in the sonicated mitoplast pellet + sonicated vesicles + sonicated high speed supernatant is taken to be 100%. The figures in parenthesis show the amount (%) that was recovered in the mitoplast fractions in Table 2 which were subjected to sonication with this activity being recovered/ distributed in the three fractions.

Thus following sonication of mitoplasts approximately the same percentage (31-34%) of malate dehydrogenase, GDH and PDG was found in the sonicated mitoplast pellet and 59-65% of the same enzymes were found in the sonicated high speed supernatant. This was in contrast to the cytochrome c oxidase which had 89% and 1% in the respective fractions.

### **3.1.2 Solubilization profile of PDG using lyophilization, detergents and sonication**

Different methods were attempted in an endeavour to solubilize PDG of rat small intestinal origin in an active and stable form. Figure 17 A below shows the total activity of PDG recovered in the supernatants (solubilized PDG) and pellets (membrane bound PDG) after rat intestinal mitochondria were treated with different methods to solubilize PDG. Figure 17 B below shows the specific activity of the fractions. The untreated mitochondria had a PDG specific activity of 77.8 nmol glutamate formed/min/mg protein. Lyophilizing mitochondria resulted in 28% of PDG total activity to be recovered in the supernatant. The pellet which resulted after lyophilization was divided into five different fractions; these were treated with different methods to solubilize PDG. When lyophilized mitochondria were sonicated 46% PDG total enzyme activity was recovered in the sonicated supernatant and its specific activity increased 3-fold compared to the specific activity of PDG found in the lyophilized supernatant. Treating lyophilized mitochondria with digitonin and digitonin combined with sonication resulted in 36% and 75% PDG total activity being recovered in the supernatant fractions, respectively. The specific activity of PDG increased 6-fold in the digitonin supernatant due to treatment with digitonin compared to the lyophilized supernatant. The highest solubilized PDG enzyme recovery was observed in the lubrol supernatant, 80% enzyme activity was recovered in this fraction and its specific activity increased 7-fold compared to lyophilized mitochondria. Due to the effects of sonication less PDG enzyme activity was recovered in the lubrol combined with sonication supernatant fraction compared to lubrol supernatant.

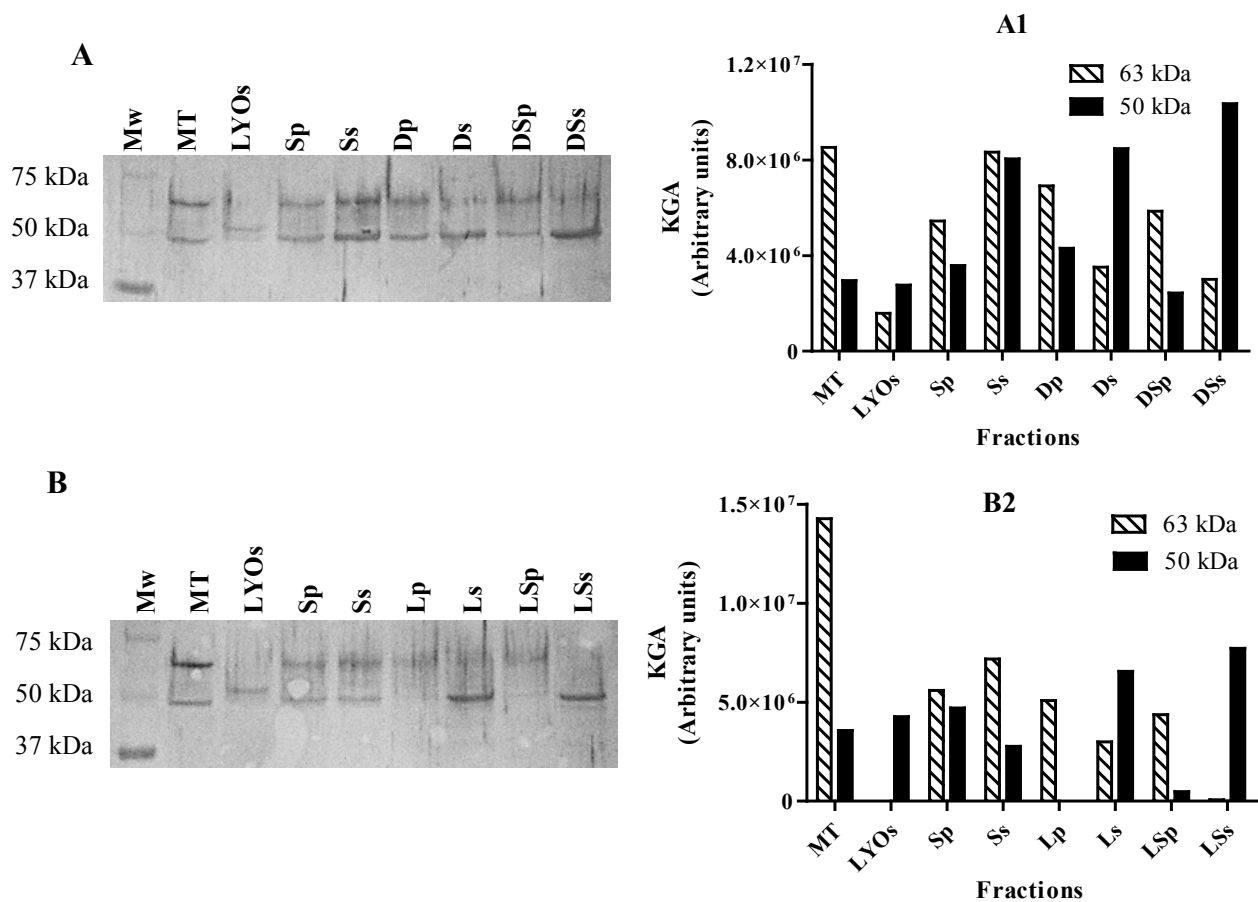


**Figure 17: Total activity percentage (A) and specific activity (B) of PDG recovered in the pellet (unsolubilized PDG) and the supernatant (solubilized PDG) fractions after rat intestinal mitochondria were subjected to different solubilizing procedures. MT : untreated intestinal mitochondria, LYO : lyophilized mitochondria, S : sonicated LYO pellet which was re-homogenized, D : digitonin treated LYO pellet, DS : digitonin + sonication treated LYO pellet, L : lubrol treated LYO pellet and LS : lubrol+ sonication treated LYO pellet.**

### 3.1.2.1 Western blot of solubilized PDG (KGA-type) from rat intestinal mitochondria

Fractions which were collected after PDG was solubilized using different procedures were separated according to their molecular weights using SDS-PAGE and PDG bands present in

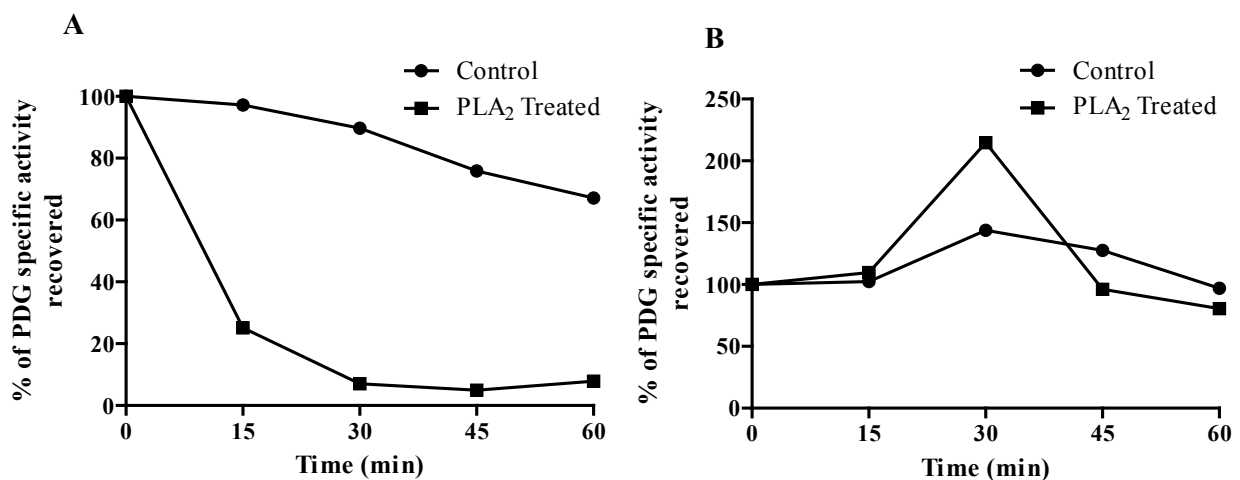
those fractions were detected using Western blotting. Figure 18 A and B show two bands of PDG with molecular weights of 63 kDa and 50 kDa which were identified using Western blot analysis. The two bands were detected in all the fractions except the lyophilized mitochondria, lubrol treated pellet and lubrol + sonicated pellet. The lyophilized mitochondria supernatant contained the 50 kDa PDG whilst the lubrol treated pellets contained the 63 kDa PDG. Densitometry analysis showed that the detergent treated supernatant fractions contained more of the 50 kDa PDG showing that it was more soluble (Figure 18 A1 and B1).



**Figure 18: Western blot analysis of solubilized and unsolubilized glutaminase from digitonin treated mitochondria (A) and lubrol treated mitochondria (B) with or without sonication. 10 µg proteins were loaded in each well. Densitometry values were used to compare the distribution of KGA bands in the digitonin and lubrol Western blot (A1) and (B1), respectively. Mw - molecular weight marker, MT - mitochondria, LYO - lyophilized mitochondria supernatant, Sp - sonicated mitochondria pellet, Ss - sonicated mitochondria supernatant, Dp - digitonin pellet, Ds - digitonin supernatant, DSP - digitonin sonicated pellet, DSs - digitonin sonicated supernatant, Lp- lubrol pellet, Ls - lubrol supernatant, LSp - lubrol sonicated pellet and LSs - lubrol sonicated supernatant.**

### 3.1.3 Effect of Phospholipase A<sub>2</sub> treatment on PDG activity

The dependency of PDG activity on the presence of phospholipids was investigated by treating intact mitochondria and solubilized PDG with phospholipase A<sub>2</sub> (Figure 19). Intact mitochondria retained 67% PDG activity after 60 minutes, while exposing the intact mitochondria to phospholipase A<sub>2</sub> for 15 minutes resulted in 80% loss of PDG activity compared to activity at 0 minutes. At 30 and 60 minutes intact mitochondria only had 7% PDG activity remaining with a further 2% activity loss observed at 45 minutes compared to activity at 0 minutes (Figure 19 A).



**Figure 19: Percentage of PDG specific activities recovered in intact rat intestinal mitochondria (A) and solubilized PDG (B) treated with phospholipase A<sub>2</sub> (PLA<sub>2</sub>). Data points are means of two determinations. The average specific activity of PDG at time zero for intact mitochondria and solubilized PDG was 20.7 and 14.7 nmol glutamate formed/min/mg protein, respectively.**

Solubilized PDG showed little increase in PDG specific activity after 15 minutes incubation compared to activity at 0 minutes (Figure 19 B). A further 44% increase was observed after 30 minutes incubation compared to activity at 0 minutes, but after 45 and 60 minutes PDG activity decreased to 116% and 97%, respectively compared to activity at 0 minutes. Solubilized PDG showed a 10% increase in PDG specific activity after 15 minutes of exposure to phospholipase A<sub>2</sub> compared to activity at 0 minutes. A further drastic increase of 105% was observed after 30 minutes of incubation. After 45 and 60 minutes the activity of solubilized PDG dropped respectively to 96 and 81% of control value (Figure 19 B). Thus, at

60 minutes the activity of solubilized PDG remained high - did not drop below 80% of the initial value regardless of whether samples were treated with phospholipaseA<sub>2</sub> or not.

### 3.1.4 Effects of sulphhydryl reagents on PDG activity

The orientation aspect of PDG localization was further evaluated using Mers (Mers) and NEM. As shown in Table 4 below shows the inhibition of PDG activity by membrane permeant (NEM) and membrane non-permeant (Mers) sulphhydryl reagents in intact and sonicated rat intestinal mitochondria. Sonication of the mitochondria lead to a significant loss of PDG activity compared to unsonicated mitochondria.

**Table 4: Effect of sulphhydryl reagents on glutaminase activity in intestinal mitochondria**

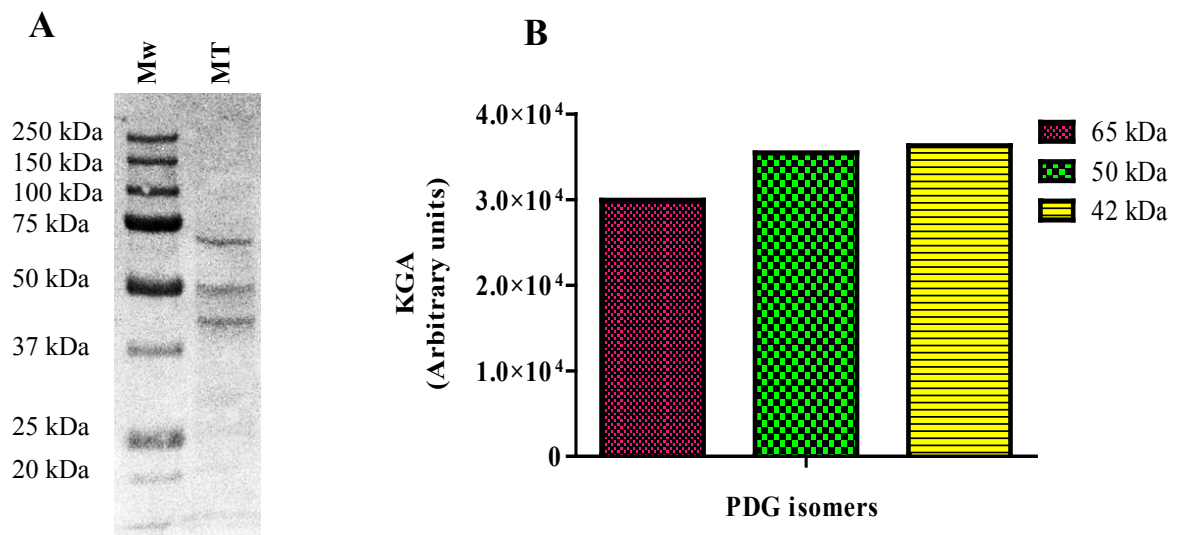
Treatment	PDG specific activity (nmol glutamate formed/min/mg)	
	Unsonicated mitochondria	Sonicated mitochondria
	131.2 ± 13.45	59.59 ± 5.59 <sup>#</sup>
<b>1 mM NEM</b>	53.25 ± 6.03 <sup>#</sup>	19.81 ± 3.10 <sup>*</sup>
<b>1 mM Mers</b>	1.80 ± 0.48 <sup>#</sup>	1.03 ± 0.36 <sup>*</sup>
<b>0.1 mM Mers</b>	34.20 ± 0.50 <sup>#</sup>	15.74 ± 0.54 <sup>*</sup>

The values above are means ± SEM (n = 4). NEM = N-ethylmaleimide and MERS = Mers. # *p* < 0.05 compared to unsonicated mitochondria and \* *p* < 0.05 compared with sonicated mitochondria.

There was also a significant decrease in PDG activity in mitochondria treated with either NEM or Mers compared to unsonicated mitochondria (Table 4). Treating mitochondria with 1 mM Mers almost completely inhibited the activity of PDG while mitochondria treated with an equal concentration of NEM retained approximately a third of PDG activity.

### 3.2 Western blot of mitochondria prepared in the presence of serine protease inhibitors

We investigated whether the two intestinal PDG bands that were previously detected using Western blotting were as a result of protease degradation. For this experiment protease inhibitors were used during the preparation of this mitochondria sample. We used PMSF and benzamidine which are serine protease inhibitors (Perez-Gomez *et al.*, 2003). Figure 20 shows three bands of PDG detected in mitochondria prepared in the presence of protease inhibitors, with a molecular weight of 65 kDa, 50 kDa and 42 kDa. The intensity of the 50 kDa and 42 kDa bands was greater than the 65 kDa band.



**Figure 20: Western blot analysis of rat intestinal mitochondria PDG prepared in the presence of 0.5 mM PMSF and 0.5 mM Benzamidine protease inhibitors (A). (B) The distribution of PDG bands evaluated using densitometry values. Mw: molecular weight marker and MT: mitochondria; 40  $\mu$ g protein was loaded. The Santa Cruz Biotechnology KGA-type antibody was used to probe the blot.**

### 3.3 Purification of PDG from rat small intestine

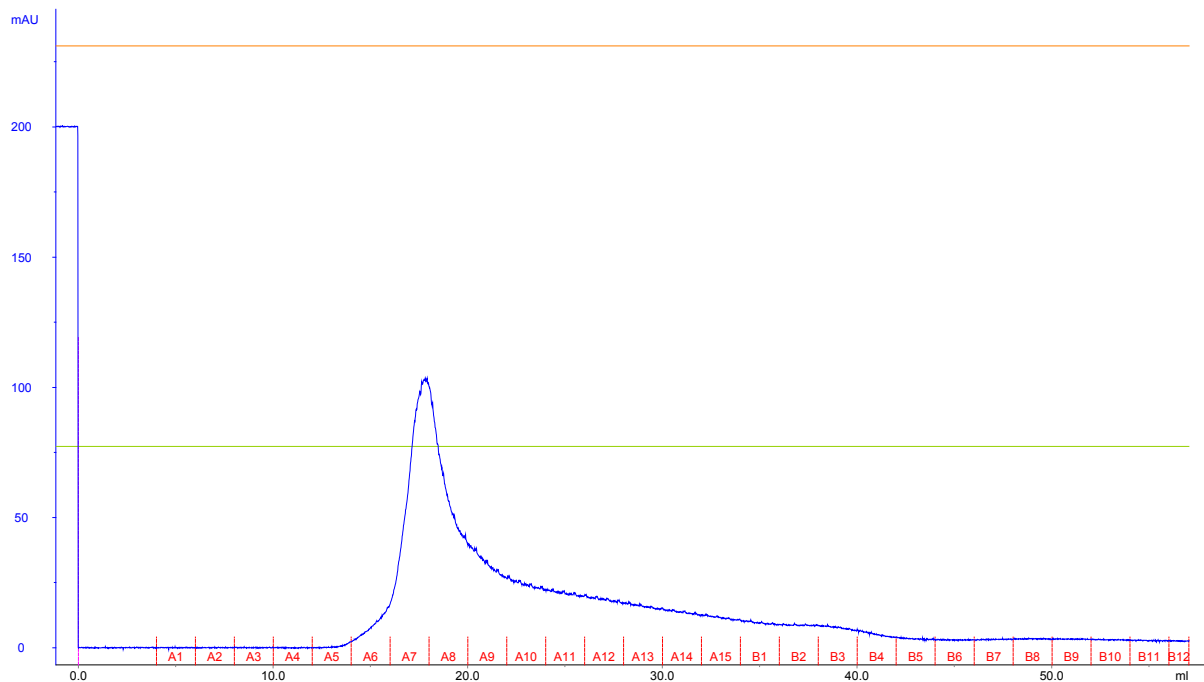
#### 3.3.1 Group 1 mitochondria

Mitochondria in this group were utilized to investigate whether ammonium sulphate fractionation may be used as an initial step towards purifying rat intestinal PDG and whether ammonium sulphate would preserve the activity of rat intestinal PDG as had been reported

for other glutaminases purified from various organs. Rat intestinal mitochondria were prepared and PDG was solubilized using the lubrol detergent method. The 1<sup>st</sup> and 2<sup>nd</sup> supernatants extracted from group 3 mitochondria were combined and fractionated using a gradient (20%, 40%, 70% and 100%) of ammonium sulphate. After dialysis of these fractions no PDG activity could be recovered. It was also observed that during dialysis, a precipitate formed that was possibly the lubrol detergent since it tended to solidify at low temperature. This characteristic did not favour any downstream purification process hence the digitonin + sonication method was utilized with group 2 mitochondria.

### **3.3.2 Purification of PDG from group 2 mitochondria on Sephacryl S-100 HR gel filtration chromatography column**

PDG from group 2 mitochondria were solubilized with digitonin + sonication and precipitated with 70% ammonium sulphate. The 1<sup>st</sup> +2<sup>nd</sup> supernatant proteins were precipitated using 70% ammonium sulphate since previously while using group 1 mitochondria most proteins were precipitated at this concentration. The precipitate was divided into two fractions and dialysed in different buffers. One fraction was dialysed against a low phosphate buffer (10 mM  $B_4Na_2O_7$ , 20 mM  $K_4P_2O_7$  and 20 mM  $KH_2PO_4$ , pH 8.6) to keep the concentration of buffer at a minimum, a condition which is compulsory for ion-exchange chromatography. There was no PDG activity that could be detected after this fraction was subjected to dialysis. The other fraction was dialysed against a phosphate-borate buffer (10 mM  $B_4Na_2O_7$ , 100 mM  $K_4P_2O_7$  and 100 mM  $KH_2PO_4$ , pH 8.6), the high phosphate amounts contained in this buffer are essential for activating PDG. The latter fraction was then loaded onto a gel filtration chromatography column. However no PDG active fraction was recovered in any of the fractions eluted from the Sephacryl S-100 HR column and 1<sup>st</sup> + 2<sup>nd</sup> supernatant proteins could not be separated/purified on Sephacryl S-100 HR as one broad peak with a drag was seen (Figure 20).

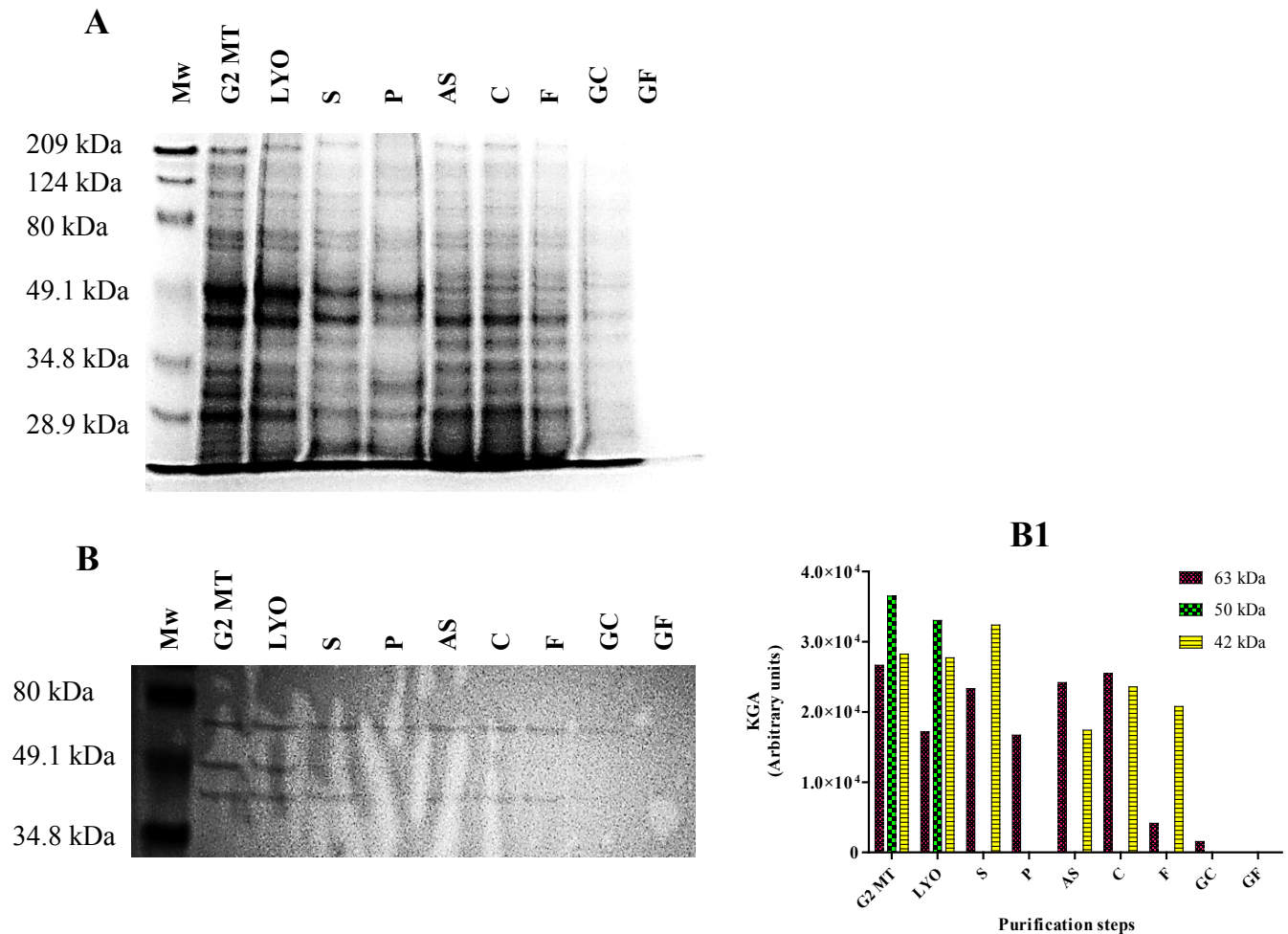


**Figure 21: Elution profile of PDG combined 1<sup>st</sup> + 2<sup>nd</sup> supernatant proteins acquired from group 2 mitochondria. Proteins were separated using a Sephacryl S-100 HR gel filtration chromatography column.**

### **3.3.2.1 SDS-PAGE and Western Blot analysis of fractions from Group 2 rat intestinal mitochondria**

Figure 22 A, below shows the distribution of proteins in a 10% polyacrylamide reducing gel after mitochondria were treated with different methods to solubilize and purify PDG. The protein bands of 1<sup>st</sup> + 2<sup>nd</sup> supernatant (S) on coomassie stained gel are less intense than those of G2 mitochondria and G2 lyophilized mitochondria. AS fractionation resulted in some proteins being lost to the filtrate (F), amongst them were KGA “isozymes” as seen on the Western blot and densitometry analysis (Figure 22 B and B1). The protein profile of GC is not directly comparable to other samples since the amount of protein loaded was lower compared to the other samples. However, it is noteworthy that after concentrating GC all proteins were retained in the concentrate, none were lost to the filtrate. KGA bands of 63 kDa, 50 kDa and 42 kDa were identified in group 2 mitochondria and group 2 lyophilized mitochondria. With subsequent solubilization and purification of PDG the 50 kDa band in the S, AS, C and F fractions disappeared, and only the 62 kDa band was retained in the P fraction. This shows that the 50 kDa isozyme was possibly more easily solubilized and was not precipitated by ammonium 70% sulphate while the 42 kDa band observed across

treatments may be as a result of proteolysis. The above mentioned method used for group 2 mitochondria was adapted and further optimized to favour purification of PDG in an active form. For group 3 mitochondria no ammonium sulphate precipitation was conducted, instead 10 kDa centrifugal filters were utilized to concentrate samples.



**Figure 22: Coomassie stained 10% polyacrylamide SDS-PAGE (A) and Western blot (B) of glutaminase isolated from rat small intestinal group 2 mitochondria. In lanes G2 MT - F: 20 µg proteins were loaded; in lane GC: 8 µg and in lane GF: 26 µl (no protein was detected therefore maximum volume in a well was loaded). Densitometry values were used to compare the distribution of PDG bands in the Western blot (B1). Mw - Molecular weight marker, G2 MT - Group 2 mitochondria, LYO - G2 lyophilized mitochondria, S- 1<sup>st</sup> + 2<sup>nd</sup> supernatant, P- Pellet recovered after removing 1<sup>st</sup> + 2<sup>nd</sup> supernatant, AS - 70% ammonium sulphate precipitate of 1<sup>st</sup> + 2<sup>nd</sup> supernatant proteins, C- Concentrate of AS sample, F - Filtrate recovered after concentrating AS, GC- Concentrate of A7-A9 fractions of Sephacryl S-100 HR column and GF- Filtrate recovered after concentrating GC.**

### 3.3.2.2 Recoveries of PDG activity in Group 2 mitochondria after solubilization and purification steps

The results in Table 5 show the recovery of PDG activity in the mitochondrial fractions resulting from the lyophilization and purification procedures for group 2 mitochondria. These results show that disrupting mitochondrial membranes by lyophilization resulted in a slight increase in PDG specific activity. Further disrupting lyophilized mitochondria membranes with digitonin released most PDG to the 1<sup>st</sup> supernatant.

**Table 5. Purification profile of PDG from group 2 rat intestinal mitochondria**

Purification step	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min/mg)	Purification (fold)	Yield (%)
<b>Group 2 mitochondria</b>	51.9	601.80	11.60		100
<b>Lyophilized mitochondria</b>	50.68	727.02	14.35	1	>100
<b>1<sup>st</sup> supernatant</b>	30.92	827.49	26.76	2	>100
<b>2<sup>nd</sup> supernatant</b>	16.63	254.45	15.31	1	42
<b>Ammonium sulphate</b>	46.98	0	0	-	-
<b>Pooled fractions A7 – A 9 from Sephacryl S-100 HR</b>	1.8	0	0	-	-

Note that 1 ml of dialysed ammonium sulphate precipitated proteins containing 3.8 mg/ml was loaded into the Sephacryl S-100 HR column.

Smaller amounts of PDG proteins were solubilized into the 2<sup>nd</sup> supernatant fraction compared to the 1<sup>st</sup> supernatant fraction (Table 5). The inhibition of PDG caused by ammonium sulphate was irreversible since even after attempting to purify PDG proteins using a phosphate-borate buffer as a running and eluting buffer in the Sephacryl S-100 HR column no PDG activity could be recovered.

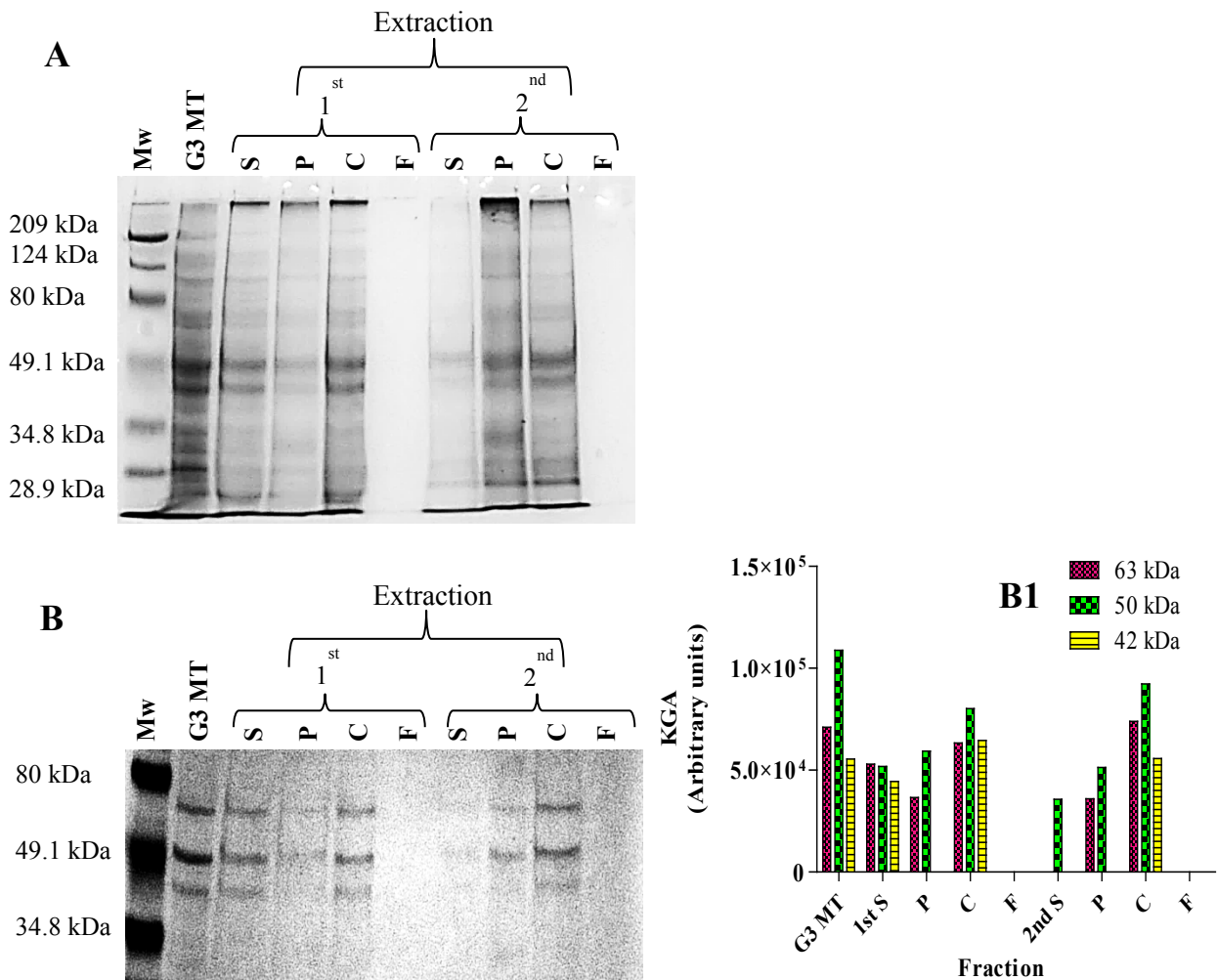
### **3.3.3 Solubilization and partial purification profile of group 3 rat intestinal mitochondria**

Figure 23 A, shows an SDS-PAGE gel of group 3 rat intestinal mitochondria proteins solubilized in an active form using an optimised method. This new optimized method involves pre-incubation of lyophilized mitochondria in a hypotonic phosphate-borate buffer for 3 hours before solubilizing PDG with digitonin detergent + sonication. A greater amount of proteins were recovered in the 1<sup>st</sup> supernatant than in the 1<sup>st</sup> pellet, denoting that most proteins were solubilized (see Table 6). The protein of 1<sup>st</sup> supernatant and 2<sup>nd</sup> supernatant were successfully concentrated without any loss of proteins to the filtrate. Fewer proteins were solubilized into the 2<sup>nd</sup> supernatant but this sample may not be directly compared to others since a lower amount of proteins was loaded into the gel. It is noteworthy that after this sample was concentrated the protein profile resembled that of the 1<sup>st</sup> supernatant concentrate. The Western blot in Figure 23 B shows that the distribution of KGA bands recovered in different fractions after solubilization of PDG are consistent with results found in Figure 20, as three bands were detected. All three bands were detected in G3 mitochondria, 1<sup>st</sup> supernatant and concentrated 1<sup>st</sup> + 2<sup>nd</sup> supernatants. In both pellets remaining after removal of the 1<sup>st</sup> and 2<sup>nd</sup> supernatant, only the 63 kDa and 50 kDa bands were observed. The dominance and presence of these bands varied from fraction to fraction but the 50 kDa band was consistently dominant in all fractions (Figure 23 B1). All three bands were observed throughout the solubilization procedure. No bands were detected in the filtrates.

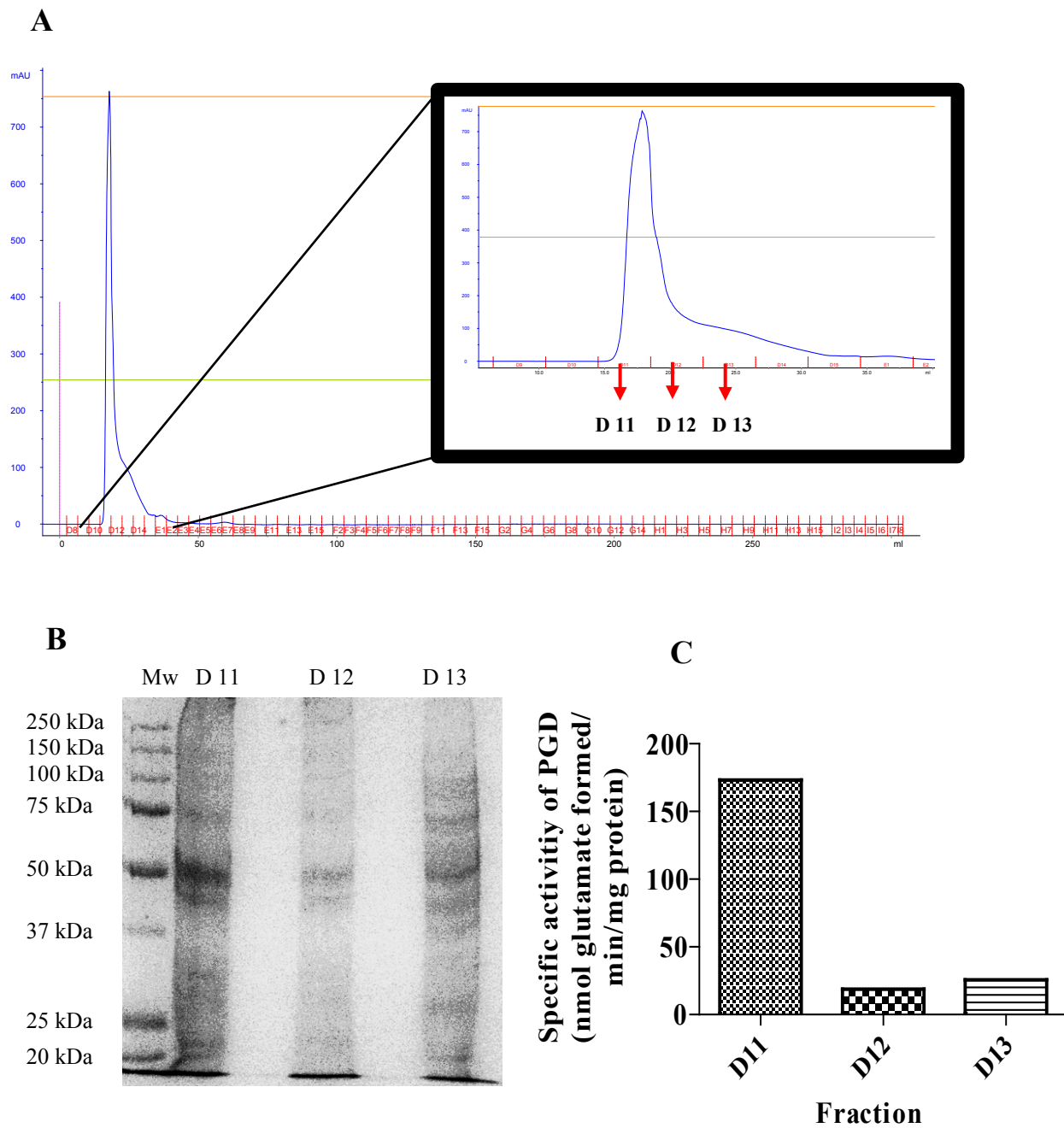
#### **3.3.3.1 Purification of PDG from group 3 mitochondria in Sephacryl S-300 HR gel filtration chromatography column, 3 ml fractions were collected**

Solubilized proteins acquired from group 3 mitochondria (1<sup>st</sup> supernatant) were separated on a Sephacryl S-300HR column. PDG was eluted starting from 16 ml up to 26 ml but most of it eluted at 18 ml (in reference to the PDG activity recovered in fraction D11) (Figure 24 C); therefore PDG was eluted in the void volume compared to the elution profile of blue dextran 2000. PDG activity was recovered in fractions D11 to D13 with the highest specific activity recovered in fraction D11 (Figure 24 C). No activity was recovered beyond fraction D13. Figure 24 B, indicates that although proteins were partially separated in the Sephacryl S-300HR column the variety and concentration of proteins which eluted into one fraction

particularly Fractions D11 and D13 was high. Therefore, in the next purification procedure all parameters were kept the same except 1 ml fractions were collected.



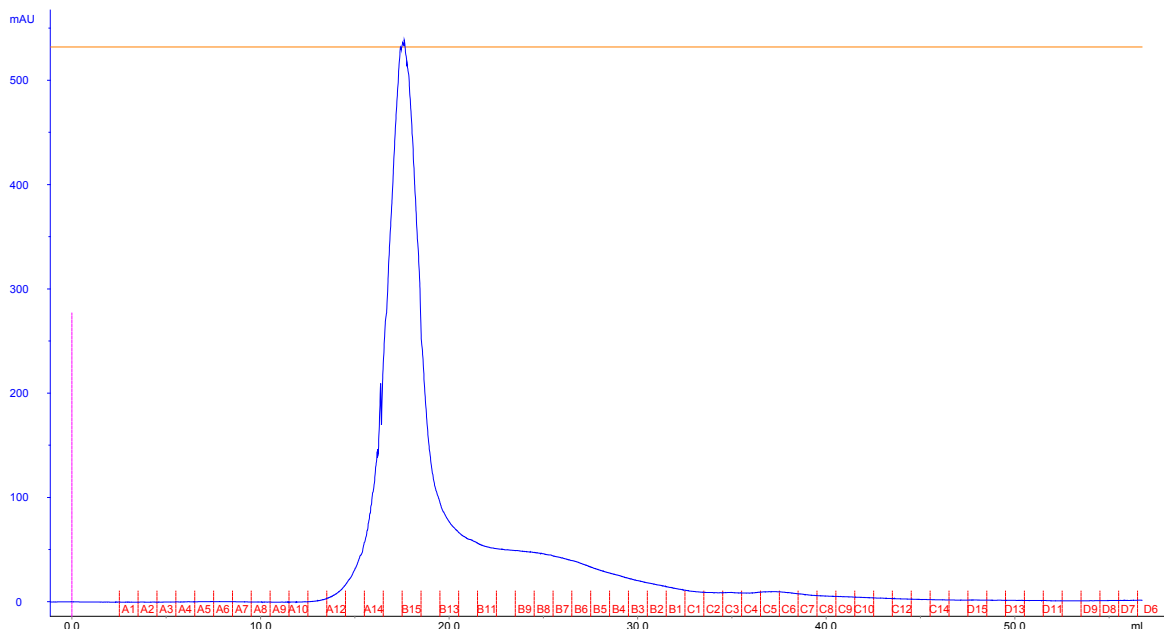
**Figure 23: Coomassie stained 10% polyacrylamide SDS-PAGE (A) and Western blot of KGA (B) solubilized from rat small intestinal group 3 mitochondria. In lanes G3 MT – 1<sup>st</sup> C and lanes 2<sup>nd</sup> P -2<sup>nd</sup> C had 30 µg protein, 2<sup>nd</sup> S had 11 µg, 1<sup>st</sup> F and 2<sup>nd</sup> F had 2 µg loaded. Densitometry values were used to show the distribution of KGA bands on the Western blot (B1). Mw - Molecular weight marker, G3 MT - Group 3 mitochondria, 1<sup>st</sup> : S- 1<sup>st</sup> supernatant , P - Pellet recovered after extraction of 1<sup>st</sup> supernatant, C - Concentrate of 1<sup>st</sup> supernatant, F - Filtrate recovered after concentrating C, 2<sup>nd</sup> : S - 2<sup>nd</sup> supernatant, P - Pellet recovered after extraction of 2<sup>nd</sup> supernatant, C - Concentrate of 2<sup>nd</sup> supernatant and F - Filtrate recovered after concentrating C.**



**Figure 24. (A)** Elution profile of 1<sup>st</sup> supernatant concentrate proteins acquired from group 3 mitochondria on a Sephacryl S-300HR gel filtration chromatography column when 3 ml fractions were collected. The volume loaded into the column was 1 ml which had 10.09 mg/ml protein. **(B)** Coomassie stained 10% polyacrylamide SDS-PAGE of fractions D11, D12 and D13 eluted from Sephacryl S-300HR column, 20  $\mu$ g protein was loaded and **(C)** PDG specific activity in D11, D12 and D13 fractions.

### 3.3.3.2 Purification of PDG on Sephacryl S-300 HR chromatography column, 1 ml fractions were collected

PDG was eluted in fractions 17 ml to 24 ml (Figure 25) as determined by the specific activity of PDG acquired in fraction A15 to B 10 (Figure 26 C), fractions beyond B 10 did not have PDG activity. Fraction A15 and B15 were partially eluted in the void volume (as determined by comparing to the elution of blue dextran 2000) and the rest of PDG proteins were continually eluted up to 24 ml. Therefore, the native molecular weight of PDG using this procedure could not be determined.

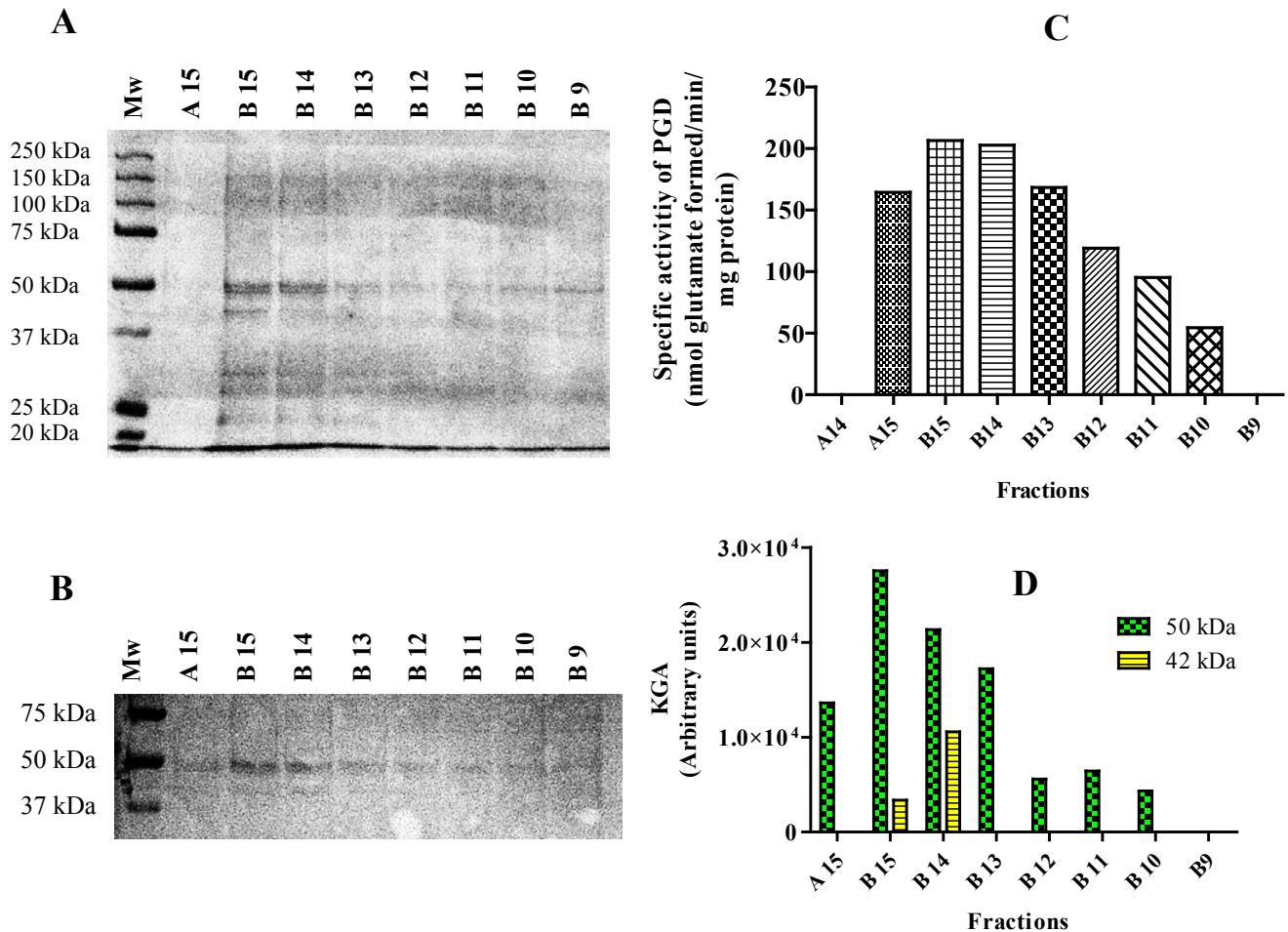


**Figure 25: Elution profile of 1<sup>st</sup> supernatant concentrate proteins acquired from group 3 mitochondria, separated on Sephacryl S-300 HR gel filtration chromatography column. The sample volume loaded into the column was 1 ml which had 10.09 mg/ml protein.**

#### 3.3.3.2.1 Investigating the presence and purity of PDG enzyme in 1 ml fraction eluted from Sephacryl S-300 HR

Figure 26 below shows the purification of PDG from solubilized 1<sup>st</sup> supernatant proteins of group 3 mitochondria on a Sephacryl S-300 HR gel filtration column. Eluates were collected as 1 ml fractions. Proteins with PDG activity were eluted in fractions A15 to B10 (Figure 26

C). A Western blot confirmed the presence of the 50 kDa and 42 kDa KGA bands in fractions A15 –B 10 (Figure 26 B). Using densitometry the 50 kDa band was observed to be the most prominent throughout all active fractions, compared to the 42 kDa band.



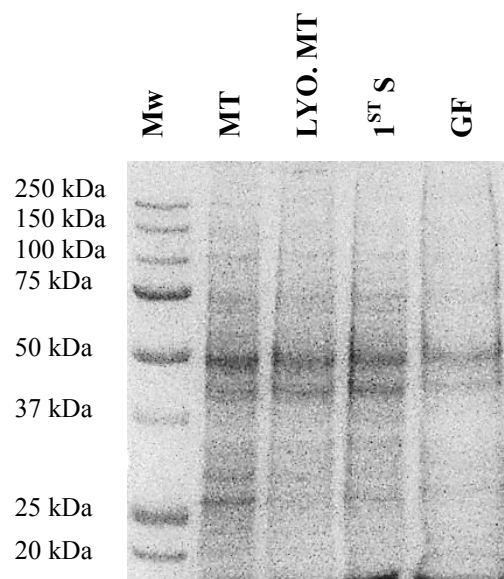
**Figure 26: Coomassie stained 10% polyacrylamide SDS-PAGE (A) and Western blot of KGA (B) from Sephacryl S-300 HR 1 ml fractions. In each lane the sample volume loaded was constant (26  $\mu$ l) but contained varying protein amount; A15: 9  $\mu$ g, B15: 25  $\mu$ g, B14: 21  $\mu$ g, B13: 17  $\mu$ g, B12: 15  $\mu$ g in B11, B10 and B9: 14  $\mu$ g. The specific activity of PDG recovered from the eluted fractions (C). Densitometry values were used to compare the distribution of KGA bands in the Western blot (D).**

To determine the purity of these fractions an SDS-PAGE gel was used. It can be seen that the intensity of protein bands in this fraction (Figure 26 A) was lower than that of D11-D13 fractions (Figure 24 B). However, for Figure 26 A, a direct comparison was not possible since different protein concentrations were loaded into the SDS-PAGE gel. A volume of 26

µl (maximum volume) as described in the legend was loaded in each lane since these samples had low protein concentrations. If equal protein amounts had been loaded in all lanes each lane would have had 9 µg protein a protein amount which could not be detected as shown in Figure 26 A and B.

### 3.3.3.3 Partial purification profile of PDG acquired from group 3 rat intestinal mitochondria

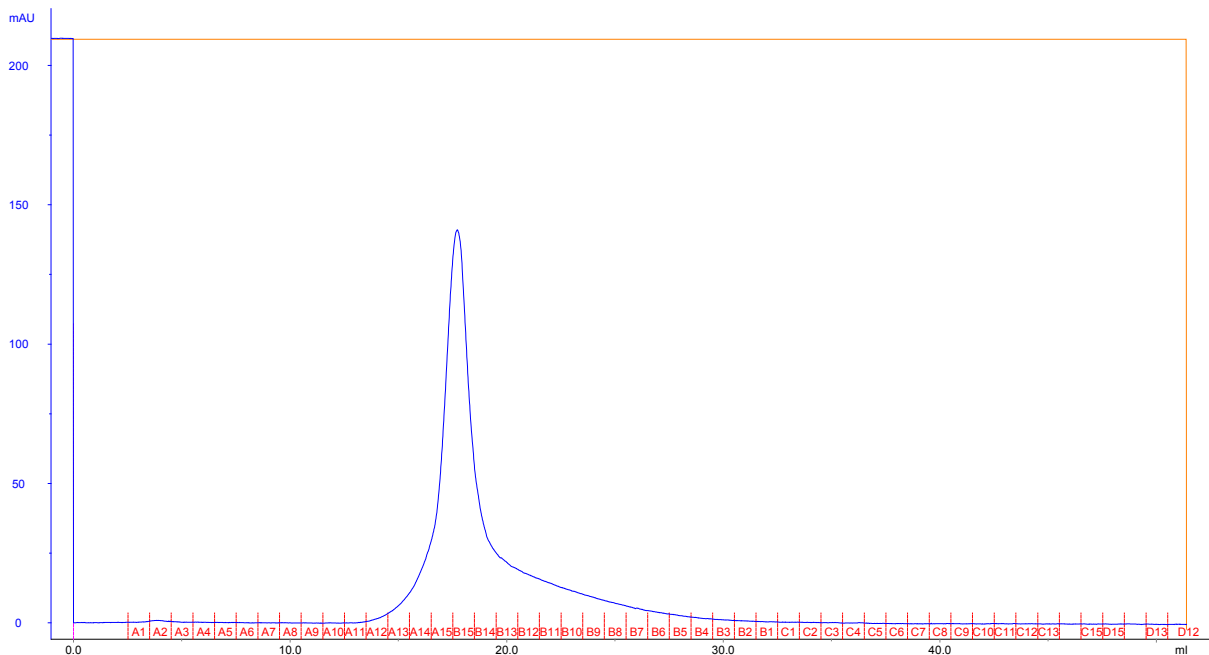
Figure 27 below shows protein distribution profile of PDG which was partially purified using a Sephacryl S-300 HR column. It is clear that with each successive step some proteins were eliminated. The GF fraction shows that after proteins were separated using Sephacryl S-300 HR gel filtration chromatography, PDG was partially purified as only two prominent bands are observed around 42 kDa - 50 kDa area.



**Figure 27: The purification profile of PDG on a 10% polyacrylamide SDS-PAGE. 20 µg protein was loaded in each lane. Mw- Molecular weight marker, MT- Mitochondria, LYO. MT - Lyophilized mitochondria, 1<sup>st</sup> S- 1<sup>st</sup> Supernatant and GF: A15-B10 concentrate.**

### 3.3.3.4 Determining the void volume of Sephacryl S-300 HR 1.0 × 60 cm column

Figure 28 below shows the void volume of a Sephacryl S-300 HR 1.0 cm × 60 cm gel filtration chromatography column as determined by the elution of Blue Dextran 2000 was determined to be 18.85 ml.



**Figure 28: Elution profile of Blue Dextran 2000 on a Sephacryl S-300 HR 1.0 × 60 cm gel filtration chromatography column.**

### 3.3.3.5 Recoveries of PDG activity in group 3 rat intestinal mitochondria after sequential methods for solubilizing and purifying PDG were employed

Pre-incubation of lyophilized mitochondria in a hypotonic phosphate-borate buffer for 3 hours before solubilizing PDG with digitonin detergent + sonication resulted in a 5 fold increase in PDG specific activity in the 1<sup>st</sup> supernatant (Table 6). This might be due to more PDG proteins being solubilized or the phosphate-borate buffer exerting stabilizing effects on PDG. Comparing the amount of protein present in lyophilized mitochondria and the 1<sup>st</sup> supernatant, it is clear that great amounts of protein were solubilized by using digitonin + sonication. The D11 fraction which was recovered after collecting 3 ml fractions on Sephacryl S-300 HR gel filtration chromatography had the highest activity of PDG compared to the D12 and D13 fractions which were also eluted in the same run. Only 8% total protein

was recovered in fraction D11 after loading 1 ml of the 1<sup>st</sup> supernatant fraction (containing 10.09 mg/ml protein) into Sephacryl S-300 HR column. The PDG recovered in D11 fraction was 37 times more pure than the Group 3 mitochondria and 7.4 times more pure than the 1<sup>st</sup> supernatant. Due to large amounts of proteins which eluted into a single tube, 1 ml fractions were collected in the subsequent Sephacryl S-300 HR run. The 1 ml fractions were assayed for PDG activity and fractions A15 - B10 which displayed PDG activity were pooled and concentrated. This combined fraction resulted in a 15-fold increase in PDG specific activity. Although the D11 sample was partially purified to a lesser degree (in terms of SDS-PAGE band profile) when compared to A15-B10 concentrate, the high specific activity indicates that higher amounts PDG protein were eluted in this fraction. Unfortunately pooling the above mentioned fractions yielded a lower specific and total PDG activity than fraction D11 (Figure 24). This may have been due to loss of proteins during concentration of pooled fractions A 15 - B 10.

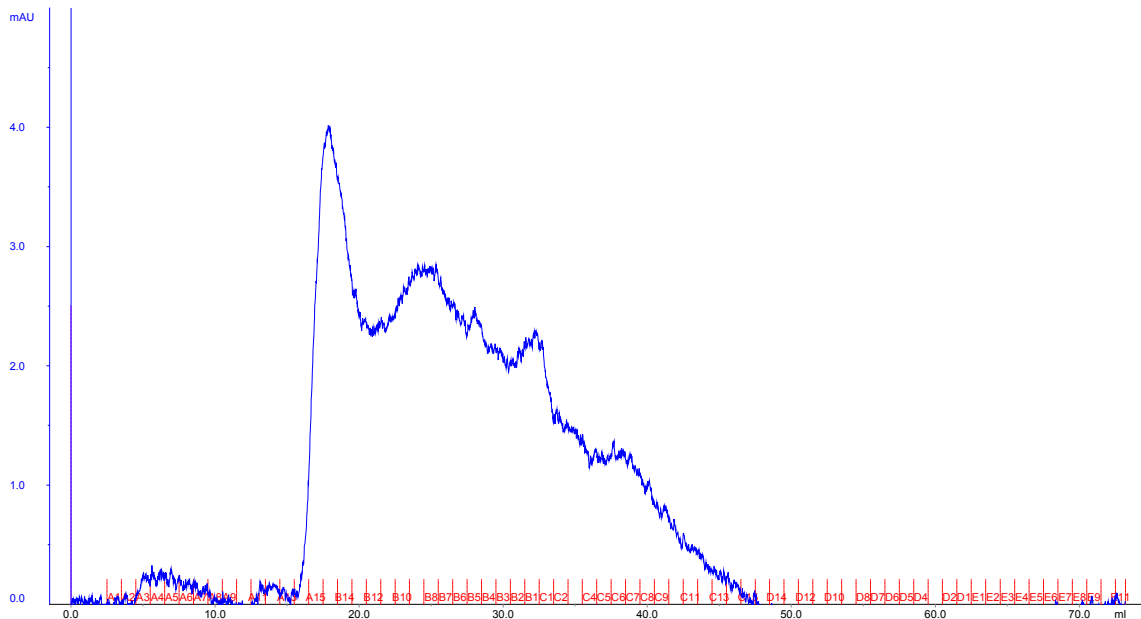
**Table 6: Purification profile of PDG from group 3 rat small intestine mitochondria**

Purification step	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min/mg)	Purification (fold)	Yield (%)
<b>Group 3 mitochondria</b>	90.40	424.67	4.70	1	100
<b>LYO mitochondria</b>	83.08	844.32	10.16	2	>100
<b>1<sup>st</sup> supernatant concentrate</b>	79.53	1872.09	23.54	5	>100
Sephacryl S-300 HR					
<b>D11 fraction (from 3 ml collections)</b>	6.87	1189.56	173.15	37	—
<b>Pooled fractions A 15 - B 10 (from 1 ml collections)</b>	11.50	809.99	70.12	15	—

Note that 1 ml of dialysed 1<sup>st</sup> supernatant proteins containing 10.09 mg/ml was loaded into Sephacryl S-300 HR column for both the runs.

### 3.3.3.6 Purification of PDG on Sephacryl S-400HR gel filtration chromatography column

Figure 29 below shows the elution profile of PDG active Sephacryl S-300 HR fractions (A15-B9) concentrate which was acquired after further attempts to purify rat intestinal PDG using a Sephacryl S-400HR column.



**Figure 29: Elution profile of fractions A15-B9 concentrated proteins eluted from Sephacryl S-300 HR as further separated on Sephacryl S-400HR gel filtration chromatography column. The volume of sample loaded into the column was 0.5 ml which contained 1.49 mg/ml.**

Due to limited sample availability this run was only performed once. The chromatogram acquired above shows that different proteins were still present in the partially purified sample that was acquired from Sephacryl S-300 HR and it is noteworthy that the tallest peak which is similar to that acquired in the Sephacryl S-300 HR chromatogram did not elute in the void volume. Unfortunately we could not determine any PDG activity or even detect any PDG (KGA) through Western blot in order to verify in which peak PDG had eluted. This was due to the very low protein concentrations achieved after running Sephacryl S-400HR chromatography.

## CHAPTER 4

### 4. Discussion

Intestinal PDG is an enzyme localized in mitochondria (Pinkus and Windmueller, 1977) which catalyses deamination of glutamine to glutamate. This reaction in the intestine is important mainly for providing enterocytes with energy (Windmueller and Spaeth, 1974). The by-product of the PDG reaction are also essential for supplying carbon and nitrogen required for synthesis of macromolecules and metabolic intermediates which help maintain the intestine structure and integrity (Watford, 2000). It has been reported that the activity of PDG in cancer cells is abnormally high not only to support cell growth and proliferation but as an adaptation strategy for cancer cells to withstand acidic environments (Medina *et al.*, 1992; Huang *et al.*, 2013). Localization of intestinal PDG and acquiring a stable partially purified enzyme will aid in studies which aim at developing therapeutic drugs that target PDG metabolism, particularly those that may target glutamine metabolism in colon and intestinal cancers. Although PDG plays such a profound role in the intestine it has never been purified from this organ and its sub mitochondrial localization is still not known. Studies used to elucidate the characteristics of rat intestinal PDG have been limited to utilizing only the small intestine as an organ, small intestine homogenates, enterocytes and isolated mitochondria.

In this study the localization of PDG was investigated and PDG of small intestine origin was partially purified. There are currently no studies that we know of which have solubilized and purified PDG from rat small intestine in an active form. The localization of rat intestine PDG was investigated by comparing the distribution of PDG in sub-mitochondrial fractions to that of mitochondria compartment markers enzymes; by treating mitochondria membranes with phospholipase A<sub>2</sub>; and by using -SH targeting reagents. Inner mitochondria membrane permeant and impermeant -SH reagents were used to investigate whether -SH groups are essential for the activity of PDG and if these -SH groups face the outer side or inner side of the inner mitochondria membrane. We also investigated whether PDG must be associated with lipids to retain activity by treating the mitochondria membrane with phospholipase A<sub>2</sub>. Phospholipase A<sub>2</sub> introduces changes to the fatty acid chain conformation; if an enzyme requires lipid-interactions such changes may drastically affect the activity of an enzyme (Carman *et al.*, 1995). Localization was further elucidated by tracking the release of PDG

after fractionation of mitochondria. The distribution of PDG in different fractions was compared to mitochondria marker enzymes to check which enzyme PDG behaves like.

In purification studies we first investigated different methods which may be utilized to solubilize rat intestine mitochondria PDG. A method which was compatible with downstream purification methods was selected and applied. Solubilized PDG was then purified using gel filtration chromatography. Usually a purification strategy involves first fractionating proteins using ammonium sulphate precipitation followed by ion-exchange chromatography then gel filtration chromatography. This strategy could not be adapted since precipitating rat intestine PDG with ammonium sulphate was not favourable for PDG activity, as described in the results section. Also PDG was found to be very unstable when the phosphate-borate buffer was substituted with a lower ionic strength buffer during dialysis and prior to application of the sample to an anion exchanger chromatography column. It is imperative that salt concentration be kept below 50 mM to optimize the binding of proteins to an anion exchanger matrix during protein purification (Acikara, 2013). Due to these limitations PDG was purified using gel filtration chromatography; this method is compatible with phosphate-borate buffer which stabilizes PDG.

The results in Table 1 for the specific activity recovered for adenylate kinase (AK) indicate that this enzyme is localized in the intermembrane space. Two isoforms of adenylate kinase (AK2 and AK3) were previously reported to be localized in the mitochondrial intermembrane space and mitochondrial matrix (Rompay *et al.*, 2000). The specific activity of malate dehydrogenase and glutamate dehydrogenase were high in the low speed supernatant compared to their activities in the mitochondria. The increase of specific activity in the low speed supernatant compared to mitochondria and in the high speed supernatant compared to the sonicated mitoplast pellet denotes that these two enzymes are associated with the matrix. The variation observed in the specific activity of malate dehydrogenase and glutamate dehydrogenase in the sonicated mitoplast pellet suggests that in intestinal mitochondria a fraction of glutamate dehydrogenase is loosely bound to the inner membrane while malate dehydrogenase is predominately found in the matrix. Brown and Chattopadhyay (2012) reported that both malate and glutamate dehydrogenase are localized in the inner mitochondrial membrane and matrix. The significant increase in specific activity of glutamate dehydrogenase observed in the high speed supernatant compared to sonicated mitoplasts and vesicles also suggests that this enzyme is associated with the matrix. The low activities of malate and glutamate dehydrogenases in sonicated vesicles may represent enzyme entrapped

within the vesicles, although the higher activity of the latter enzyme still suggests a small fraction being attached to the membrane.

The high specific activity of cytochrome c oxidase observed in the mitoplasts, sonicated mitoplasts and sonicated vesicles compared to all other elucidated cellular marker enzymes shows that this enzyme is tightly bound to the inner membrane. Further we observed that the specific activity of cytochrome c oxidase is decreased in sonicated vesicles compared to sonicated mitoplast pellet. These results show that cytochrome c oxidase is localized on the outer side of the inner mitochondrial membrane since turning mitoplasts inside out through sonication caused a decrease in enzyme activity, probably due to limited access of the substrate to interact with the active site of cytochrome c oxidase. The sonicated mitoplast fraction represents mitoplasts that are still largely intact and have not been turned into vesicles by sonication. These results are similar to those reported by Masola and Devlin (1995) for liver mitochondria, who showed that 77% of cytochrome c oxidase activity was retained in the sonicated vesicles after mitoplasts were sonicated. A review by Schneider (2013) also supports that this enzyme is localized in the inner membrane.

The specific activity of rat intestinal PDG in the mitoplast and low speed supernatant was similar although slightly higher (1.2-fold) in the mitoplasts. This was in contrast to malate and glutamate dehydrogenase activity which were 56-fold and 5-fold higher in low speed supernatant compared to the mitoplasts, respectively; and cytochrome c oxidase whose mitoplast specific activity was 10-fold that in the low speed supernatant. These results suggest that although PDG has a strong association with the inner membrane, some of enzyme is soluble or at least loosely attached to the membrane. The percentage of total activity recovered in the mitoplast and low speed supernatant shows that the distribution of PDG resembles that of cytochrome c oxidase to some extent compared with other mitochondria compartment marker enzymes (Table 2). The percentage of total PDG activity recovered in the sonicated mitoplast pellet, sonicated vesicles and high speed supernatant shows that the distribution of PDG correlates to that of glutamate dehydrogenase and malate dehydrogenase (Table 3). These results suggest that rat intestinal PDG is localized in the matrix with another fraction bound to the mitochondrial inner membrane. The same localization was observed in rat kidney by Shapiro *et al.* (1985). We could not distinguish whether membrane bound PDG is localized on the outer face of the inner mitochondrial membrane or on the inner face of the inner mitochondrial membrane. This was due to that  $\beta$ -hydroxybutyrate dehydrogenase activity (a marker enzyme for the inner face of the inner

mitochondria membrane) could not be detected in rat intestinal mitochondria or their sub-mitochondrial fractions. Rat liver mitochondria were prepared and used as a positive control; we were able to detect the activity of  $\beta$ -hydroxybutyrate dehydrogenase (results not shown) using the same method we used for rat intestinal mitochondria.

We investigated how to solubilize rat intestinal PDG while conserving its activity using different methods. In Figure 17 A and B, the osmotic imbalance caused by resuspending lyophilized mitochondria in water to dilute the hypertonic buffer and destabilize mitochondrial membranes was not sufficient for the solubilization of rat intestinal PDG as more than 70% of PDG activity was retained in the pellet. These results differ from those reported by Curthoys *et al.* (1976a) who successfully solubilized renal PDG using the above mentioned method. The pellet acquired after the lyophilization step was re-homogenized to enable further solubilization of PDG enzyme but this method failed to solubilize membrane-bound PDG as seen by the high total activity of PDG recovered in the unsonicated lyophilized pellet (US pellet) compared to the unsonicated lyophilized supernatant (Figure 17 A). Our results show that sonication of mitochondria resulted in approximately 30-50% of activity being lost depending on the level or intensity of sonication (results not shown). This loss may be due to the phospholipid dependent PDG species being detached from the mitochondrial membrane or sonication affecting the tertiary structure of PDG. It has been reported that although sonication results in more assayable PDG (Kenny *et al.*, 2003), disrupting intact intestinal mitochondria with sonication leads to a partial loss of enzyme activity (Masola and Ngubane, 2010). Even though a loss of total recovered enzyme activity was observed due to sonication, it proved essential for disrupting the mitochondrial membrane in order to detach membrane bound PDG which was still retained within the pellet of lyophilized rat intestinal mitochondria (US pellet).

Sonication resulted in a 3-fold increase of solubilized PDG specific activity when lyophilized mitochondria were sonicated (Figure 16 B). In lyophilized mitochondria (LYO) the bulk of the enzyme was found in the pellet fraction which also had a higher specific activity. This indicates that most of the PDG proteins are membrane bound and could not be easily solubilized using mild methods. There was a 5-fold and 6-fold increase in specific activity observed after lyophilized mitochondria were treated with digitonin and digitonin combined with sonication (D and DS supernatants), respectively compared to untreated lyophilized mitochondria. This increase in specific activity in the DS supernatant was thought to be due to the combined effect of sonication effectively disrupting the integrity of the mitochondria

membranes enabling digitonin to efficiently integrate into the mitochondria membrane therefore solubilizing membrane bound PDG while conserving its native structure.

The treatment of lyophilized mitochondria with lubrol solubilized/detached most of PDG protein from intestinal mitochondrial membranes compared to all other treatments. This slight variation in the percentage of PDG solubilized using lubrol and digitonin could be due to the higher concentration of lubrol detergent (1 mg lubrol/mg digitonin) used to solubilize PDG compared to 4 mg digitonin/10 mg protein for digitonin. The combination of lubrol with sonication caused the PDG specific activity in the supernatant to be halved when compared to the supernatant recovered after lubrol treatment only. Schaitman and Greenwalt (1968) utilized lubrol instead of sonication to activate membrane bound enzymes. It therefore appears that the mitochondria used in the current study were already leaky due to lyophilisation and thus lubrol treatment enabled more of the PDG protein to become solubilized, but combining lubrol with sonication lowered PDG activity possibly due to the partial deactivation of the PDG.

The dependence of PDG on lipids was investigated by treating intact rat intestinal mitochondria with phospholipase A<sub>2</sub>. The drastic decrease of PDG specific activity observed in intact mitochondria treated with phospholipase (Figure 19) may be due to the digestion of phospholipid in membrane where the membrane bound PDG is localized. Alterations to the mitochondrial membranes by phospholipase A<sub>2</sub> may be damaging vital lipid-enzyme interactions essential for PDG activity. These results suggest that PDG requires the presence of phospholipids to retain full activity. These results are almost similar to those attained by Aledo *et al.* (1997) who reported a complete loss of activity in Ehrlich ascites tumour cell glutaminase when they were treated with > 80 U/ml phospholipase A<sub>2</sub> for 10 minutes. They also reported a drastic loss of PDG activity after treating Ehrlich ascites tumour mitoplasts with approximately 15 U/ml phospholipase A<sub>2</sub>. At a concentration of 15-50 U/ml phospholipase A<sub>2</sub> they observed a slight increase in PDG activity before it was completely lost at 80 U/ml phospholipase A<sub>2</sub>. In the current study 25 U/ml phospholipase A<sub>2</sub> was tested over different incubation periods and a similar trend of a slight increase in PDG activity was observed.

A totally different trend was observed when solubilized PDG was treated with phospholipase A<sub>2</sub> (Figure 19 B). When membrane proteins are solubilized using non-ionic detergents they tend to integrate into the detergent forming an enzyme-detergent complex. Some

phospholipids from the membrane are stripped along with the enzyme (Maire *et al.*, 2000; Luckey, 2014). When the phospholipids associated with solubilized PDG were digested by phospholipase A<sub>2</sub> an increase in PDG specific activity was observed after 15 minutes (Figure 19 B). The increase of solubilized PDG specific activity in the control from 15 - 30 minutes might be due to thermal activation of the enzyme over time or might be as a result of the enzyme changing its conformation. Therefore, the greater activation from 15 - 30 minutes after incubating solubilized PDG with phospholipase could be due to a dual effect: thermal activation and phospholipase releasing PDG which was encapsulated in an enzyme-detergent complex. The released PDG enzymes then allow better access of substrate to PDG resulting in an increased activity. After 45 minutes of treating solubilized PDG with phospholipase, the activity of soluble PDG dropped below the activity of the control. This may be due to phospholipase having completely degraded the phospholipid membrane which had been solubilized along with the membrane bound form of PDG. It is encouraging that the solubilized PDG still retained over 80% PDG activity after 60 minutes of incubation. These results also support the existence of two species of PDG in intestinal mitochondria as observed during localization of PDG by comparing PDG distribution to marker enzymes. They suggest a species of PDG that is tightly membrane bound and which is dependent on phospholipids for activity and another which is localized in the matrix and might be less dependent on phospholipids for activity. The matrix PDG is thought to be to some extent less affected by the digestion of mitochondria membrane with phospholipase since it is not entirely attached to the phospholipid membrane. Therefore, the decrease in PDG activity might be due to lipid dependent PDG losing its activity.

Studies using -SH inhibitors showed that PDG harbours free -SH residues which are involved in its catalytic potential. Treatment with membrane impermeant Mers with or without sonication affected the activity of PDG more than membrane permeant NEM (Table 4). Since our results suggest that rat intestinal PDG is localized in the matrix and inner mitochondrial membrane the inhibition of PDG by membrane impermeant Mers suggests an inhibitory effect on a transport system which delivers glutamine inside the mitochondria. Several studies have reported the existence of a Mers sensitive electroneutral glutamine uniport transport system, in rat liver mitochondria, kidney mitochondria and in kidney sub-mitochondrial particles (Kvamme *et al.*, 1991; Kilberg and Haussinger, 1992). Kvamme *et al.* (1991) also showed that on addition of 0.03% Triton X, the inhibitory effect exerted by Mers treatment was reversed. This Mers induced inhibition therefore indicates that the transport

system involved in the transport of glutamine across the mitochondria membrane possesses –SH groups located on the outer mitochondria membrane which are essential for its proper functioning. It was observed that a higher concentration of Mers (1 mM) inhibited PDG activity to a greater extent compared to 0.1 mM Mers with almost complete loss of PDG activity. The trend of PDG inhibition by 0.1 mM Mers compared to 1 mM NEM is different than that claimed by Aledo and colleagues, (1997). They compared the inhibition of Ehrlich tumour PDG activity by 0.1 mM Mers to 1 mM NEM (different reagent concentrations) and claimed that the same inhibition pattern would be observed even if 1 mM Mers was utilized, but did not show the results.

Although the permeability of NEM was not investigated in the current study, other studies have shown that NEM was not able to permeate mitochondrial membranes from Ehrlich tumour cells (Aledo *et al.*, 1997; Gomez-Fabre *et al.*, 2000) and renal mitochondrial membranes (Kvamme *et al.*, 1991). Membrane permeant NEM inhibited rat intestine PDG to a lesser extent than Mers. This inhibition implies that intestinal mitochondrial PDG has –SH groups which are essential for PDG activity. The inhibition of PDG activity observed in sonicated mitochondria treated with NEM compared to untreated sonication mitochondria seems to support that rat intestinal PDG has a dual localization. The inhibition observed in unsonicated NEM treated mitochondria was most probably due to NEM going through the inner mitochondria membrane and binding to the –SH group found in the active site of membrane bound PDG as had been suggested by Campos *et al.* (1998) after working with tumour cells. While the inhibition observed in mitochondria treated with sonication and NEM might be as a result of disrupting the mitochondria membrane and making it more permeable allowing NEM to interact with matrix localized PDG. Roberg *et al.* (1997) reported that there seems to exist two species of PDG in pig and rat kidney, one which is sensitive to NEM and another which was only inhibited after intact mitochondria had been frozen and thawed. It has also been shown that PDG of different origin were inhibited differently by 1 mM NEM, that from liver completely lost activity while those from rat brain synaptosomal and pig renal mitochondrial were only partially inhibited (Campos *et al.*, 1998). The chemical structure of sulphhydryl reagents used in this study may explain why a membrane permeant NEM inhibited PDG to a lesser degree than membrane impermeant Mers. Mers harbours a hydrophobic tail in its structure which may assist it to quickly integrate into mitochondrial membranes while NEM has no tail (Figure 5 and 6). These characteristics suggest that NEM may require a longer reaction time to permeate the inner membrane than it would take Mers

to integrate into the mitochondrial membrane. Therefore, the results which were acquired by using sulphhydryl reagents can't be used to conclude on the orientation of PDG in the inner mitochondria membrane.

After investigating methods which may be used to solubilize PDG of rat intestinal origin based on the highest yield of the recovered solubilized PDG specific activity a method was developed that yielded partial purification of PDG. The method of solubilizing PDG using lubrol yielded the highest recovery of solubilized PDG but its limitation was later revealed. Samples of mitochondria which were solubilized using lubrol could not be used for PDG purification studies since during the dialysis process, lubrol precipitated to form a white insoluble precipitate. Precipitation of proteins during dialysis may be observed when a high ionic strength buffer is used or if the detergent is removed from the native membrane protein (Schagger, 2013). A precipitate formed during dialysis using a high ionic strength buffer can be re-solubilized after dialysis by diluting the buffer. Therefore, it is clear that the white precipitate which was observed was due to lubrol since it is known that the small micelles formed by non-ionic detergents are not removable by dialysis and we observed that lubrol at low temperature solidifies (Koval and Sprott, 2007). Concentrating the sample with ammonium sulphate and dialyzing to remove lubrol and ammonium sulphate rendered a completely inactive PDG. The loss in PDG activity may be due to the ammonium ions which are present in ammonium sulphate inactivating PDG irreversibly since even after dialysis of this sample in a hypotonic buffer no PDG activity was recovered. The inhibition of intestinal PDG by ammonium ions has also been reported by Masola and Zvinavashe (2003).

Since the lubrol method was not compatible with downstream purification processes another method, involving digitonin treatment with and without sonication, from the PDG solubilization studies was employed. PDG from group 2 mitochondria was then solubilized using 4 mg digitonin/10 mg protein combined with sonication after mitochondria were lyophilized. A low phosphate buffer was used during dialysis to produce a sample for ion exchange chromatography. However, changing the buffer system was not favourable to conserving the activity of PDG thus the strategy to employ this purification method fell through.

Although this fraction was now inactive it was used to investigate whether PDG could be successfully desalted and purified on a Sephacryl S-100 HR gel filtration column. Phosphate-borax buffer was used as a running and eluting buffer during gel filtration chromatography.

One of the functions of gel filtration chromatography is to dialyze protein samples while separating them according to their size (Luckey, 2014). This characteristic was manipulated to investigate if the inactive PDG could be reactivated while proteins were being separated according to their size. Still no PDG activity could be recovered indicating that the inhibition by ammonium ions was irreversible.

Since concentrating the sample with ammonium sulphate leads to loss of PDG activity another procedure was adapted for protein concentration. Centrifuge filter units (Millipore Ultrafree-15 with Biomax-30K and another with Biomax-10K membrane) were used as an alternative to concentrating our sample while conserving PDG activity. It was observed that when the ammonium sulphate (AS) sample was concentrated with a Biomax-30K membrane filter some KGA proteins went through the filter and were lost to the filtrate (Figure 22 B). Therefore, from this point onwards only the Biomax-10K membrane filter unit was utilized to concentrate samples since it showed to conserve all proteins in the concentrate fraction (Figure 22 B).

For reasons stated previously only gel filtration chromatography was used to purify rat intestinal PDG. The protein profile acquired on Sephacryl S-100 HR (Figure 21) indicates that most proteins eluted in the void volume in one broad peak. An active fraction of solubilized rat intestine PDG was separated using Sephacryl S-300 HR gel filtration chromatography. As observed in Figure 24 C, the highest PDG active fraction was D11. Although D11 was partially purified there were still other contaminating proteins in the sample as observed in a coomassie stained SDS-PAGE (Figure 24 B). This was due to the fact that 3 ml fractions were collected resulted in more proteins falling into one tube at a given time. Therefore, for other group 3 mitochondria the solubilized PDG fraction was separated on a Sephacryl S-300 HR column and eluted proteins were collected in 1 ml fractions. This allowed purer fractions to be attained as observed in the coomassie stained SDS-PAGE gel in Figure 26 A compared to D11, D12 and D13 fractions in Figure 24 B which were eluted as 3 ml fractions. It was also observed that the fraction which had PDG activity was eluted in the tall peak which fell in the void volume as determined using the elution profile of blue dextran 2000. Therefore, the native molecular weight of rat intestinal PDG could not be determined due to apparent polymerization which resulted in the protein eluting in the void volume, such has also been observed in PDG from other tissues (Curthoys *et al.*, 1976a; Curthoys *et al.*, 1976b). Curthoys *et al.*, 1976 b reported that a purified native Tris-form KGA (KGA in a Tris buffer) lacking a polyvalent ion exists as an inactive protomer with a molecular weight range

of 90-137 kDa, which upon addition of phosphate forms a dimer and experiences reversible polymerization into a high molecular mass aggregate in borate buffer.

Our results suggest that the 50 kDa band is the matrix PDG enzyme as it could be detected in all the supernatants containing solubilized PDG regardless of the treatment ( Figure 18 A and B). The 63-65 kDa band may be denoted as the membrane-bound PDG since, as observed in Figure 18 A and B, all pellets regardless of the treatment either had both the 63-65 kDa and 50 kDa bands detected or only the 63-65 kDa. There is no pellet which was devoid of the 63-65 kDa. This shows that the 63-65 kDa band is less soluble than the 50 kDa band. A fraction of the 63-65 kDa form is loosely attached to the membrane and can be easily solubilized using a mild method like lyophilization as observed in Figure 18 A1 and A2. Several authors have postulated that the 50 kDa band observed in Western blot analysis of KGA could be a result of alternative splicing or simply a non-specific band (Elgadi *et al.*, 1999; Miller *et al.*, 2012). We believe, however, through the results generated that the 50 kDa is rather a true isoform of KGA that is localized in the matrix. Klein *et al.* (2002) only detected a 55 kDa KGA band after isolating purified phosphate dependent glutaminase from *Bacillus pasteurii* that they reported as a novel type of glutaminase. Miller *et al.* (2012) detected a dominant 65 kDa KGA band in rat brain, spinal cord, kidney and dorsal root ganglion. A second 53 kDa band was attributed to be the result of non-specific binding of the KGA antibody. Others have detected KGA bands of 68 and 65 kDa from rat brain and rat neutrophils (Curi *et al.*, 1997) and in addition to these two bands a 72 kDa band in rat intestine (Shenoy *et al.*, 1996). The three KGA species may be isozymes of PDG. The 63-65 kDa representing a membrane bound possibly an integral protein, while the 50 kDa being a loosely membrane bound enzyme which is missing an anchor which is embedded in the inner mitochondria membrane (contains a soluble and insoluble portion). The 42 kDa band detected by Western blotting may be due to unspecific binding of the antibody or protease degradation.

## CHAPTER 5

### 5. Conclusion

The study suggests that intestinal PDG is partly localized in the mitochondrial matrix with another fraction bound to the inner membrane. The phospholipase A<sub>2</sub> experiment also supported the existence of two pools of intestinal PDG which are localized in different compartments within the mitochondria and that their requirement for phospholipids to retain enzyme activity varies. The matrix PDG was found to be less dependent on phospholipids than membrane bound PDG. The orientation of intestinal PDG could not be clearly distinguished using sulphhydryl reagents. For PDG solubilization experiments, lubrol detergent was found to be the most efficient method which solubilized intestinal PDG in an active state; followed by the digitonin sonication method however solubilization of the enzyme was incompatible with subsequent purification procedures. Intestinal PDG was partially purified using Sephacryl S-300 HR. A 63-65 kDa band along with 50 kDa and 42 kDa bands were detected using Western blots. The 63-65 kDa band is membrane bound PDG; the 50 kDa band corresponds to a soluble matrix PDG while the 42 kDa may arise from non-specific binding or proteolysis. In conclusion intestinal PDG is localized in the mitochondrial matrix with a mass of 50 kDa as well as bound to the inner membrane with a size of 63-65 kDa on SDS-PAGE.

### Further studies

- Investigate the orientation of PDG by employing other methodologies besides –SH targeting agents.
- Further purify intestinal PDG using Sephacryl-S400 HR.
- Characterize a pure intestinal PDG enzyme in terms of its structure and kinetic properties.

## CHAPTER 6

### 6. References

- Acikara, O.B. (2013). Column chromatography. (*InTech*). pp. 46-47.
- Ahluwalia, G.S., Grem, J.L., Hao, Z., and Cooney, D.A. (1990). Metabolism and action of amino acid analog anti-cancer agents. *Pharmacology and Therapeutics* 46, 243-271.
- Aledo, J.C., de Pedro, E., Gómez-Fabre, P.M., de Castro, I.N., and Márquez, J. (1997). Submitochondrial localization and membrane topography of Ehrlich ascitic tumour cell glutaminase. *Biochimica et Biophysica Acta - Biomembranes* 1323, 173-184.
- Ardawi, M.S. (1987). The maximal activity of phosphate dependent glutaminase and glutamine metabolism in late-pregnant and peak-lactating rats. *Biochemical Journal* 242, 75-80.
- Ardawi, M.S., Majzoub, M.F., Kateilah, S.M., and Newsholme, E.A. (1991). Maximal activity of phosphate-dependent glutaminase and glutamine metabolism in septic rats. *The Journal of Laboratory and Clinical Medicine* 118, 26-32.
- Bak, L.K., Schousboe, A., and Waagepetersen, H.S. (2006). The glutamate/GABA-glutamine cycle: aspects of transport, neurotransmitter homeostasis and ammonia transfer. *Journal of Neurochemistry* 98, 641-653.
- Bak, L.K., Zieminska, E., Waagepetersen, H.S., Schousboe, A., and Albrecht, A. (2008). Metabolism of [U-13C]glutamine and [U-13C]glutamate in isolated rat brain mitochondria suggests functional phosphate-activated glutaminase activity in matrix. *Neurochemical Research* 33, 273-278.
- Bergmeyer, H.U. (1974). Myokinase, adenylate kinase from rabbit muscle. In: methods of enzymatic analysis. (New York: *Academic Press*). Second edition pp. 486-487.
- Bhagavan, N.V. (2002). Medical Biochemistry. (Canada: *Academic Press*). Fourth edition p. 1016.

- Bonner, P.L.R. (2007). Protein purification. (United Kingdom: *Garland Science*).
- Bradford, H.K., Ward, H.K., and Sandberg, M. (1984). Kinetic-properties of glutaminase from cerebral-cortex *Neurochemical Research* 9, 751-757.
- Brosnan, J.T., and Brosnan, M.E. (2002). Hepatic glutaminase-a special role in urea synthesis? *Nutrition* 18, 455-457.
- Brown, H.D., and Chattopadhyay, S.K. (2012). Chemistry of the Cell Interface - Part 1. (United States of America: *Academic Press*). p. 352.
- Burgess, R.R. (2009). Methods in enzymology-guide to protein purification. (United States of America: *Academic Press*). Second edition pp. 332-338.
- Cabrera, R.A., Usry, J.L., Arrellano, C., Nogueira, E.T., Kutschenko, M., Moeser, A.J., and Odle, J. (2013). Effects of creep feeding and supplemental glutamine or glutamine plus glutamate (Aminogut) on pre- and post-weaning growth performance and intestinal health of piglets. *Journal of Animal Science and Biotechnology* 4, 1-12.
- Calder, P.C., and Yaqoob, P. (1999). Glutamine and the immune system. *Amino Acids* 17, 227-241.
- Campos, J.A., Aledo, J.C., del Castillo-Olivares, A., del Valle, A.E., de Castro, I.N., and Marquez, J. (1998). Involvement of essential cysteine and histidine residues in the activity of isolated glutaminase from tumour cells. *Biochimica et Biophysica Acta* 1429, 275-283.
- Carman, G.M., Deems, R.A., and Dennis, E.A. (1995). Lipid signaling enzymes and surface dilution kinetics *Journal of Biological Chemistry* 270, 18711-18714.
- Carr, E.L., Kelman, A., Wu, G.S., Gopaul, R., Senkevitch, E., Aghvanyan, A., Turay, A.M., and Frauwirth, K.A. (2010). Glutamine uptake and metabolism are coordinately regulated by ERK/MAPK during T lymphocyte activation. *The Journal of Immunology* 185, 1097-1044.
- Cassagoa, A., Ferreiraa, A.P.S., Ferreiraa, I.M., Fornezaria, C., Gomesa, E.R.M., Greeneb, K.S., Pereirac, H.M., Garrattc, R.C., Diasa, S.M.G., and Ambrosioa, A.L.B. (2012).

Mitochondrial localization and structure-based phosphate activation mechanism of glutaminase c with implications for cancer metabolism. *Proceedings of the National Academy of Sciences* 109, 1092-1097.

Chelius, D., Loeb-Hennard, C., Fleischer, S., and McIntyre, J.O. (2000). Phosphatidylcholine activation of human heart (R)-3-hydroxybutyrate dehydrogenase mutants lacking active center sulfhydryls: site-directed mutagenesis of a new recombinant fusion protein. *Biochemistry* 39, 9687-9697.

Chiu, J.F., and Boeker, E.A. (1979). Cow brain glutaminase: partial purification and mechanism of action. *Archives of Biochemistry and Biophysics* 196, 493-500.

Curi, T.C.P., De Melo, M.P., De Azevedo, R.B., Zorn, T.M.T., and Curi, R. (1997). Glutamine utilization by rat neutrophils: presence of phosphate-dependent glutaminase. *American Journal of Cell Physiology* 273 C1124-C1129.

Curthoys, N.P. (2001). Role of mitochondrial glutaminase in rat renal glutamine metabolism. *The Journal of Nutrition* 131, 2491S-2495S.

Curthoys, N.P., Kuhlenschmidt, T., and Godfrey, S.S. (1976a). Regulation of renal ammoniogenesis. Purification and characterization of phosphate-dependent glutaminase from rat kidney. *Archives of Biochemistry and Biophysics* 174, 82-89.

Curthoys, N.P., Kuhlenschmidt, T., Godfrey, S.S., and Weiss, R.F. (1976b). Phosphate-dependent glutaminase from rat kidney. Cause of increased activity in response to acidosis and identity with glutaminases from other tissues. *Archives of Biochemistry and Biophysics* 172, 162-167.

Curthoys, N.P., and Watford, M. (1995). Regulation of glutaminase activity and glutamine metabolism. *Annual Reviews of Nutrition* 15, 133-159.

Curthoys, N.P., and Weiss, R.F. (1974). Regulation of renal ammoniogenesis. Subcellular localization of rat kidney glutaminase isoenzymes. *Journal of Biological Chemistry* 249, 321-326.

- Danbolt, N.C. (2001). Glutamate uptake. *Progress in Neurobiology* 65, 101-105.
- Dang, C.V. (2013). MYC, metabolism, cell growth and tumorigenesis. *Cold Spring Harbor Perspectives in Medicine* 3, a014217.
- Davila, A., Blachier, F., Gotteland, M., Andriamihaja, M., Benetti, P., Sanz, Y., and Toméa, D. (2013). Intestinal luminal nitrogen metabolism: Role of the gut microbiota and consequences for the host. *Pharmacological Research* 68, 95-107.
- De-Souza, D.A., and Greene, L.J. (2005). Intestinal permeability and systemic infections in critically ill patients: effect of glutamine. *Critical Care Medicine* 33, 1125-1135.
- Dumont, M.E., Cardillo, T.S., Hayes, M.K., and Sherman, F. (1991). Role of cytochrome c heme lyase in mitochondrial import and accumulation of cytochrome c in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* 11, 5487-5496.
- Duong-Ly, K.C., and Gabelli, S.B. (2014). Salting out of proteins using ammonium sulfate precipitation. *Methods in Enzymology* 541, 85-94.
- Durá, M.A., Flores, M., and Toldrá, F. (2002). Purification and characterisation of a glutaminase from *Debaryomyces* spp.. *International Journal of Food Microbiology* 76, 117-126.
- Durán, R.V., Oppliger, W., Robitaille, A.M., Heiserich, L., Skendaj, R., Gottlieb, E., and Hall, M.N. (2012). Glutaminolysis activates rag-mTORC1 signaling. *Molecular Cell* 47, 349-358.
- Elgadi, K.M., Meguid, R.A., Qian, M., Souba, W.W., and Abcouwer, S.F. (1999). Cloning and analysis of unique human glutaminase isoforms generated by tissue-specific alternative splicing. *Physiological Genomics* 1, 51-62.
- Elia, M. (1992). Energy expenditure in the whole body. (New York: *Raven*). pp. 19-59.
- Fritz, J.S., and Gjerd, D.T. (2009). Ion chromatography. (Germany: *John Wiley & Sons*).

Gerich, J.E., Meyer, C., and Stumvoll, M.W. (2000). Glutamate and glutamine in metabolism. Hormonal control of renal and systemic glutamine metabolism. *The American Journal of Nutrition* 130, 995S-1001S.

Godfrey, S.S., Kuhlenschmidt, T., and Curthoys, N.P. (1977). Correlation between activation and dimer formation of rat phosphate-dependent glutaminase. *Journal of Biological Chemistry* 252, 1927-1931.

Gomez-Fabre, P.M., Aledo, J.C., Del castillo-Olivares, A., Alonso, F.J., Nuez DE Castro, I., Campos, J.A., and Marquez, J. (2000). Molecular cloning, sequencing and expression studies of the human breast cancer cell glutaminase. *Biochemistry Journal* 345 365-375.

Gotterer, G.S. (1967). Rat liver D- $\beta$ -hydroxybutyrate dehydrogenase. I. Partial purification and general properties. *Biochemistry* 6, 2139-2152.

Grohmann, U., and Bronte, B. (2010). Control of immune response by amino acid metabolism. *Immunological Reviews* 236, 243-264.

Gupta, P.K. (2008). Molecular biology and genetic engineering. (India: *Rastogi Publications*). pp. 370-371.

Hahn, P., Taller, M., and Chan, H. (1988). Pyruvate carboxylase, phosphate-dependent glutaminase and glutamate dehydrogenase in the developing rat small intestinal mucosa. *Journal of Neonatal Biology* 53, 362-366.

Haque, S.M., Chen, K., Usui, N., Liboshi, Y., Okuyama, H., Masunari, A., Cui, L., Nezu, R., Takagi, Y., and Okada, A. (1996). Alanyl-glutamine dipeptide- supplemented parenteral nutrition improves intestinal metabolism and prevents increased permeability in rats. *Annals of Surgery* 223, 334-341.

Haser, W.G., Shapiro, R.A., and Curthoys, N.P. (1985). Comparison of the phosphate-dependent glutaminase obtained from rat brain and kidney. *Biochemical Journal* 229, 399-408.

- Häussinger, D. (1998). Hepatic glutamine transport and metabolism. *Advance in Enzymology and Related Areas of Molecular Biology* 72, 43-86.
- Hawkins, R.A. (2009). The blood-brain barrier and glutamate. *The American Journal of Clinical Nutrition* 90, 867S-874S.
- Heini, H.G., Gebhardt, R., Brecht, A., and Mecke, D. (1987). Purification and characterization of rat liver glutaminase. *European Journal of Biochemistry* 162, 541-546.
- Hensley, C.T., Wasti, A.T., and DeBerardinis, R.J. (2013). Glutamine and cancer: cell biology, physiology, and clinical opportunities. *The Journal of Clinical Investigation* 123, 3678-3684.
- Holcomb, T., Taylor, L., Trohkimoinen, J., and Curthoys, N.P. (2000). Isolation, characterization and expression of a human brain mitochondrial glutaminase cDNA. *Molecular Brain Research* 76, 56-63.
- Houdijk, A.P., Van Leeuwen, P.A., Boermeester, M.A., Van Lambalgen, T., Teerlink, T., Flinkerbusch, E.L., Sauerwein, H.P., and Westorp, R.I. (1994). Glutamine-enriched enteral diet increases splanchnic blood flow in the rat. *American Journal of Physiology - Gastrointestinal and Liver Physiology* 267, G1035-G1040.
- Hu, W., Zhang, C., Wu, R., Sun, Y., Levine, A., and Feng, Z. (2010). Glutaminase 2, a novel p53 target gene regulating energy metabolism and antioxidant function. *Proceedings of the National Academy of Sciences of the United States of America* 107, 7455-7460.
- Huang, W., Choi, W., Chen, Y., Zhang, Q., Deng, H., He, W., and Shi, Y. (2013). A proposed role for glutamine in cancer cell growth through acid resistance. *Cell Research* 23, 724-727.
- Hughey, R.P., Rankin, B.B., and Curthoys, N.P. (1980). Acute acidosis and renal arteriovenous differences of glutamine in normal and adrenalectomized rats. *The American Journal of Physiology* 238, F199-F204.

James, L.A., Lunn, P.G., and Elia, M. (1998). Glutamine metabolism in the gastrointestinal tract of the rat assessed by the relative activities of glutaminase (EC 3.5.1.2) and glutamine synthetase (EC 6.3.1.2). *British Journal of Nutrition* 79, 365-372.

Janson, J., and Jonsson, J. (2011). Protein purification: principles, high resolution methods and applications. (New Jersey: *John Wiley & Sons*). Third edition pp. 26-32.

Jeong-Min, J., Hae-In, L., Sang-Hyun, H., Chung-Soon, C., and Jae-Seong, S. (2010). Partial purification and characterization of glutaminase from *Lactobacillus reuteri* KCTC3594. *Applied Biochemistry and Biotechnology* 162, 146-154.

Kalra, J., and Brosnan, J.T. (1973). Localization of glutaminase in rat liver. *FEBS Letters* 37, 325-328.

Kaneko, T., Urade, Y., Watanabe, Y., and Mizuno, N. (1987). Production, characterization and immunohistochemical application of monoclonal antibodies to glutaminase purified from rat brain. *The Journal of Neuroscience* 7, 302-309.

Kenny, J., Bao, Y., Hamm, B., Taylor, L., Toth, A., Wagers, B., and Curthoys, N.P. (2003). Bacterial expression, purification, and characterization of rat kidney-type mitochondrial glutaminase. *Protein Expression and Purification* 31, 140-148.

Kilberg, M.S., and Haussinger, D. (1992). Mammalian amino acid transport mechanisms and control. (New York: *Plenum Press*). pp. 105-107.

Klein, M., Kaltwasser, H., and Jahns, T. (2002). Isolation of a novel, phosphate-activated glutaminase from *Bacillus pasteurii*. *FEMS Microbiology Letters* 206, 63-67.

Klimberg, V.S., Souba, W.W., Salloum, R.M., Holley, D.T., Hautamaki, R.D., Dolson, D.J., and Copeland, E.M. (1990). Intestinal glutamine metabolism after small bowel resection. *The American Journal of Surgery* 159, 27-32.

Klingman, J.D., and Handler, P. (1958). Partial purification and properties of renal glutaminase *Journal of Biological Chemistry* 232, 369-380.

Kong, S., Hall, J.C., Cooper, D., and McCauley, R.D. (2000). Starvation alters the activity and mRNA level of glutaminase and glutamine synthetase in the rat intestine. *The Journal of Nutrition* 11, 393-400.

Koval, S.F., and Sprott, G.D. (2007). Methods for general and molecular microbiology. (United States of America: *American Society for Microbiology Press*). Third edition.

Krebs, H.A. (1935). Metabolism of amino acids. The synthesis of glutamine from glutamic acid and ammonia and the enzymic hydrolysis of glutamine in animal tissues. *Biochemical Journal* 29, 1951-1969.

Kvamme, E., Roberg, B., and Torgner, I.A. (1991). Effects of mitochondrial swelling and calcium on phosphate-activated glutaminase in pig renal mitochondria. *European Journal of Biochemistry* 197, 675-680.

Kvamme, E., Svenneby, G., Hertz, L., and Schousboe, A. (1982). Properties of phosphate activated glutaminase in astrocytes cultured from mouse brain. *Neurochemical Research* 7, 761-770.

Kvamme, E., Torgner, I.A., and Roberg, B. (2001). Kinetics and localization of brain phosphate activated glutaminase. *Journal of Neuroscience Research* 66, 951-958.

Kvamme, E., Tveit, B., and Svenneby, S. (1970). Glutaminase from pig renal cortex: I. Purification and general properties. *Journal of Biological Chemistry* 245, 1871-1877.

Labow, B.A., and Souba, W.W. (2000). Glutamine. *World Journal of Surgery* 24, 1503-1513.

Lacey, J.M., and Wilmore, D.W. (1990). Is glutamine a conditional essential amino acid. *Nutrition Reviews* 48, 297-309.

Langhans, W. (2010). The enterocyte as an energy flow sensor in the control of eating. *Forum of Nutrition* 63, 75-83.

Lee, Y., Yang, C., Chang, C., Hsu, H., Chen, I., Chang, H., Lee, C., Lee, J., Kumar, C.R., Qiu, Y., et al. (2014). Discovery of selective inhibitors of glutaminase-2, which inhibit

mTORC1, activate autophagy and inhibit proliferation in cancer cells. *Oncotarget* 5, 6087-6101.

Linke, D. (2009). *Methods in enzymology. Guide to protein purification.* (United States of America: *Academic Press*). Second edition pp. 603-617.

Luckey, M. (2014). *Membrane structural biology: with biochemical and biophysical foundations.* (New York: *Cambridge University Press*). Second edition p. 400.

Macintyre, A.N., and Rathmell, J.C. (2013). Activated lymphocytes as a metabolic model for carcinogenesis. *Cancer and Metabolism* 1, 1-12.

Maire, M., Champeil, P., and Möller, J.V. (2000). Interaction of membrane proteins and lipids with solubilizing detergents. *Biochimica et Biophysica Acta* 1508, 86-111.

Manso, H.E.C., Manso Filho, H.C., de Carvalho, L.E., Kutschenko, M., Nogueira, E.T., and Watford, M. (2012). Glutamine and glutamate supplementation raise milk glutamine concentrations in lactating gilts. *Journal of Animal Science and Biotechnology* 3, 1-7.

Márquez, J., Cardona, C., Campos-Sandoval, J.A., Peñalve, A., Tosina, M., Matés, J.M., and Martín-Rufián, M. (2013). Mammalian glutaminase isozymes in brain. *Metabolic Brain Disease* 28, 133-137.

Márquez, J., Tosina, M., de la Rosa, V., Segura, J.A., Alonso, F.J., Matés, J.M., and Campos-Sandoval, J.A. (2009). New insights into brain glutaminases: beyond their role on glutamatergic transmission. *Neurochemistry International* 55, 64-70.

Martín-Rufián, M., Tosina, M., Campos-Sandoval, J.A., Manzanarez, E., Lobo, C., Segura, J.A., Alonso, F.J., Mates, J.M., and Marquez, J. (2012). Mammalian glutaminase Gls2 gene encodes two functional alternative transcripts by a surrogate promoter usage mechanism. *PLoS One* 7, e38380.

Masola, B., and Devlin, T.M. (1995). Intramitochondrial localization of alanine aminotransferase in rat-liver mitochondria: comparison with glutaminase and aspartate aminotransferase. *Amino Acids* 9, 363-374.

Masola, B., and Evered, D.F. (1984). Preparation of rat enterocyte mitochondria. *Biochemical Journal* 218, 441-447.

Masola, B., and Ngubane, N.P. (2010). The activity of phosphate-dependent glutaminase from the small rat intestine is modulated by ADP and is dependent on integrity of mitochondria. *Archives of Biochemistry and Biophysics* 504, 197-203.

Masola, B., Peters, T.J., and Evered, D.F. (1985). Transamination pathways influencing L-glutamine and L-glutamate oxidation by rat enterocyte mitochondria and the subcellular localization of L-alanine aminotransferase and L-aspartate aminotransferase. *Biochimica et Biophysica Acta* 843, 137-143.

Masola, B., and Zvinavashe, E. (2003). Phosphate-dependent glutaminase in enterocyte mitochondria and its regulation by ammonium and other ions. *Amino Acids* 24, 427-434.

McCauley, R., Heel, K., and Hall, J.C. (1997). Enteral branched chain amino acids increase the specific activity of jejunal glutaminase and reduce jejunal atrophy. *Journal of Gastroenterology and Hepatology* 12, 429-433.

McCauley, R., Kong, S., Heel, K., and Hall, J.C. (1999). The role of glutaminase in the small intestine. *The International Journal of Biochemistry and Cell Biology* 31, 405-413.

McGivan, J.D., Lacey, J.H., and Joseph, S.K. (1980). Localization and some properties of phosphate-dependent glutaminase in disrupted liver mitochondria. *Biochemistry Journal* 192, 537-542.

Medina, M.A., Sánchez-Jiménez, F., Márquez, J., Quesada, A.R., and de Castro Núñez, I. (1992). Relevance of glutamine metabolism to tumor cell growth. *Molecular and Cellular Biochemistry* 113, 1-15.

Michalik, M., Nelson, J., and Erecinska, M. (1992). Glutamate production in islets of Langerhans: properties of phosphate-activated glutaminase. *Metabolism* 41, 1319 -1326.

Mihara, K., Omura, T., Harano, T., Brenner, S., Fleischer, S., and Rajagopalan, K.V. (1982). Rat liver l-glutamate dehydrogenase, malate dehydrogenase, d-β-hydroxybutyrate

dehydrogenase and sulfite oxidase are each synthesized as larger precursors by cytoplasmic free polysomes. *Journal of Biological Chemistry* 257, 3355-3358.

Miller, K.E., Balbás, J.C., Benton, R.L., Lam, T.S., Edwards, K.M., Kriebel, R.M., and Schechter, R. (2012). Glutaminase immunoreactivity and enzyme activity is increased in the rat dorsal root ganglion following peripheral inflammation. *Pain Research and Treatment* Article ID 414697, 9.

Mintz-Oron, S., Aharoni, A., Ruppin, E., and Shlomi, T. (2009). Network-based prediction of metabolic enzymes subcellular localization. *Bioinformatics* 25, I247-I252.

Mueller, A.R., Nalesnik, M.A., Langreh, J.M., Rao, P.N., Synder, J.T., Hoffman, R.A., and Schraut, W.H. (1993). Evidence that small bowel preservation causes primarily basement membrane and endothelial rather than epithelial cell injury. *Transplantation* 56, 1499-1504.

Nagy, L.E., Pittler, A., and Kretchmer, N. (1988). Development of glutaminase along the villus-crypt axis in the jejunum of rat. *Journal of Pediatric Gastroenterology and Nutrition* 7, 907-913.

Nelson, D., Rumsey, W.L., and Erecinska, M. (1994). Glutamine catabolism by heart muscle: regulation of phosphate-activated glutaminase by ATP, citrate, and chloride. *Archives of Biochemistry and Biophysics* 314, 376-383.

Newsholme, E.A., Crabtree, B., and Ardawi, M.S. (1985). The role of high rates of glycolysis and glutamine utilization in rapidly dividing cells. *Bioscience Reports* 5, 393-400.

Newsholme, P., Lima, M.M.R., Procopio, J., Pithon-Curi, T.C., Doi, S.Q., Bazotte, R.B., and Curi, R. (2003a). Glutamine and glutamate as vital metabolites. *Brazilian Journal of Medical and Biological Research* 36, 153-163

Newsholme, P., Procopio, J., Lima, M.M.R., Pithon-Curi, T.C., and Curi, R. (2003b). Glutamine and glutamate-their central role in cell metabolism and function. *Cell Biochemistry Function* 21, 1-9.

Nigam, A., and Ayyagari, A. (2007). Lab manual in biochemistry: immunology and biotechnology. (New Dehli: *Tata McGraw-Hill Education*). pp. 110-112.

Nimmo, G.A., and Tipton, K.F. (1980). Purification of soluble glutaminase from pig brain. *Biochemical Pharmacology* 29 359-367.

Nussbaum, M.S., and Berry, S.M. (1996). Parenteral nutrition. Nutrition and metabolism in the surgical patients. Second edition pp. 715-759.

Olalla, L., Aledo, J.C., Bannenberg, G., and Marquez, J. (2001a). The C terminus of human glutaminase L mediates association with PDZ domain-containing proteins. *FEBS Letters* 488, 116–122.

Olalla, L., Aledo, J.C., G., G.B., and J., M. (2001b). The C-terminus of human glutaminase L mediates association with PDZ domain-containing proteins. *FEBS Letters* 488, 116-122.

Paila, Y.D., Pucadyil, T.J., and Chattopadhyay, A. (2005). The cholesterol-complexing agent digitonin modulates ligand binding of the bovine hippocampal serotonin<sub>1A</sub> receptor. *Molecular Membrane Biology* 22, 241-249.

Patel, M., and McGivan, J.D. (1984). Partial purification and properties of rat liver glutaminase. *Biochemical Journal* 220, 583-590.

Perez-Gomez, C., Campos-Sandoval, J.A., Alonso, F.J., Segura, J.A., Manzanares, E., Ruiz-Sanchez, P., Gonzalez, M.E., Marquez, J., and Mates, J.M. (2005). Co-expression of glutaminase K and L isoenzymes in human tumour cells. *Biochemical Journal* 386, 535-542.

Perez-Gomez, C., Mates, J.M., Gomez-Fabre, P.M., Castillo-Olivares, A.D., Alonso, F.J., and Marquez, J. (2003). Genomic organization and transcriptional analysis of the human L-glutaminase gene. *Biochemical Journal* 370, 771-784.

Pinkus, L.M., and Windmueller, H.G. (1977). Phosphate-dependent glutaminase of small intestine: localization and role in intestinal glutamine metabolism. *Archives of Biochemistry and Biophysics* 182, 506-517.

Poll, M.C.G., Soeters, P.B., Deutz, N.E.P., Fearon, K.C.H., and Dejong, C.H.C. (2004). Renal metabolism of amino acids: its role in interorgan amino acid exchange. *The American Journal of Clinical Nutrition* 79 185-197.

Porter, L.D., Ibrahim, H., Taylor, L., and Curthoys, N.P. (2002). Complexity and species variation of the kidney type glutaminase gene. *Physiological Genomics* 9, 157-166.

Quesada, A.R., Sanchez-Jimenez, F., Perez-Rodriguez, J., Marquez, J., Medina, M.A., and Nunez de Castro, I. (1988). Purification of phosphate-dependent glutaminase from isolated mitochondria of Ehrlich ascites-tumour cells. *Biochemistry Journal* 255, 1031-1036.

Rajagopalan, K.N., and DeBerardinis, R.J. (2011). Role of glutamine in cancer: therapeutic and imaging implications. *The Journal of Nuclear Medicine* 52, 1005-1008.

Rao, R.K., and Samak, G. (2012). Role of glutamine in protection of intestinal epithelial tight junctions. *Journal of Epithelial Biology and Pharmacology* 5, 47-54.

Reeds, P.J., and Burrin, D.G. (2001). Glutamine and the bowel. *The Journal of Nutrition* 131, 2505S-2508S.

Rizzo, D. (2009). Fundamentals of anatomy and physiology. (United States of America: Cengage Learning). Third edition p. 529.

Roberg, B., Torgner, I.A., and Kvamme, E. (1995). The orientation of phosphate activated glutaminase in the inner mitochondrial membrane of synaptic and non-synaptic rat brain mitochondria. *Neurochemical International* 27, 367-376.

Roberg, B., Torgner, I.A., and Kvamme, E. (1997). Soluble and membrane-bound forms of phosphate activated glutaminase with different kinetic characteristics in pig and rat kidney mitochondria. *Contributions to Nephrology* 121 11-18.

Roberg, B., Torgner, I.A., Laake, J., Takumi, Y., Ottersen, O.P., and Kvamme, E. (2000). Properties and submitochondrial localization of pig and rat renal phosphate-activated glutaminase. *American Journal of Cell Physiology* 279 C648-C657.

Rompay, A.R.V., Johansson, M., and Karlsson, A. (2000). Phosphorylation of nucleosides and nucleoside analogs by mammalian nucleoside monophosphate kinases. *Pharmacology and Therapeutics* 87, 189-198.

Roos, P.H. (2000a). Protein liquid chromatography. (*Elsevier*). pp. 16-32.

Roos, P.H. (2000b). Protein liquid chromatography. (*Elsevier*). pp. 64-66.

Rosenberg, I.M. (2006). Protein analysis and purification: benchtop techniques. (*Springer Science and Business Media*). p. 509.

Rudermann, N.B., and Lund, P. (1972). Amino acid metabolism in skeletal muscle: regulation of glutamine and alanine release in perfused rat hindquarters. *Journal of Biological Chemistry* 8, 295-302.

Salleh, M., Ardawi, M.S., Majzoub, M.F., and Newsholme, E.A. (1988). Effect of glucocorticoids on glucose and glutamine metabolism by the small intestine of the rat. *The Journal of Clinical Science* 75, 93-100.

Salloum, R.M., Copeland, E.M., and Souba, W.W. (1991). Brush border transport of glutamine and other substrates during sepsis and endotoxemia. *Annals of Surgery* 213, 401-410.

Sarantos, P., Abouhamze, A., and Souba, W.W. (1992). Glucocorticoids regulate intestinal glutaminase expression. *Surgery* 112, 278-283.

Schagger, H. (2013). A practical guide to membrane protein purification-separation, detection and characterization of biological macromolecules. (United States of America: *Elsevier*). pp. 31-34.

Schaitman, C., and Greenawalt, J.W. (1968). Enzymatic properties of the inner and outer membranes of rat liver mitochondria. *The Journal of Cell Biology* 38, 158-175.

Schneider, D.L. (2013). Progress in surface and membrane science. (*Elsevier*). pp. 228-229.

Scopes, R.K. (1993). Protein purification: principles and practice. (United States of America: *Springer Science and Business Media*). Third edition p. 399.

Seddon, A.M., Curnow, P., and Booth, P.J. (2004). Membrane proteins, lipids and detergents: not just a soap opera. *Biochimica et Biophysica Acta* 1666, 105-117.

Shapiro, R.A., Farrell, L., Srinivasan, M., and Curthoys, N.P. (1991). Isolation, characterization, and in vitro expression of a cDNA that encodes the kidney isoenzyme of the mitochondrial glutaminase. *Journal of Biological Chemistry* 266, 18792-18796.

Shapiro, R.A., Haser, W.G., and Curthoys, N.P. (1985). The orientation of phosphate-dependent glutaminase on the inner membrane of rat renal mitochondria. *Archives of Biochemistry and Biophysics* 243, 1-7.

Shenoy, V., Roig, J.C., Kubilis, P., and Neu, J. (1996). Characterization of glutaminase in the developing rat small intestine. *Journal of Nutrition* 126, 1121S -1130S.

Simpson, R.J. (2004). Purifying proteins for proteomics: a laboratory manual. *CSHL Press*, 801.

Smith, E.M., and Watford, M. (1988). Rat hepatic glutaminase: purification and immunochemical characterization. *Archives of Biochemistry and Biophysics* 260, 740-751.

Sottocasa, G.L., Kuylenstierna, B., Ernster, L., and Bergstrand, A. (1967). An electron-transport system associated with the outer membrane of liver mitochondria. A biochemical and morphological study *The Journal of Cell Biology* 32, 415-438.

Souba, W.W., Herskowitz, K., Kllimberg, V.S., Salloum, R.M., Plumley, D.A., Flynn, T.C., and Copeland III, E.M. (1990a). The effect of sepsis and endotoxemia on gut glutamine utilization. *Annals of Surgery* 163, 543-549.

Souba, W.W., Klimberg, V.S., Plumley, D.A., Salloum, R.M., Flynn, T.C., Bland, K.I., and Copeland III, E.M. (1990b). The role of glutamine in maintaining a healthy gut and supporting the metabolic response to injury and infection. *Journal of Surgical Research* 48, 383-391.

Stumvoll, M., G., P., C., M., and J., G. (1999). Role of glutamine in human carbohydrate metabolism in kidney and other tissues. *Kidney International* 55 778-792.

Svenneby, G., Roberg, B., Hogstad, S., Torgner, I.A., and Kvamme, E. (1986). Phosphate-activated glutaminase in the crude mitochondrial fraction (P2 fraction) from human brain cortex *Journal of Neurochemistry* 47, 1351-1355.

Svenneby, G., Torgner, I.A., and Kvamme, E. (1973). Purification of phosphate dependent pig brain glutaminase *Journal of Neurochemistry* 20, 1217-1224.

Svinivasan, M., Kalousek, F., and Curthoys, N.P. (1995). In vitro characterization of the mitochondrial processing and potential function of the 68 kDa subunit of renal glutaminase. *Journal of Biological Chemistry* 270, 1185-1190.

Szeliga, M., Matyja, E., Obara, M., Grajkowska, W., Czernicki, T., and Albrecht, J. (2008). Relative expression of mRNAs coding for glutaminase isoforms in CNS tissues and CNS tumors. *Neurochemical Research* 33, 808-813.

Taylor, L., and Curthoys, N.P. (2004a). Glutamine metabolism. Role in acid-base balance *Biochemistry and Molecular Biology Education* 32, 291-304.

Taylor, L., and Curthoys, N.P. (2004b). Glutamine metabolism: Role in acid-base balance. *Biochemistry and Molecular Biology Education* 32, 291-304.

Trivedi, M.V., Laurence, J.S., and Siahhan, T.J. (2009). The role of thiols and disulfides in protein chemical and physical stability. *Current Protein and Peptide Science* 10, 614-625.

Vermeulena, T., Görgb, B., Voglc, T., Wolfc, M., Vargac, G., Toutaine, A., Paulf, R., Schliessb, F., Häussingerb, D., and Häberlea, J. (2008). Glutamine synthetase is essential for proliferation of fetal skin fibroblasts. *Archives of Biochemistry and Biophysics* 478, 96-102.

Walker, J. (2010). Principles and techniques of biochemistry and molecular biology. (United States of America: New York: *Cambridge University Press*). Seventh edition pp. 315-316.

Walsh, G. (2007). *Pharmaceutical biotechnology: concepts and applications*. (England: *John Wiley & Sons*). p. 142.

Warburg, O., Wind, F., and Negelein, E. (1927). The metabolism of tumors in the body. *The Journal of General Physiology*, 519-530.

Watford, M. (1993). Hepatic glutaminase expression: relationship to kidney-type glutaminase and to the urea cycle. *FASEB Journal Review* 7, 1468-1474.

Watford, M. (2000). Glutamine and glutamate metabolism across the liver sinusoid. *The Journal of Nutrition* 130, 983S-987S.

Windmueller, H.G., and Spaeth, A.E. (1974). Uptake and metabolism of plasma glutamine by the small intestine. *Journal of Biological Chemistry* 249, 5070-5079.

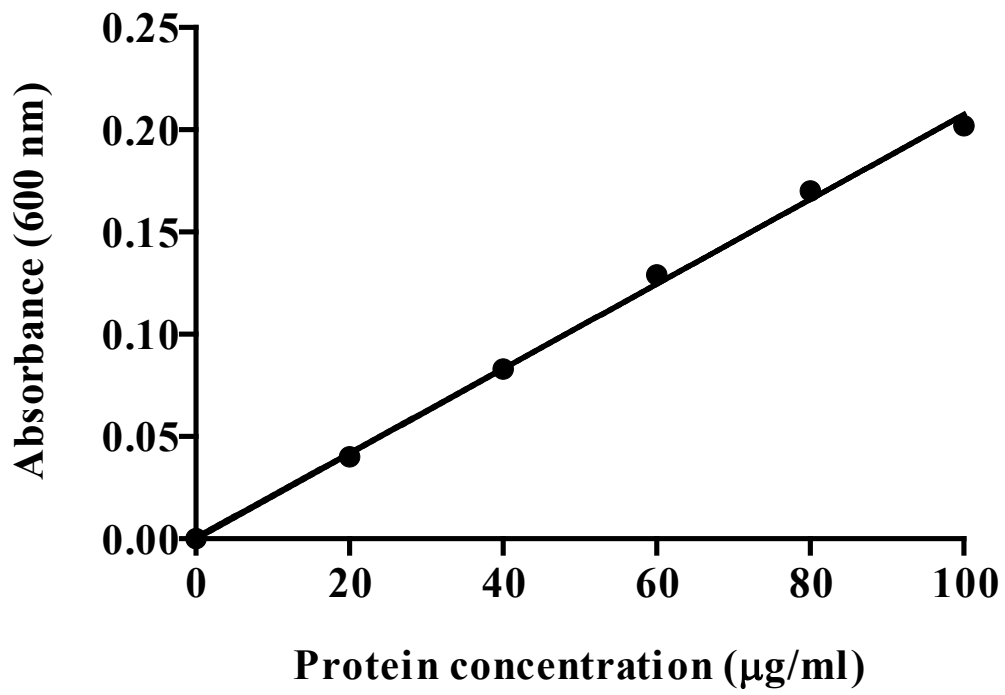
Yuneva, M., Zamboni, N., Oefner, P., Sachidanandam, R., and Lazebnik, Y. (2007). Deficiency in glutamine but not glucose induces MYC-dependent apoptosis in human cells. *The Journal of Cell Biology* 178, 93-105.

Zeidel, M.L. (1997). *Diuretic Agents: Clinical Physiology and Pharmacology*. (United States of America: *Academic Press*). p. 128.

Zhao, L., Huang, Y., Tian, C., Taylor, L., Curthoys, N., Wang, Y., Vernon, H., and Zheng, J. (2012). Interferon- $\alpha$  regulates glutaminase 1 promoter through STAT1 phosphorylation: relevance to HIV-1 associated neurocognitive disorders. *PLoS One* 7, e32995.

Zhao, Y., Butler, E.B., and Tan, M. (2013). Targeting cellular metabolism to improve cancer therapeutics. *Cell Death and Disease* 4, e532.

## **Appendix 1: Folin-Lowry assay standard curve**



$$Y = 0.0021x + 0.0007, R^2 = 0.99977$$

## Appendix 2: Formula for calculating PDG activity

$$\text{Glutaminase activity} = \frac{\Delta \text{ Abs.} \times \text{Reaction vol. (ml)} \times 1000 \times 1000 \times 20}{6.22 \times 1000 \times \text{Time (min)} \times \text{mg/ml enzyme}}$$

Units: **nmoles glutamate formed/min/mg protein**

- $\Delta \text{ Abs.}$  = change in absorbance when glutamate is converted to  $\alpha$ -ketoglutarate
- Reaction vol. = Final volume of the reaction mixture in ml
- $\times 1000 \times 1000$  = so that the answer would be expressed in nano moles
- 20 = sample volume multiplied by a factor to express the volume as if in 1 ml
- $6.22 \times 10^3$  = NADH molar extinction coefficient ( $\text{Moles}^{-1}\text{cm}^{-1}$ ) at 340 nm
- Time = how long was the reaction catalysed by glutamate dehydrogenase to yield  $\beta$ -NADH in minutes
- mg enzyme = mg/ml protein as determined using the Lowry's method

### Appendix 3: Formula for calculating cytochrome c oxidase activity

$$\text{Cytochrome c oxidase} = \frac{\Delta \text{ Abs. min}^{-1} \times \text{Reaction vol. (ml)} \times 1000 \times 1000 \times 20}{18.7 \times 1000 \times \text{mg/ml enzyme}}$$

Units: **nmoles cytochrome c oxidized/min/ml**

- Molar  $\epsilon$  of reduced Cytochrome c is  $27.7 \times 10^3 \text{ Mole}^{-1} \text{ cm}^{-1}$
- Molar  $\epsilon$  of oxidized Cytochrome c is  $\frac{9.0 \times 10^3 \text{ Mole}^{-1} \text{ cm}^{-1}}{\text{Difference} = 18.7 \times 10^3 \text{ Mole}^{-1} \text{ cm}^{-1}}$
- $\Delta \text{ Abs.}$  = the change in absorbance per minute when reduced cytochrome c is oxidation
- Reaction vol. = Final volume of the reaction mixture in ml
- $\times 1000 \times 1000$  = so that the answer would be expressed in nano moles
- 20 = sample volume multiplied by a factor to express the volume as if in 1 ml
- $18.7 \times 10^3$  = the difference of cytochrome c oxidase molar extinction coefficient ( $\text{Moles}^{-1} \text{ cm}^{-1}$ ) at 550 nm
- mg enzyme = mg/ml protein as determined using the Lowry's method

#### **Appendix 4: Formula for calculating malate dehydrogenase and glutamate dehydrogenase activity**

$$\text{Activity} = \frac{\Delta \text{ Abs. min}^{-1} \times \text{reaction volume (ml)} \times 1000 \times 1000 \times 10}{6.22 \times 1000 \times \text{mg/ml enzyme}}$$

Units for malate dehydrogenase activity: **nmoles malate formed/ min/mg protein**

Units for glutamate dehydrogenase activity: **nmol 2-oxoglutarate formed/min/mg protein**

- $\Delta \text{ Abs.}$  = for malate dehydrogenase, change in absorbance per minute acquired when oxaloacetate was converted to malate  
= for glutamate dehydrogenase change in absorbance per minute acquired when 2-oxoglutarate is converted to glutamate
- Reaction vol. = final volume of the reaction mixture in ml
- $\times 1000 \times 1000$  = so that the answer would be expressed in nano moles
- 10 = sample volume multiplied by a factor to express the volume as if in 1 ml
- $6.22 \times 10^3$  = NADH molar extinction coefficient ( $\text{Moles}^{-1}\text{cm}^{-1}$ ) at 340 nm
- mg enzyme = mg/ml protein as determined using the Lowry's method