PROLINE CATABOLISM IN LIVER

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by

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Abstract

The goal of this work was to localize one of the enzymes involved in proline oxidation, Δ^1-pyrroline-5-carboxylate dehydrogenase (P5CDh) and to gain an understanding of the factors affecting proline catabolism in rat liver. In this regard we performed a systematic subcellular localization for P5CDh and studied proline catabolism in response to dietary protein and exogenous glucagon. Our results indicate that P5CDh is located solely in mitochondria in rat liver. With respect to factors affecting proline catabolism we observed that rats fed a diet containing excess protein (45% casein) display a 1.5 fold increase in activity of P5CDh and proline oxidase (PO), and a 40% increase in flux through the pathway resulting in complete oxidation of proline in isolated mitochondria. We also observed that rats administered exogenous glucagon exhibit a 2 fold increase in PO activity and a 1.5 fold increase in P5CDh activity, and a 2 fold increase in flux through the pathway resulting in complete oxidation of proline in isolated mitochondria. $^{14}CO_2$ production from ^{14}C-proline in the isolated nonrecirculating perfused rat liver was also elevated 2 fold in the glucagon treated rat. We also studied the transport of proline into isolated hepatocytes and observed a 1.5 fold increase in the transport of proline in rats given exogenous glucagon.

Conclusions:

Subcellular localization:

a) The spectrophotometric assay is valid and provides a quick, easy method for assays of P5CDh

b) P5CDh is located strictly in the mitochondrial matrix

High protein diet increases:

a) flux through the proline catabolic pathway in mitochondria resulting in the production of CO_2

b) activity of PO, P5CDh, and ornithine aminotransferase (OAT) in rat liver mitochondria

Glucagon increases:

a) proline transfer from plasma to hepatocytes

b) oxidation of proline by perfused liver

c) flux through the proline catabolic pathway resulting in the production of CO_2

d) activity of PO, P5CDH and glutamate dehydrogenase (GDH) in rat liver mitochondria

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Abbreviations

α-KG	alpha ketoglutarate
ATA1	system A transporter subtype 1
ATA2	system A transporter subtype 2
ATA3	system A transporter subtype 3
BSA	bovine serum albumin
^{14}C-proline	[U]- ^{14}C-proline
CNS	central nervous system
DNA	deoxyribonucleic acid
cDNA	complementary deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylen glycol-bis(β-aminoethyl ether) *N,N,N,',N'*-tetra-acetic acid
Fp	flavoprotein
FpH_2	flavoprotein reduced
GDH	glutamate dehydrogenase
GS	glutamine synthetase
Kb	kilobase
LDH	lactate dehydrogenase
MeAIB	*N*-methylamino-α-isobutyric acid
NAD^+	oxidized nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
$NADP^+$	oxidized nicotinamide adenine dinucleotide phosphate

NADPH	reduced nicotinamide adenine dinucleotide phosphate
OAT	ornithine aminotransferase
P2C	Δ^1-pyrroline-2-carboxylic acid
P5C	(D,L) Δ^1-pyrroline-5-carboxylic acid
P5CDh	Δ^1-pyrroline-5-carboxylate dehydrogenase
P5C reductase	Δ^1-pyrroline-5-carboxylic acid reductase
P5C synthase	Δ^1-pyrroline-5-carboxylic acid synthase
PO	proline oxidase
ROS	reactive oxygen species

Chapter 1: Introduction

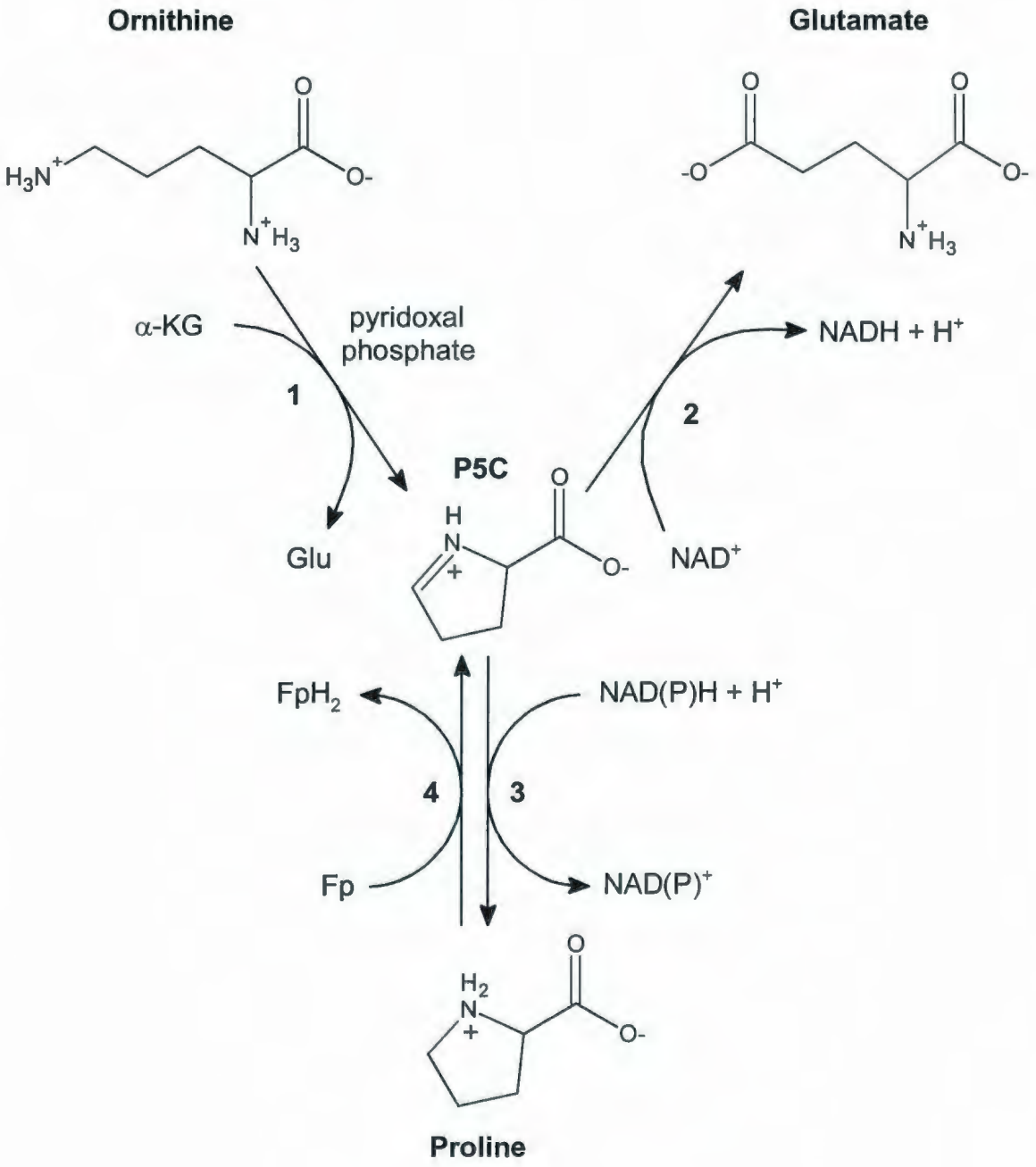
Proline is a dietary non-essential amino acid that is found in significant amounts in mammalian body fluids. In fact proline in extracellular fluid can reach concentrations as high as 0.35 mM and is exceeded only by those of glutamine and alanine (85). Proline is considered to be a neutral molecule in free solution at physiologic pH and is a secondary amine because the nitrogen and alpha carbon atoms are part of a pyrrolidine ring.

Proline (figure 1.1) is a unique amino acid due to the presence of this cyclic pyrrolidine ring. The ring structure of proline gives proline characteristics that dictate the role of proline as a component of proteins as well as in regard to metabolism. The presence of the secondary amino group excludes proline from the pyridoxal-5'-phosphate dependent transamination reactions that are so important for general amino acid metabolism. In regard to incorporation into proteins, proline is an integral component of numerous proteins and is often termed as an α-helix breaking amino acid (109). That is, the structure of proline does not easily fit into the α-helix motif that is such a common structural component of proteins. The most common protein that is associated with proline is collagen where proline is present in a ratio of approximately 110-130 residues per 1000 residues (85).

The relatively large amount of proline present in collagen is largely responsible for the left-handed helix for which collagen is known. This type of

Figure 1.1: Enzymes involved in metabolism of Δ^1-pyrroline-5-carboxylic acid in rat liver.

Enzymes: 1, ornithine aminotransferase; 2, Δ^1-pyrroline-5-carboxylic acid dehydrogenase; 3, Δ^1-pyrroline-5-carboxylic acid reductase; 4, proline oxidase. Abbreviations; α-KG, α-ketoglutarate; Glu, glutamate; Fp, flavoprotein.


Ornithine
Glutamate
α-KG
pyridoxal phosphate
1
2
NADH + H+
P5C
Glu
NAD+
FpH2
NAD(P)H + H+
4
3
Fp
NAD(P)+
Proline

helix is commonly referred to as a poly-pro helix which signifies the importance of proline residues in determining the secondary structure of this protein. A derivative of proline, 4-hydroxy-L-proline is also present in mammalian body fluids as well as in collagen. The presence of hydroxyproline in collagen is due to hydroxylation catalyzed by prolyl hydroxylase following synthesis of the collagen peptides (106).

1.1 Proline catabolism

The catabolism of proline in liver is a multi-step process. The first step, the oxidation of proline to Δ^1-pyrroline-5-carboxylic acid (P5C), is catalyzed by proline oxidase (PO) (EC number not assigned) while the second step is the conversion of P5C to glutamate by Δ^1-Pyrroline-5-carboxylic acid dehydrogenase (P5CDh) (EC1.5.1.12) which uses $NAD(P)^+$ as a cofactor (Figure 1).

1.1.1 Proline oxidase

Proline oxidase is located in mitochondria in liver and is associated with the inner mitochondrial membrane. PO is thought to transfer electrons generated from the oxidation of proline to the electron transport chain via an intervening flavoprotein (63). Human cDNAs for PO have been identified and the gene has been located at 22q11.2 (82). Proline oxidase in humans is a 561 amino acid protein that is located primarily in liver, kidney and brain tissue with liver displaying the highest activity (35).

The product of the reaction catalyzed by PO, P5C is in spontaneous equilibrium with an open chain tautomer known as glutamic-γ-semialdehyde. It is not known with certainty whether P5C or glutamic-γ-semialdehyde is the preferred substrate for enzymes that utilize this compound; however the equilibrium is known to favour P5C under conditions *in vivo* (23). P5C is present in body fluids such as plasma, cerebrospinal fluid, urine and saliva. Interestingly, plasma concentrations have been observed to follow a diurnal pattern (22).

The plasma concentration of P5C appears to fluctuate from a baseline level of approximately 0.40 μM to values that are in the order of 3-5 fold higher. The highest peak in P5C concentration appears to occur at approximately 08:00 and does not necessarily correspond strictly to a fed or fasted state (22). The fact that plasma concentrations of P5C may fluctuate considerably and that fluctuations of this nature are generally not observed with other amino acids has lead to the thought that P5C may function as a regulator of various cellular processes (22). At this time however there is no substantial evidence to implicate P5C as a regulator of cellular metabolism.

1.1.2 Δ^1-pyrroline-5-carboxylate dehydrogenase

The second step in the proline catabolic pathway is catalyzed by Δ^1-pyrroline-5-carboxylate dehydrogenase. P5CDh catalyzes the NAD^+-dependent conversion of P5C to glutamate and has been purified from rat liver mitochondria (99). It has long been felt that P5CDh is located in both mitochondria and the cytosol (12). There is some controversy, however, regarding the presence of a

cytosolic enzyme (see chapter 3) (12). P5CDh is thought to be present in most mammalian tissues (82).

P5CDh isolated from rat liver mitochondria is thought to be an α-2-dimer with subunits of 59,000 daltons while the human protein which is also thought to be an α2-dimer may be slightly larger at 70,600 daltons (23). The larger size of the human enzyme has been challenged by Small and Jones (99). Western blot analysis of tissue extracts obtained from various rat and human tissues illustrated that P5CDh from both species gave bands corresponding to a protein with a mass of 59,000 daltons.

P5CDh is considered to be a non-reversible enzyme, as the results of *in-vitro* assays were unable to show the production of P5C from glutamate (99). The purified rat enzyme has a K_m of 0.09 mM and a V_{max} of 9.3 nmol/min with P5C as a substrate but does exhibit the ability to catalyze reactions involving other semialdehydes although they occur at a much reduced rate (99).

In 1996 two full length P5C dehydrogenase cDNAs were generated that differ only in the presence of a 1-kb intron located in the 3' untranslated region (37). Although the significance of the difference between the two cDNAs is not known at present, the longer transcript is more prevalent in most tissues. The gene for human P5CDh has been mapped to chromosome 1 and encodes a protein of 563 residues and also contains a putative N-terminal mitochondrial targeting sequence (37).

1.1.3 Glutamate dehydrogenase

Glutamate dehydrogenase (GDH) (EC 1.4.1.2) is a mitochondrial matrix enzyme that catalyzes a reaction that produces α-ketoglutarate and free NH_3 from glutamate using NADH as a cofactor (8). GDH appears to be freely reversible in mammalian liver and is known to be allosterically inhibited by GTP and allosterically activated by ADP (45). GDH is an important enzyme in the continuing metabolism of proline since GDH connects glutamate produced from proline with the Kreb's cycle and also is an important early step in the production of glucose from proline.

GDH in mammals is a homohexamer with the GLUD1 gene being located on chromosome 10q (73). GDH is found in liver, brain, kidney and the pancreas (45). A second gene for GDH, GLUD2 has also been found on the X chromosome that codes for a protein with 95% homology with that of GLUD1 (45). The tissue expression and function of GLUD2 has not been demonstrated.

1.1.4 Other enzymes involved in P5C metabolism

Proline oxidase and P5CDh are not the only enzymes that form and/or utilize P5C. Depending on the tissue and its requirements at any given time (eg. ATP production, gluconeogenesis, protein synthesis) there are other enzymes that may play important roles in P5C metabolism.

1.1.4.1 Δ^1-pyrroline-5-carboxylate reductase

Δ^1-pyrroline-5-carboxylate reductase (P5C reductase) (EC 1.5.1.2) is a cytosolic enzyme that catalyzes the conversion of P5C to proline using NAD(P)H as a cofactor (65). P5C reductase is commonly found in all mammalian tissues and catalyzes the final step in proline synthesis.

P5C reductase isolated from different tissues appears to exhibit quite different characteristics which has led to the prediction that there may be numerous isoenzymes of P5C reductase which display differing affinities for cofactors and are inhibited by different metabolites (65). For example, P5C reductase from human erythrocytes preferentially oxidizes NADPH exhibiting a 10-20 fold higher affinity than for NADH. The enzyme also displays a 5-10 fold increase in affinity for P5C as a substrate when NADPH is used as a cofactor as compared to NADH (65). This does not, however, appear to be the case with the enzyme expressed in liver. The hepatic P5C reductase demonstrates a preference for NADH, exhibiting activity with NADPH that is one tenth that observed with NADH as a cofactor (35).

1.1.4.2 P5C synthase

P5C synthase is a vital enzyme in the endogenous synthesis of proline. Glutamate is converted to P5C by P5C synthase, an enzyme that in mammals exhibits highest activity in the small intestine. P5C synthase activity has also been detected in colon, thymus, pancreas and brain but not in liver (112). Proline has been implicated as a potential neurotransmitter in the CNS and as

such the activity of P5C synthase in brain may play a role in producing the low concentration of proline (<5μM) observed in the extracellular fluid of the CNS (75).

1.1.4.3 Ornithine aminotransferase

Ornithine aminotransferase (OAT) (EC 2.6.1.13) is a member of the family of pyridoxal phosphate-dependent transaminases and is located in the mitochondrial matrix (60). OAT catalyzes the transamination of ornithine with the production of glutamate and P5C, and is thought to be freely reversible (35). However McGiven et al., (60) showed that in intact mitochondria ornithine was not produced from P5C unless oxidative phosphorylation was inhibited by the inclusion of rotenone in the assay. The presence of rotenone would inhibit complete oxidation of proline, which in turn would lead to increased concentrations of P5C in the mitochondrion. The increased availability of P5C could then allow OAT to function in the direction of ornithine production. This would suggest that in liver, in vivo, OAT functions mainly in catabolism and not synthesis of ornithine in actively respiring mitochondria and that inhibition of proline oxidation at the level of P5CDh or beyond must occur to produce ornithine from proline. OAT is present in numerous tissues with the kidney, small intestine and liver all having high activity. Recent work in our lab also supports the fact that OAT from liver does not appear to be reversible as it was not possible to show the production of ornithine from P5C in isolated hepatocytes (87).

1.2 Regulation of proline catabolism

Regulation of proline catabolism has been described in response to hormones, diet and various metabolites. In many cases however proline catabolism has been studied as a very small part of a larger study focusing on other amino acids or metabolites. The effect of this is that there is not a tremendous amount known about the regulation of proline catabolism in mammalian liver and what is known is often conflicted in published data. As an example of this, Kowaloff et al., (48) reported that proline oxidase activity is increased in response to glucocorticoids while Kawabata et al., (44) could not duplicate this result. In the present study we have focused our attention solely on the proline catabolic enzymes.

1.2.1 Proline catabolism in response to diet

Hormonal response to diet composition is thought to play an important role in determining the fate of ingested nutrients. Two hormones released from the islets of Langerhans of the mammalian pancreas, insulin and glucagon, appear to play a key role in regulating pathways of amino acid metabolism. The plasma concentration of the glucogenic hormone glucagon is known to be increased in response to starvation, diet and decreasing levels of glucose in plasma (51;53;71). It has been observed that the plasma concentration of glucagon can increase as much as 3-fold following four days on a high protein diet (78).

The maintenance of nitrogen balance is an important function of tissues such as kidney, muscle, intestine and liver with the latter playing by far the

largest role in nitrogen homeostasis. A diet high in protein increases the demand on enzymes involved in the catabolism of amino acids and in order to remain in nitrogen balance it is necessary to dispose of the increased quantity of nitrogen. It has been observed in man that the ingestion of a normal protein load of approximately 100 g per day would require an amount of oxygen in excess of what the liver is known to consume on a daily basis in order to completely oxidize the protein content in the diet (11). Given this fact it is obvious then that the liver must utilize the ingested amino acids for other purposes such as gluconeogenesis, ketogenesis, protein synthesis and production of other nitrogen containing macromolecules. Regulation of amino acid catabolic pathways is therefore likely to occur in order to shuttle carbon skeletons away from pathways that lead to complete oxidation.

The catabolism of amino acids produces the toxic weak acid NH_4^+ that is incorporated into the non-toxic forms of urea and glutamine by the liver for disposal (2). Urea and glutamine can be exported by the liver and later removed from circulation by the kidneys with urea being directly excreted in urine while NH_3 is added to urine following cleavage of glutamine by renal glutaminase (32). The production of urea is known to increase in response to a high protein diet as are the activities of various enzymes involved in amino acid catabolism (36;111).

An increase in the catabolism of proline in response to a diet high in protein could be expected to increase the rate of urea synthesis by supplying glutamate for the production of N-acetylglutamate which is a known activator of hepatic glutaminase (106). Proline catabolism as previously mentioned could

also provide glutamate for the production of glutamine and aid in the scavenger pathway of NH_4^+ detoxification.

It has been shown that the activities of PO (44) and P5CDh (57) are increased in rat liver mitochondria in response to a diet high in protein. However, the protein content of the diets employed in these studies was not a true reflection of physiologically relevant situations (60% and 70% respectively). Our goal was therefore to investigate the role of dietary protein in the catabolism of proline under conditions that were physiologically attainable.

1.2.2 Proline metabolism in response to glucagon

Hormonal regulation of amino acid catabolism is a common theme in the mammalian liver. Insulin, glucagon, glucocorticoids, catecholamines and thyroid hormone have all been reported to influence amino acid catabolism (49;54;101). Specifically the regulatory effects of glucagon on amino acid metabolism are well known. For example, in liver it can activate the glycine cleavage system, stimulate amino acid transport, and induce the five urea-cycle enzymes (29;55;100). It has also been observed that patients with a glucagonoma have diminished plasma amino acid levels that appear to be related to increased clearance by the liver (4). Given the effects of glucagon on amino acid metabolism, it appears likely that proline catabolism could be similarly regulated, since it is an important intermediate in various pathways as previously discussed.

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The response of the proline catabolic enzymes to long-term exposure to glucagon has not been studied to date. However in experiments with isolated hepatocytes incubated for 30 minutes in the presence of glucagon (300 nm) it has been shown that glucagon increases gluconeogenesis from proline by

approximately 30% (104). The increase in catabolism of proline in response to glucagon is felt to be due to a cAMP mediated effect and while the duration of the effect was not determined beyond 30 minutes, it is believed to be of relatively short duration (104).

The increase in proline catabolism observed via short term incubation of hepatocytes in the presence of glucagon is not a unique finding as results of this nature have been reported for other amino acids such as threonine, glycine, and arginine (36;42;77). It is worthy to note that acute regulation of amino acid catabolism by glucagon is not simply a characteristic of amino acid catabolism in general since ornithine catabolism does not exhibit such an increase in the presence of glucagon in perfused liver (77).

In addition to acute modification of enzyme activities, exposure to glucagon for longer periods of time is known to induce various enzymes related to amino acid catabolism. For example enzymes of the urea cycle are known to be induced by long-term glucagon treatment (111). In the case of the urea cycle enzymes, increased transcription of mRNA for arginase and carbamyl phosphate synthase has been observed (111). An increase in the flux through the transsulfuration pathway in liver has also been reported in response to long-term treatment with glucagon (40).

1.3 Possible roles for proline catabolism (old and new)

The production of glutamate from proline following the reactions catalyzed by PO and P5CDh provides a number of possibilities as to the final catabolic product of proline. With regard to catabolism, proline is a very versatile amino acid which given the correct conditions supplies needed substrates for synthesis of glucose, glutamate, glutamine, ATP and proteins. Proline as a substrate for the production of reactive oxygen species (ROS) has also been implicated as a mediator of apoptosis (18;58;59).

The catabolism of proline could supply glutamate for glutamine synthesis and in so doing provide an auxiliary source of glutamine which is considered to be a conditionally essential amino acid during trauma, major surgery or during other times of stress (110). Glutamine, a common extracellular amino acid reaching a plasma concentration of 0.7 mM is also utilized extensively by cells of the immune system and the intestine (74). Hepatic production of glutamine by glutamine synthase (GS) could occur utilizing glutamate obtained from the oxidation of proline. The glutamine produced in this manner could subsequently be exported to the circulation and taken up by lymphocytes. It is known that the enzymes required occur in rat liver and O'Sullivan et. al., (76) demonstrated that P5CDh must occur in the same intrahepatic zone as GS.

Glutamine production has also been described as a potential method to decrease the amount of toxic NH_3 released from the liver as a by-product of amino acid catabolism. In this manner glutamine synthesis functions in a so-called scavenger fashion to remove endogenously produced NH_3^+ (32).

Glutamate supplied from proline could also be oxidized via Kreb's cycle following conversion to α-ketoglutarate by GDH, or be converted to glucose via the gluconeogenic pathway starting once again with conversion to α-ketoglutarate by GDH and subsequent production of oxaloacetate via Kreb's cycle enzymes.

As previously mentioned proline has been implicated in the induction of apoptosis in colorectal cancer cells. Maxwell and Davis (58) have shown that induction of proline oxidase occurs in response to the tumour suppressor protein p53. The increased activity of PO in response to p53 is postulated to play a role in pathways that are designed to prevent the generation of mutations and eventually tumours.

The tumour suppressor protein p53 functions in various manners, one of which is to arrest the cell cycle at G_1 in response to DNA damage (59). The result of halting the cell cycle is to allow the DNA damage to be repaired prior to replication. The induction of proline oxidase is felt to possibly play two roles, one of which may aid in repair of DNA while the other is implicated in the induction of apoptosis should DNA repair fail.

1.3.1 Proline and DNA repair

The pathway of proline induction that may aid in DNA repair is based on an intracellular proline/P5C cycle between mitochondria and cytosol that generates $NADP^+$ in the cytosol (figure 1.2) (80). The increased concentration of $NADP^+$ in the cytosol is proposed to lead to an increase in the oxidation of

glucose through the pentose phosphate pathway and eventually increased production of nucleotides. $NADP^+$ is a cofactor required by the first enzyme of the pentose phosphate pathway, glucose-6-phosphate dehydrogenase and NADPH is known to be an inhibitor of the enzyme (21). The increased production of nucleotides provides needed substrates for DNA repair. Increased activity of the pentose phosphate pathway has been observed in response to P5C in erythrocytes and fibroblasts and although it has not been demonstrated in liver the complement of enzymes required is present (81;114).

The proposed proline/P5C cycle transfers redox potential into mitochondria in the form of proline that is then oxidized by PO with the transfer of electrons into the electron transport chain and the generation of ATP (84). The molecule of P5C formed by PO is then transported into the cytosol and reduced to proline by P5C reductase with the production of $NADP^+$. Induction of PO and increased production of nucleotides may play a role in DNA repair but it is felt that the potential contribution of PO to apoptosis is of greater importance since the availability of nucleotides and ATP have been implicated in the expression and activation of caspases that are required for apoptosis (59).

1.3.2 Proline and apoptosis

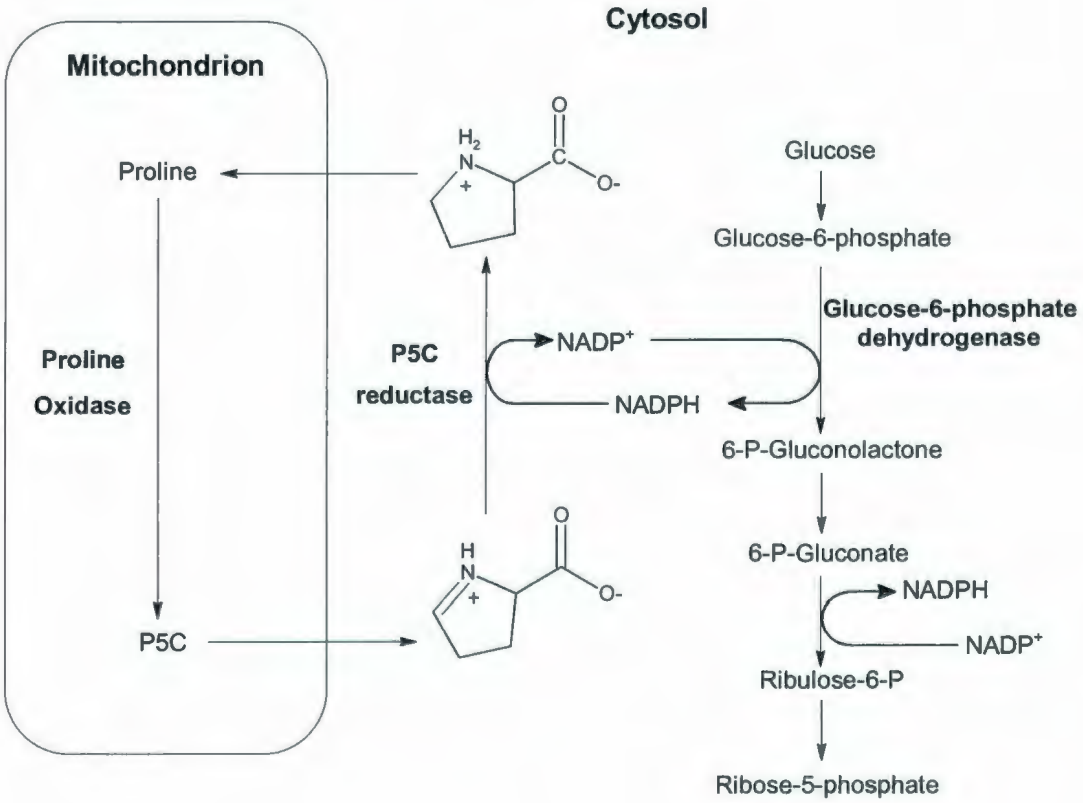
The role of PO in apoptosis has been investigated in bladder cell carcinomas, renal carcinomas, non-small cell lung carcinoma and colorectal carcinomas. Although studies have not been conducted with hepatoma cell lines,

it is possible that some of the findings described may apply to cancers of the liver (18;58;59).

Initial investigations in cells that are susceptible or resistant to p53-mediated apoptosis led to the observation that induction of PO occurs in cells susceptible to p53-mediated apoptosis (58). The generation of ROS via PO is felt to occur by disruption of the electron transport chain and an interruption of the normal flow of electrons generated by the oxidation of proline. PO donates electrons to cytochrome c and one of the characteristics of apoptosis is the mitochondrial membrane permeability transition that results in the release of cytochrome c into the cytosol (18). The release of cytochrome c into the cytosol could disrupt the flow of electrons from proline oxidation and lead to the production of ROS.

Figure 1.2: Proposed proline/P5C cycle.

Proline enters the mitochondrion and is oxidized by proline oxidase to P5C which leaves the mitochondrion. Cytoplasmic P5C is then reduced by P5C reductase to proline with the concomitant oxidation of NADPH. Proline formed in the cytosol can then re-enter the mitochondrion to start a new cycle. The increased ratio of $NADP^+$/NADPH stimulates flux through the pentose phosphate pathway, which in turn increases synthesis of ribose-5-phosphate and thus phosphoribosyl pyrophosphate (81).


Cytosol
Mitochondrion
Proline
Proline
Oxidase
P5C
P5C
reductase
NADP+
NADPH
Glucose
Glucose-6-phosphate
Glucose-6-phosphate
dehydrogenase
6-P-Gluconolactone
6-P-Gluconate
NADPH
NADP+
Ribulose-6-P
Ribose-5-phosphate

The actual role of PO in inducing apoptosis *in vivo* has not been determined. However mitochondrially derived ROS do appear to play a substantial role in the pathway of apoptosis (94) and given the fact that PO under the correct conditions may produce ROS it is not inconceivable that p53 induction of PO may have a role in this pathway. To exactly what extent PO contributes to apoptosis if at all is yet to be determined.

1.4 Proline transport

1.4.1 Transport across the plasma membrane

Transport across the plasma membrane is a vital step in the catabolism of amino acids. Given the correct circumstances this process could exert considerable control over the rate of amino acid catabolism. For example, transport of alanine, a major gluconeogenic amino acid, into hepatocytes is thought to be the rate-controlling step for the catabolism of this amino acid (97). It has also been shown that the transport of glutamine across the plasma membrane of hepatocytes may be an important factor in determining the rate of glutamine breakdown and synthesis (33). Given the fact that the liver is a major organ of amino acid metabolism the importance of amino acid transport cannot be discounted.

System A is almost ubiquitous in regard to distribution and transports small neutral amino acids showing a preference for alanine, serine and glutamine (62). System A is characterized by its ability to transport *N*-methyl amino acid derivatives and the non-metabolized alanine analogue *N*-methylamino-α-

isobutyric acid (meAIB), tolerance of substitution of Li^+ for Na^+ (depending on the isoform), trans-inhibition and sensitivity to pH (62). The stoichiometry of the reaction catalyzed by the system A family of transporters is 1 Na^+:1 amino acid (31).

Proline transport has classically been considered to occur by the transport system known as system A (46). In mammals system A is a Na^+ dependent co-transport system and is found in numerous tissues including liver (62). The family of amino acid transporters previously known as system A is now known to consist of at least three transporters, ATA1, ATA2, and ATA3. The members of this family of transporters have been cloned and although they are classified together they exhibit differing characteristics in regard to specificity, and tissue distribution (9). ATA2 and ATA3 are present in mammalian liver and while both transport the usual substrates reported for system A transporters, ATA3 also transports arginine and, in fact, may preferentially transport arginine (31).

System ATA2 is thought to be the major inducible transporter of small neutral amino acids in the mammalian liver (46). System A activity is known to be induced in response to amino acid starvation (adaptive response), various hormones (glucagon, insulin and growth hormone) and hyperosmotic stress (1;5).

1.4.2 Mitochondrial transport

Transport of proline into the mitochondrion is an obligate step in the oxidation of proline and as such is also a possible point of control. The transport

of amino acids across mitochondrial membranes has not been studied in depth and does not appear to be considered as an important step in controlling amino acid metabolism. The outer mitochondrial membrane is not considered to be a barrier to amino acid transport due to the presence of the protein complex porin that provides channels for the free movement of molecules (106). The inner mitochondrial membrane does, however, provide a barrier to amino acid movements and thus the transport of various amino acids into the mitochondrial matrix is a vital component of hepatic metabolism. The presence of mitochondrial transport proteins or carriers for some amino acids has been known for some time as is the case for biologically important molecules such as ADP/ATP and carnitine/acylcarnitine (69).

It is not known at the present time exactly what role the transport of proline across the inner mitochondrial membrane may play in the regulation of proline catabolism. However transport of amino acids into the mitochondrial matrix has been suggested as a potential rate-limiting step for catabolism. McGiven et al., (60) have suggested that the transport of ornithine across the inner mitochondrial membrane is the rate-limiting step in the catabolism of ornithine and/or urea synthesis. The data supporting this conclusion include the observation that in intact mitochondria K_m and V_{max} values are very similar for both transport and ornithine catabolism while in solubilized mitochondria the V_{max} for ornithine aminotransferase is considerably higher (60).

In regard to proline transport across the inner mitochondrial membrane it was believed for quite some time that proline simply diffused into the

mitochondrial matrix (28). However the findings by Meyer (66) that proline transport into mitochondria is stereospecific, subject to inhibition and able to create a concentration gradient of proline which favours the intramitochondrial accumulation of proline would certainly seem to disprove the thought that proline simply diffuses into liver mitochondria. The nature and identity of the transporter responsible for proline entry into mitochondria has not be determined to date.

1.4.3 Regulation of proline transport

System A (ATA2) is often termed the major inducible amino acid transporter of eukaryotes (46). Numerous investigators have described increases in system A activity in response to stimuli such as amino acid starvation and hormones. At present it appears as though only ATA2 is subject to regulation and that the other two members of the system A transport family may function in a strictly constitutive manner (91).

System ATA2 is subject to control by glucagon, insulin, and glucocorticoids (46). Data on glucocorticoid control of system ATA2 is not conclusive but appear to point to a permissive role for glucocorticoids as has been observed in other situations when glucocorticoids are combined with either insulin or glucagon (96).

In regard to system ATA2 induction by glucagon LeCam and Freychet (52) observed that half maximal stimulation is observed at a concentration of 1.5 nM and appears to occur via a cAMP-dependent mechanism as cAMP is able to invoke the same effect as glucagon itself (46;52). The effect of glucagon requires

approximately sixty minutes to occur and is detected even if the hormone is removed shortly after exposure to isolated hepatocytes which has been interpreted to mean that protein synthesis is occurring (46;52). A similar result was reported by Edmondson and Lumeng (20). In addition to the supposed protein synthesis dependent increase in activity of system A, it was also observed that there was a small (20% compared to control) increase in activity during the first sixty minutes which was cycloheximide insensitive (20).

Further evidence for increased synthesis of ATA2 in response to glucagon is provided by Barber et al, (5) who used tunicamycin, an inhibitor of asparagine-linked glycoprotein synthesis to demonstrate that by blocking protein synthesis there is no increase in system A activity in response to glucagon following that observed in the first sixty minutes. It has also been proposed that a regulatory protein may be involved in system ATA2 induction in response to glucagon and other hormones (5). The existence of a regulatory protein has not been demonstrated to date.

1.5 Disorders of proline catabolism

The importance of proline catabolism is readily illustrated by considering disorders of proline catabolism that result in increased plasma concentrations of proline. Hyperprolinemia has been associated with renal disease, seizures and mental retardation. Type I hyperprolinemia is associated with decreased or absent proline oxidase activity while type II hyperprolinemia is associated with decreased or absent P5CDh activity (82). Type I and type II hyperprolinemia are

both autosomal recessive diseases and are due to mutations/deletions at different loci (82).

Proline has been implicated as a possible neurotransmitter in the CNS and has been shown to act as an excitatory neurotransmitter in rat spinal neurons (3). With this in mind, Nadler et al. (72) demonstrated that proline is toxic towards rat hippocampal neurons and have hypothesized that this may be the reason for neurological and cognitive deficits seen with hyperprolinemia. The concentration of proline present in the extracellular fluid of the CNS in individuals afflicted with hyperprolinemia is markedly increased reaching levels that are well above the normal level of < 5 μM.

1.5.1 Type I hyperprolinemia

Type I hyperprolinemia has generally been regarded as a benign disease with a possible relation to renal disease and relatively little or no neuropathy but as of this time there is no direct correlation between the observed increase in plasma proline and renal dysfunction. There does, however, appear to be a degree of variability with respect to the phenotype displayed by affected individuals as a report by Humbertclaude et al., (38) reported a case of type I hyperprolinemia in which the individual displayed severe neurologic dysfunction with no impairment of renal function.

1.5.2 Type II hyperprolinemia

Type II hyperprolinemia as previously mentioned is a disease caused by a deficiency in P5CDh and is characterized by increased incidences of seizures, often in childhood, and mental retardation (24). The phenotype of this disease is an increase in the plasma and urine concentrations of both proline and P5C. Levels of proline in plasma of affected individuals have been reported as high as 2.3 mM (normal <0.30 mM) while urinary concentrations of 40.0 mM (normal <0.1 mM) have been observed (24). The plasma level of proline in individuals with type I hyperprolinemia rarely reaches 2.0 mM while in type II hyperprolinemia the plasma level of proline is virtually never below 1.0 mM (95). The higher plasma levels of proline and the accumulation and excretion of P5C are indicators of type II hyperprolinemia.

Geraghty et al., (24) have determined that there are various mutations that can cause type II hyperprolinemia ranging from deletions of a single base pair that causes a frameshift mutation and a non-functional protein, to what appears to be a neutral mutation in the putative mitochondrial targeting sequence. In regard to the neutral mutation in the gene for P5CDh it is not known how this relates to a deficiency in functional protein, but it would appear the mutation would lead to a decrease in functional protein in the mitochondrial matrix. Type II hyperprolinemia has an estimated frequency of 1 in 200,000 which translates to a carrier frequency of 1 in 223 (24).

1.6 Problem of investigation

The first step in understanding the factors that influence the catabolism of any substance is to determine the location of the enzymes metabolizing that substance. In that regard our first task was to determine the location of P5CDh. The subcellular location of this enzyme has not been definitively shown. In order to perform a true subcellular location of an enzyme a valid assay for the enzyme in question is required. Therefore we first had to develop an assay that would allow us to assay the activity of P5CDh in rat liver so we could subsequently determine the subcellular location of P5CDh.

In order to gain an understanding of the changes that may occur in proline catabolism in liver we have studied enzymes involved in proline degradation under varying conditions. The two major enzymes involved in proline catabolism in the mammalian liver, PO and P5CDh, catalyze sequential reactions that result in the production of glutamate from proline. Glutamate produced from proline can subsequently be combined with ammonia to form glutamine via glutamine synthetase (GS), enter Kreb's cycle as α-ketoglutarate via glutamate dehydrogenase (GDH), or supply carbons for glucose production via gluconeogenesis. Given that proline is one of the most abundant free amino acids in plasma (90) and that proline degradation can supply substrates for a diverse range of products, it is probable that regulation of the proline catabolic enzymes occurs in response to a variety of metabolic signals. Specifically it has been observed that significant changes in the catabolism and/or transport of

amino acids can occur in response to changes in the level of ingested protein (53) and in response to the catabolic hormone glucagon (4;56). It has also been observed that the plasma level of glucagon is increased in response to a diet high in protein (4). Given the implication of proline in the induction of apoptosis and the diversity that proline exhibits in regard to the formation of metabolic products, the regulation of the proline catabolic enzymes is of increasing importance. Therefore we have studied the response of proline catabolism in liver to a diet containing excess protein and injections of glucagon.

Chapter 2: Materials and methods

2.1 Materials

All chemicals were of the highest quality available and were purchased from Sigma (Oakville, ON, Canada), except where noted in the text.

2.2 Animals

Male Sprague-Dawley rats (supplied by the University's breeding colony) weighing between 200-300 grams were used in all studies and were fed laboratory chow (Lab diet, 5P00 Prolab RMH 3000) *ad libitum* except when protein was varied, and had free access to water. The rats were exposed to a 12-hour light: 12-hour dark cycle beginning with lights on at 8 am. All studies were conducted 2 hours following termination of the dark cycle. All procedures were approved by Memorial University's Institutional Animal Care Committee and are in accordance with the principles and guidelines of the Canadian Council on Animal Care.

2.3 High protein diet

Male Sprague-Dawley rats weighing 210-285g were housed separately in polycarbonate cages and fed a modified AIN-93G diet (89) that contained 15% (control) or 45% (high) protein for 7 days. The modified diet conformed to AIN-93G specifications for energy, fat and micronutrients but varied in the amount of protein (casein) (table 2.1). Energy density was kept equal to that in the original AIN-93G diet by substituting protein (casein) for cornstarch, sucrose and

Table 2.1: Contents of diets

	45% protein (g/1000g)	**15% protein** (g/1000g)
Casein	450	150
Corn starch	238	429
dextrinized-corn starch	79	143
Sucrose	60	108
Soya bean oil	70	70
Alpha cell (AIN)	50	50
Mineral mix (AIN)	35	35
Vitamin mix	10	10
Cysteine	3	3
Choline Bitartrate	2.5	2.5
T-butyl hydroquinone	0.014	0.014

dextrinized cornstarch (removed in proportion to their concentration in the original formulation). All animals were weighed daily at 8:00 AM. The amount of the diet that each animal consumed was also tracked by daily weighing of the feeders assigned to each animal. Animals were sacrificed at 10:00 AM on the last day of the study.

Using data obtained from our study in regard to food intake (chapter 4) and data obtained from ICN Biomedicals (Aurora, Ohio) with respect the amino acid composition of the casein (Cat # 960128) that we used in our study it was possible to determine the approximate intake of proline per day by the study animals (table 2.2). As well using data obtained for the food intake of control animals (table 4.1) fed a standard chow diet and data obtained from Lab diet (Richmond, IN) regarding the amino acid content of the Prolab RMH 3000 rodent diet we also calculated the intake of proline in animals consuming a regular chow diet (table 2.2).

2.4 Glucagon treatment

Glucagon treatment followed the procedure of Jacobs et al., (40). Glucagon (Eli Lilly Canada Inc.) (4mg/kg body weight/24 hrs, subcutaneously) was administered in three injections at eight-hour intervals for two days while control animals received the vehicle (diluting solution provided by Eli Lilly Canada Inc.). All animals were fasted overnight on the last day of the study. Two hours following the last injection, rats were anesthetized using sodium pentobarbital (6.5mg/100g body wt.). Following a midline abdominal incision, a blood sample

Table 2.2: Content and amount consumed of proline, arginine and glutamate in standard rat chow and modified AIN 93G diets.

	Content (g/100 g of diet)			Consumption (g/day)		
	Proline	**Arginine**	**Glutamate**	**Proline**	**Arginine**	**Glutamate**
Standard chow	1.73	1.37	5.33	0.45	0.36	1.47
Modified 45% diet	4.95	1.67	9.50	1.20	0.38	2.29
Modified 15% diet	1.65	0.56	3.17	0.42	0.14	0.84

was collected from the abdominal aorta. The liver was then rapidly removed, a portion freeze-clamped at –70°C while the remaining tissue was used to isolate mitochondria. Heparinized tubes containing the blood samples were placed on ice until plasma was separated by centrifugation in a clinical centrifuge (3700xg for 15min). The plasma was stored at -70°C until required.

2.5 Subcellular fractionation of liver

Rats were anesthetized using 6.5 mg of sodium pentobarbital per 100 gram. Following a midline incision the liver was quickly removed, weighed, and placed in ice-cold homogenization buffer, consisting of 5 mM Hepes (pH 7.4), 1 mM EGTA, and 0.33 M sucrose to a final volume of 5 mL of buffer per gram of liver. The liver was finely minced with scissors, washed three times in ice-cold homogenization buffer and homogenized at approximately 500 rev/min by 5-6 strokes of a motor driven loose fitting teflon pestle (clearance 0.13-0.18 mm). The homogenate was diluted to 10 mL per gram of liver with ice-cold homogenization buffer and filtered through two layers of cheesecloth. Following filtration 10mL of the homogenate were removed and labelled as total homogenate (TH) while the remaining filtrate was fractionated by differential centrifugation into a nuclear fraction (N), a heavy mitochondrial fraction (M1), a light mitochondrial fraction (M2), a lysosomal fraction (L), a microsomal fraction (P), and a cytosolic fraction (S). The method proposed by DeDuve et al. (17) was followed with the following modifications. All procedures were completed at 4°C.

The nuclear fraction was obtained by centrifugation of the homogenate at 384xg for 2 minutes. The resulting pellet (intermediate) was resuspended (by manual shaking) in 20.0 ml of ice-cold homogenization buffer and centrifuged again at 384xg for 2 minutes. The final nuclear pellet was resuspended in 10.0 ml of ice-cold homogenization buffer and the supernatant pooled with the previous supernatant, to give the post nuclear supernatant. The heavy mitochondrial fraction was isolated employing two centrifugations of the post nuclear supernatant at 1000xg for 10 minutes. The same procedure for resuspension of the intermediate and final pellets was employed as for the nuclear fraction. The resultant supernatants were again pooled. The light mitochondrial fraction was obtained by an initial centrifugation of the post heavy mitochondrial supernatant at 3500xg for 10 minutes, followed by resuspension of the intermediate pellet as above and a second centrifugation at 3000xg for 10 minutes. The lysosomal and microsomal fractions were obtained by single centrifugations of 9090xg for 20 minutes and 100,000xg for 60 minutes respectively and resuspension of the resulting pellets as before. The supernatant obtained following the centrifugation at 100,000xg represents the cytosolic fraction. Exact volumes were noted for all fractions and they were packaged in 1.0 ml aliquots and stored at -70°C until analysis.

2.6 Preparation of mitochondria

Rats were anesthetized using 6.5 mg of sodium pentobarbital per 100 grams. Following a midline incision the liver was quickly removed, weighed, and

placed in ice-cold homogenization buffer, consisting of 5 mM Hepes at pH 7.4, 1.0 mM EGTA, 0.225 M mannitol and 0.075 M Sucrose, to a final volume of 5.0 ml per gram of liver. The liver was finely minced with scissors, then homogenized at approximately 500 revs/min by 5-6 strokes of a motor driven loose fitting teflon pestle (clearance 0.13-0.18 mm). The homogenate was filtered through two layers of cheese-cloth and mitochondria were isolated according to the method of Jois et al., (42) with minor modifications. Briefly, the homogenate obtained above was centrifuged at 2250xg for 10 minutes and the resulting pellet was discarded (nuclei/cellular debris) while the supernatant was transferred to clean 50 ml centrifugation tubes and centrifuged at 8200xg for 10 minutes. The pellet obtained was resuspended in homogenization medium and the centrifugation and resuspension steps repeated three times. The final pellet was resuspended in a small volume of homogenization medium (1.0-1.5 ml) which routinely resulted in a mitochondrial protein concentration of 60-80 mg/ml as determined via the Biuret method (25). Mitochondrial integrity was assessed via determination of respiratory control ratios using a Clark oxygen electrode with 10mM α-ketoglutarate as the substrate according to (42) and was always greater than 4. Samples of mitochondria were stored at -70°C until required for measuring enzyme activity or were used freshly prepared for oxidation of proline.

2.7 Proline oxidation by isolated mitochondria

Aliquots of the freshly prepared mitochondria were added to 25mL Erlenmeyer flasks to give a final concentration of 0.25 mg/mL of mitochondrial

protein and were pre-incubated for 15 minutes in a shaking water bath at 25°C in a buffer solution consisting of 100 mM potassium chloride, 50 mM mannitol, 20 mM sucrose, 10 mM potassium phosphate, 0.1 mM EGTA, 1.0 mM magnesium chloride, 1.0 mM ADP, 25 mM Hepes at pH 7.4 (final volume 3.0 ml). Following the 15-minute pre-incubation 20 μl of the appropriate concentration of [U]-^{14}C-proline[1] (286.0 mCi/mmol) (PerkinElmer Life Sciences Inc. Boston, MA) was added to the flasks. Each flask was then fitted with a centre well containing a small piece of fluted filter paper and 0.30 ml of NCS tissue solubilizer to trap $^{14}CO_2$ released after the reaction was stopped following a 10 minute incubation. The assay was terminated by the addition of 0.30 ml of ice-cold 30% (w/v) perchloric acid by syringe through the centre well. $^{14}CO_2$ was collected for 60 minutes at which time the centre wells were removed from the flasks and the piece of filter paper was transferred to a scintillation vial containing 15 ml of ScintiSafe Plus™ 50%, (Fisher Scientific, Nepean, ON) and radioactivity was determined in a LKB 1214 Rackbeta Liquid Scintillation Counter. All samples were run in triplicate as were zero time blanks in which the acid was added prior to the addition of proline and zero protein blanks in which no mitochondrial protein was added to the appropriate flasks which were then subjected to the same procedure as all other samples.

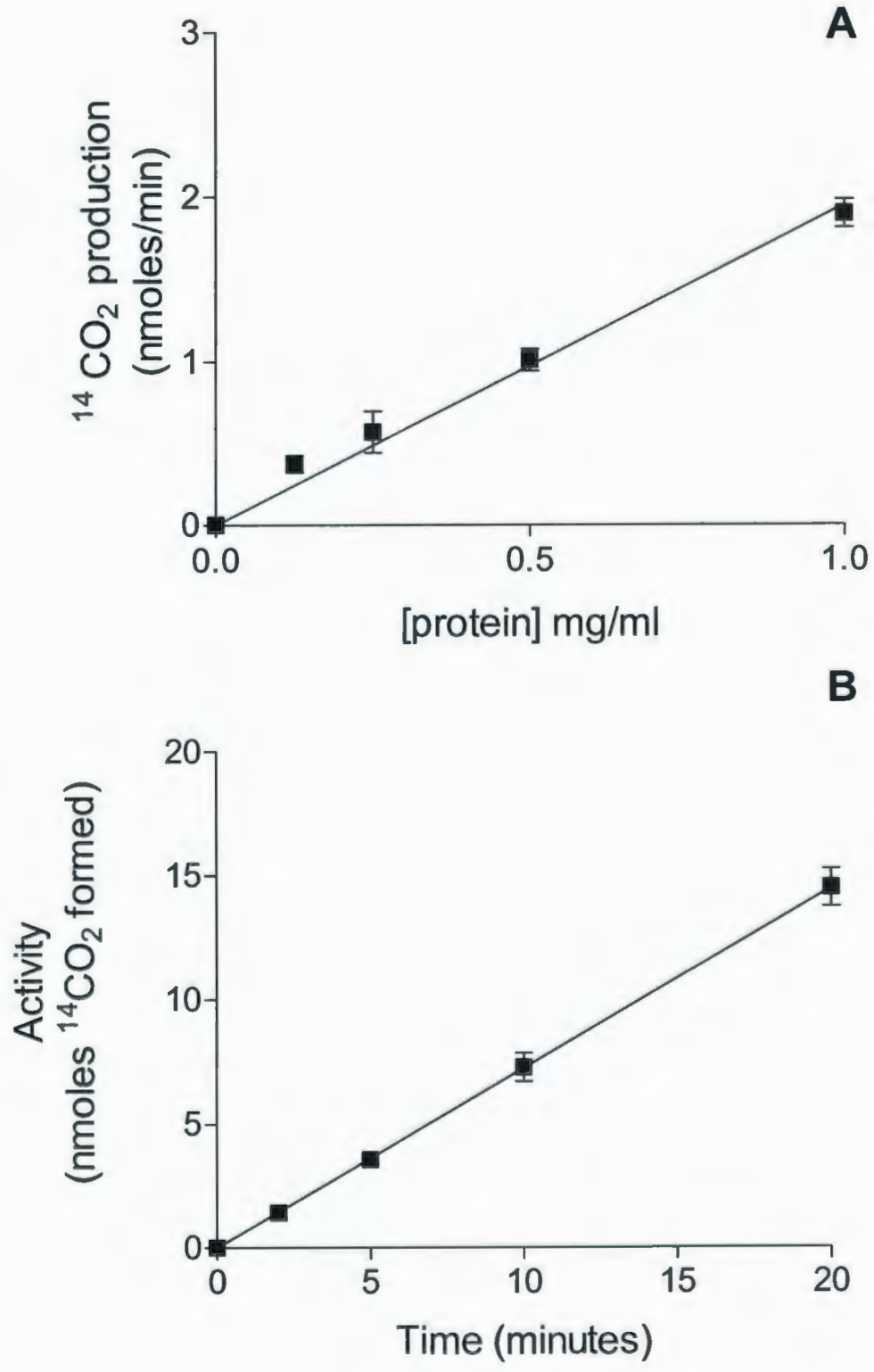
[1] ^{14}C-proline is taken to represent [U]-^{14}C-proline unless stated in the text

2.7.1 Linearity with time and protein and kinetic assays

To determine linearity with time and protein for the production of $^{14}CO_2$ from ^{14}C-proline in isolated mitochondria a series of experiments were conducted in which the concentration of protein was varied from 0.10 mg/ml to 1.0 mg/ml (figure 2.1 A) and for times ranging from 2 to 20 minutes (figure 2.1 B). Once it was determined over which intervals the reaction was linear in regard to time and protein the concentration of proline was varied over the range of 0.25 mM to 5.0 mM using 0.25 mg of mitochondrial protein and an incubation time of 10 minutes in both control and animals receiving exogenous glucagon. All analyses were conducted in triplicate.

Figure 2.1: Linearity of proline oxidation in mitochondria with time and protein

Oxidation of [U]-^{14}C-proline in freshly isolated, actively respiring mitochondria. Mitochondria were isolated from the livers of animals that had received exogenous glucagon (4mg/kg body weight/24 hrs for two days, subcutaneously). Control animals received the vehicle (diluting solution provided by Eli-Lily). A: Linearity with protein: incubations were conducted for 10 minutes at 25°C with concentration of mitochondrial protein from 0.0-1.0 mg/ml and 1.0 mM proline.
B: Linearity with time: Incubations were conducted with 0.25 mg/ml of mitochondrial protein for up to 20 minutes at 25°C with 1.0 mM proline. Data presented are means ± SD, n=4.


A
3
2
1
0
0.0
0.5
1.0
$^{14}CO_2$ production
(nmoles/min)
[protein] mg/ml
B
20
15
10
5
0
0
5
10
15
20
Activity
(nmoles $^{14}CO_2$ formed)
Time (minutes)

2.8 Amino acid, glucose and hormonal analyses

For amino acid determination, plasma and freezed-clamped liver were deproteinized with 10% sulfosalicylic acid. Following centrifugation, the resulting supernatant was adjusted to pH 2.2 with lithium citrate buffer. The samples were analysed on a Beckman 121 MB Amino Acid Analyser using Benson D-X, 0.25 Cation Xchange Resin and a single column, three buffer lithium method as per Beckman 121MB-TB-017 application notes. Results were quantified using a Hewlett Packard Computing Integrator Model 3395A. Plasma glucose concentrations were determined enzymatically (hexokinase/glucose-6-phosphatase) according to the method of (6). Plasma insulin and glucagon levels were measured by Linco Research Inc (St. Charles, MO) using rat insulin and glucagon, respectively, as standards. Intracellular hepatic amino acid concentrations were calculated according to the method of Jacobs et al. (40).

2.9 Enzyme assays

2.9.1 Δ^1-pyrroline-5-carboxylic acid dehydrogenase

P5CDh activity was measured, using either NAD^+(Roche Biochemicals) or $NADP^+$ (Roche Biochemicals) as a cofactor. The method of Strecker (105) was used as a basis for designing the assay conditions used in this study. The assay was modified by the presence of 10 μM rotenone in the reaction mixture to prevent reoxidation of NADH. The reaction mixture consisted of 260 μL of a solution containing 1mM EDTA and 12 mM Hepes at pH 7.8, 10 μL of 0.300 mM rotenone (dissolved in dimethyl sulfoxide), 10 μL of a 0.12 mg/ml suspension of

mitochondrial protein and 10 μL of 100 μM NAD^+ or $NADP^+$. The reaction was started by the addition of 10μL of 12 mM DL-P5C. The resultant final concentrations in the assay were: EDTA 0.87 mM, Hepes 10.4 mM, 0.010 mM rotenone, 0.033 μM $NAD(P)^+$, 0.40 mM P5C and 1.2 μg of protein. All samples were run in triplicate and the progress of the reaction at 37°C was measured by recording the production of NADH/NADPH at 340 nm.

2.9.2 Preparation of Δ^1-pyrroline-5-carboxylic acid

P5C was obtained as the 2,4-dinitrophenylhydrazone derivative and prepared for use in enzyme assays according to the method of Mezl and Knox (67). Determination of DL-P5C concentration was carried out according to the method of Piez et al. (86).

2.9.3 DNA and protein analysis

DNA was extracted from the subcellular fractions using the method of Schneider (93), and the concentration of DNA in each sample was determined using the method described by Burton (13) with calf thymus DNA as standard. Protein concentration in each fraction was determined by the biuret method (25) using Bovine serum albumin as standard, following solubilization of membranous material for 15 minutes with 5% deoxycholate (39).

2.9.4 Other enzyme assays

LDH activity was measured according to Morrison et al. (70), succinate cytochrome C reductase and NADPH cytochrome C reductase activity were measured according to Sottocassa et al. (102), proline oxidase according to Herzfeld et al. (35), ornithine aminotransferase according to Herzfeld and Knox, (34) with the addition of 0.05 mM pyridoxal-5-phosphate, glutaminase according to Lacey et al. (50), glutamate dehydrogenase according to Morrison et al., (70) and β-glucuronidase according to deDuve (17). Zero time, zero protein and/or zero substrate blanks were completed as appropriate. All enzyme assays were completed in triplicate, conducted at 37°C following disruption of organelles via three cycles of freezing and thawing, and were linear with time and protein.

2.10 Preparation of hepatocytes

Primary rat hepatocytes were isolated via the method of Berry *et al.*, (7). An anaesthetised rat was injected with 0.10 ml of heparin (1000 units/ml) through the femoral vein. A cannula was then inserted into the portal vein and non-recirculating perfusion commenced immediately, (the inferior vena cava was cut below the kidney), with 500 ml calcium-free Krebs-Henseleit medium (144 mM Na^+, 6 mM K^+, 1.2 mM Mg^{2+}, 126 mM Cl^-, 1.2 mM $H_2PO_4^-$, 1.2 mM SO_4^{2-}, 25 mM HCO_3^-) containing 2 mM EGTA, 20 mM glucose, 2.1 mM lactate, 0.3 mM pyruvate (pH 7.4). The flow rate was 40 ml/minute and the medium was gassed with 19:1 O_2/CO_2 for 20 minutes before use. Following a 12-minute flow through period, the medium was switched to 500 ml Krebs-Henseleit medium containing

1.3 mM Ca^{2+}, 20 mM glucose, 2.1 mM lactate, and 0.3 mM pyruvate (also gassed for 20 min before use). A cannula was then inserted through the right atrium and into the inferior vena cava and the inferior vena cava was tied off above the renal artery to stop flow through the previously made cut. Krebs-Henseleit medium supplemented with 0.25 % BSA and collagenase (50 mg/100 mL) was then recirculated through the liver. The recirculation of perfusate continued until the liver became soft (15-20 minutes), at which point the liver was transferred to a petri dish containing approximately 20ml of the collagenase containing medium and massaged gently to liberate cells. The resulting suspension was incubated at 37°C for 10 minutes in a shaking water bath under constant gassing (19:1 O_2/CO_2). Following filtration through cheesecloth, the cells were centrifuged at 600 rpm for 2 minutes and then resuspended in Krebs-Henseleit medium containing calcium, and the process was repeated. A final wash with Krebs-Henseleit medium containing 2.5% BSA was completed, and the cell pellet was resuspended in this medium. Hepatocytes were quantified by drying 3.0 ml of resuspended cells or 3.0 ml of 2.5% BSA Krebs-Henseleit medium in pre-weighed foil pans at 50°C for 24 hours. The difference in the weights represented the dry weight of the cells. Cell viability was determined by 0.1% trypan blue exclusion and was always greater than 95%.

2.11 Proline transport into hepatocytes

Proline transport into isolated hepatocytes was measured as described by Salter *et al.,* (92). Freshly prepared hepatocytes (~5.0 mg/ml) were pre-incubated for 20 minutes in Krebs-Henseleit medium (pH 7.4), gassed with O_2-CO_2 (19:1) with added lactate and pyruvate (2.1 and 0.3 mM, respectively). After pre-incubation ^{14}C-proline was added to give final concentrations of 0.5 mM, 1.0 mM, 2.0 mM, 3.0 mM, 4.0 mM, 5.0 mM, 6.0 mM, 7.0 mM, 8.0 mM and 9.0 mM. At 30 seconds following the addition of proline, 1 ml aliquots were transferred to 1.5 ml microcentrifuge tubes containing 0.25 ml of silicone oil mixture (2:1 (v/v) Dow Corning 550 silicone oil and dionony phthalate) layered on top of 0.1 ml of 6% (v/v) perchloric acid. The tubes were centrifuged at 14,000g for 15 seconds to pellet cells through the silicone oil and into the acid layer, leaving the extracellular component on top of the oil. Following centrifugation, the tubes were frozen in liquid nitrogen and were then cut through at the bottom of the silicone oil layer. The bottom layer, containing the intracellular ^{14}C-proline, was placed in a scintillation vial containing 10 ml of ScintiSafe Plus™ (Fisher Scientific, Nepean, ON) and radioactivity was determined in a LKB 1214 Rackbeta Liquid Scintillation Counter. The volume of extracellular space that was carried through the silicone oil was determined by measuring the bottom layer following parallel cell incubations with carboxyl-^{14}C-inulin. This value was used to correct rates of proline transport. Proline transport rates were also corrected by subtracting zero time rates determined from samples placed on ice which represent amino acids

non-specifically binding to membranes, from the values obtained at subsequent time points. All samples were run in triplicate.

2.11.1 Linearity with time

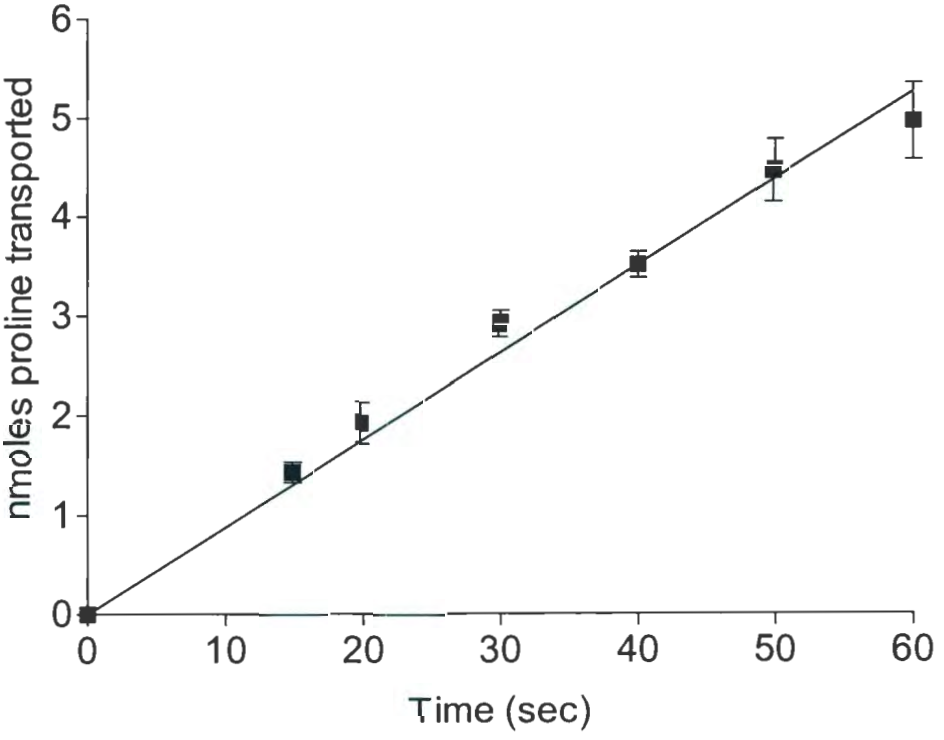
The exact method as described above was employed but the incubations were allowed to continue for times ranging from 5 to 60 seconds (figure 2.2) with a final concentration of ^{14}C-proline of 1.0 mM and a concentration of cells of approximately 5.0 mg/ml.

2.12 Proline oxidation by perfused liver

Nonrecirculating perfusions of rat livers were carried out as described by O'Sullivan et al., (76). Briefly, Krebs-Henseleit medium (pH 7.4), gassed with O_2-CO_2 (19:1) with added lactate and pyruvate (2.1 and 0.3 mM, respectively), served as the basic perfusion medium. The flow rate was maintained at 40.0 mL/min. ^{14}C-proline was added at a final concentration of 0.5 mM, and the production of $^{14}CO_2$ was determined in the effluent. The rate of infusion was such that no change in pH or P_{CO2} was discernible. To ensure that livers were viable throughout the procedure, oxygen consumption, perfusate (P_{CO2}), and pH were monitored by means of a blood gas analyzer (model 238, Ciba Corning, Bayer, Toronto, On, Canada). Oxygen consumption was approximately 2.5 µmol/min/g liver. An initial sample of each influent medium was taken. Effluent samples were collected at 5-min intervals after that. Samples for $^{14}CO_2$ analysis were taken under mineral oil. To measure $^{14}CO_2$ production, 25 ml Erlenmeyer flasks

Figure 2.2: Proline transport (linearity with time).

The transport of proline into isolated hepatocytes was conducted at a final concentration of [U]-^{14}C-proline from 1.0 mM. Incubation times ranged from 0-60 seconds and contained ~5mg of cells (dry weight). All assays were conducted in triplicate and were completed at 37°C. Data presented are means ± SD.


nmoles proline transported
0
1
2
3
4
5
6
0
10
20
30
40
50
60
Time (sec)

containing 0.40 ml of 1 N HCL were fitted with center wells containing filter paper and 0.40 ml of NCS tissue solublilizer. Into each flask, 5 ml of perfusate was injected through the stopper. The flasks were incubated in a shaking water bath at 37°C for 1 hour to ensure that all of the evolved CO_2 would be trapped in the center wells. The center wells were transferred to scintillation vials containing 10 ml of scintillation fluid and radioactivity was determined. Medium blanks were prepared to ensure that no preformed $^{14}CO_2$ was present in the radioactive compounds.

2.13 Statistical analysis

Data are presented as means ± SD unless otherwise noted. Student's unpaired t test was performed to compare means unless otherwise noted in the text. A p value of <0.05 was taken to indicate a significant difference.

Chapter 3: Localization of Δ^1-pyrroline-5-carboxylate dehydrogenase

3.1 Assay of Δ^1-pyrroline-5-carboxylate dehydrogenase

Small and Jones (98) have stated that spectrophotometric assays for P5CDh activity that rely on the reduction of NAD^+ to assess the progress of the reaction are unacceptable due to a lack of linearity with time and protein. In this study, 10 μM rotenone was used to inhibit oxidation of NADH by the electron transport chain. The spectrophotometric assay was found to be linear with time for a minimum of 15 minutes following the addition of P5C (figure 3.1 A) and with protein up to 3.4 μg of mitochondrial protein in a 300 μL assay volume (figure 3.1 B).

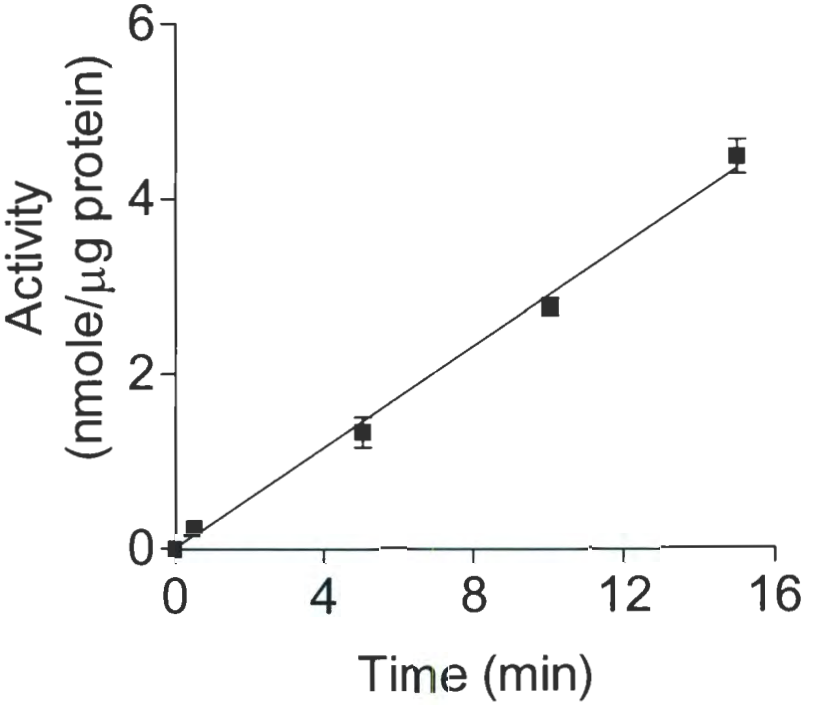
3.2 Subcellular localization of P5CDh

Table 3.1 shows the distribution of P5CDh activity as compared to that of markers in the subcellular fractions isolated from a homogenate of rat liver in the manner proposed by deDuve (16). We have added an extra mitochondrial fraction because we were consistently getting a significant amount of cross contamination within the nuclear and mitochondrial fractions obtained using the classical method which yields one mitochondrial fraction. The recoveries of enzyme activity, DNA and protein ranged from 87-103% (Table 3.1). The distribution patterns of typical nuclear (DNA), mitochondrial (succinate cytochrome C reductase), lysosomal (β-glucuronidase), microsomal (NADPH cytochrome C reductase) and cytosolic (lactate dehydrogenase) markers, shown in figure 3.2, are similar to those reported by deDuve et al. (17).

Figure 3.1: Linearity with time and protein for P5CDh assay

Assay conditions are given in Materials and Methods. A: P5CDh activity as a function of time of assay. Assays were conducted with 1.2 μg of mitochondrial protein and allowed to continue for 15 minutes. B: P5CDh assay as a function of mitochondrial protein in the assay. Assays were conducted for 10 minutes with the amounts of mitchondrial protein indicated. Values represent mean ± SD, n=3.

A


Activity
(nmole/µg protein)
6
4
2
0
0
4
8
12
16
Time (min)

B

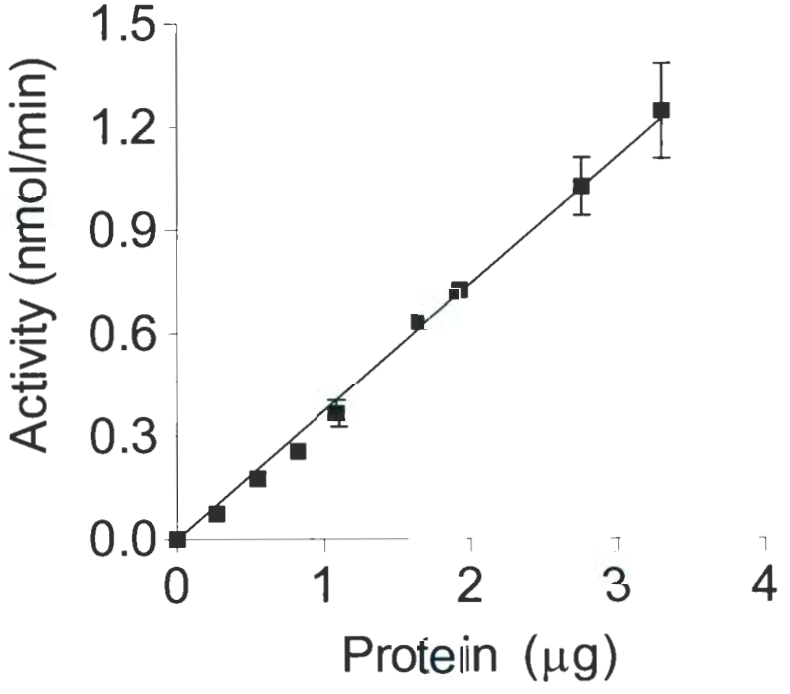

Activity (nmol/min)
1.5
1.2
0.9
0.6
0.3
0.0
0
1
2
3
4
Protein (µg)

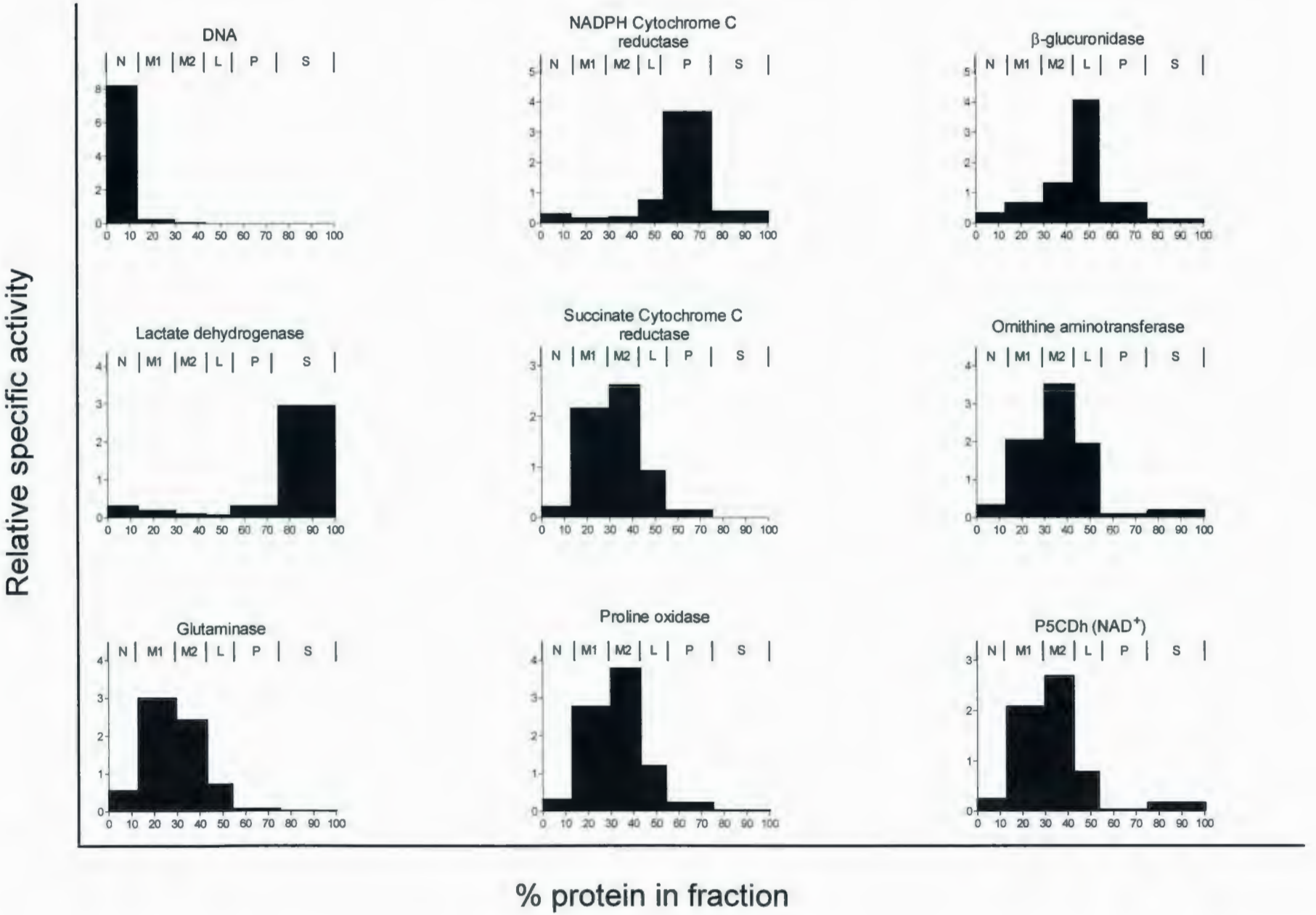
Table 3.1: Specific activities of markers and P5CDh from subcellular fractionation

Specific activities and recovery of P5CDh, marker enzymes, DNA and protein from subcellular fractions of rat liver. Specific activities of enzymes are given as nmol of product formed per minute per μg of protein. DNA is expressed as μg per mg of protein. All values are presented as means ± SD, n=6. Abbreviations; N, nuclear fraction; M1, first mitochondrial fraction; M2, second mitochondrial fraction; L, lysosomal fraction; P, microsomal fraction; S, cytosolic fraction. Recoveries are based on homogenate values as 100%.

Enzyme	Fraction						
	N	M1	M2	L	P	S	Recovery %
Ornithine aminotransferase	1.29±0.63	7.51±1.43	12.90±2.64	7.07±1.19	0.40±0.31	0.81±0.57	100
P5CDh (NAD^+)	0.030±0.006	0.229±0.037	0.299±0.037	0.079±0.019	0.006±0.001	0.020±0.004	92
P5CDh ($NADP^+$)	0.019±0.003	0.149±0.024	0.188±0.037	0.052±0.015	0.005±0.003	0.016±0.001	93
Glutaminase	5.33±3.75	47.54±10.17	39.58±6.05	11.57±6.8	1.05±1.28	0.09±0.22	96
Proline oxidase	0.64±0.38	5.94±2.23	8.42±5.00	2.74±1.51	0.50±0.37	0.10±0.21	103
Succinate cytochrome c reductase	5.15±1.79	52.28±20.72	62.71±22.39	21.12±7.84	3.27±4.85	0.00±0.00	89
β-glucuronidase	0.12±0.06	0.22±0.15	0.43±0.27	1.21±0.42	0.29±0.30	0.04±0.03	93
NADPH cytochrome c reductase	7.46±2.12	4.30±2.37	5.31±3.05	18.69±7.58	86.71±14.75	9.54±9.19	87
Lactate dehydrogenase	0.54±0.21	0.33±0.10	0.20±0.13	0.13±0.11	0.52±0.73	4.98±1.14	98
DNA	612.4±168	18.1±4.5	3.4±2.6	0.0±0.0	0.0±0.0	0.0±0.0	102

Figure 3.2: Relative specific activities of markers and P5CDh in subcellular fractionation

Distribution pattern of P5CDh, DNA, and marker enzymes in fractions for rat liver. Ordinate: mean relative specific activity of markers (specific activity in fraction / specific activity in homogenate, calculated from data given in Table 3.1). Absicca: relative protein content of fractions (cumulatively from left to right). N, nuclear fraction; M1, first mitochondrial fraction; M2, second mitochondrial fraction; L, lysosomal fraction; P, microsomal fraction; S, cytosolic fraction, n=6.


DNA
NADPH Cytochrome C reductase
β-glucuronidase
Lactate dehydrogenase
Succinate Cytochrome C reductase
Ornithine aminotransferase
Glutaminase
Proline oxidase
P5CDh (NAD+)
N
M1
M2
L
P
S
0 10 20 30 40 50 60 70 80 90 100
Relative specific activity
% protein in fraction

In our study we have used several marker enzymes for mitochondria: succinate cytochrome C reductase, an intrinsic protein in the mitochondrial inner membrane (17); glutaminase (61) and proline oxidase (41), extrinsic proteins associated with the inner mitochondrial membrane; ornithine aminotransferase, a soluble enzyme located in the mitochondrial matrix (34). The distribution pattern of P5CDh closely follows that of these mitochondrial enzymes (figure 3.2).

There is no leakage of the membrane-associated enzymes into the cytosolic fraction, but approximately 7-8% of the matrix enzymes do appear in this compartment, indicating a slight release of soluble enzymes from mitochondria. A similar amount of P5CDh also appears in the cytosolic fraction, whether assayed with NAD^+ or $NADP^+$ as cofactor. It can therefore be concluded that P5CDh occurs solely in mitochondria in liver.

It has been proposed by Phang (79) that the presence of P5CDh in the cytosol may represent an isoenzyme that could show a preference for $NADP^+$ versus NAD^+ (79). The results obtained do not support this and in fact show that the specific activity of P5CDh decreases when $NADP^+$ is used as a cofactor in all fractions (table 3.1). The fact that the specific activity for P5CDh in the cytosol does not increase when $NADP^+$ is used as a cofactor also suggests that the activity observed in the cytosol is due to leakage of the enzyme during fractionation and not to a true cytosolic enzyme or isoenzyme.

The finding in this study that P5CDh is located solely in mitochondria is in contrast to data from Brunner and Neupert (12). Rat liver also contains the cytosolic enzyme P5C reductase which catalyzes the conversion of P5C to proline (83). The assay employed by Brunner and Neupert followed the disappearance of P5C and assumed that this would be due only to P5CDh. When Brosemer and Veerabhardrappa (10) originated this assay for P5CDh in insects, more P5C disappeared than could be accounted for as glutamate, although they inhibited further glutamate metabolism. The assay as conducted by Brunner and Neupert (12) would not discriminate between the loss of P5C due to glutamate synthesis by P5CDh or proline synthesis by P5C reductase. It is likely that the activity which was attributed to the presence of P5CDh in the cytosol was in fact due to the presence of P5C reductase which occurs in this compartment (83).

In regard to the proposed cycle between proline and P5C (figure 1.2), the activity of P5CDh and its subcellular location are quite important. The reduction of P5C in the cytosol by P5C reductase is dependent on the presence of P5C. If P5CDh were in fact located in the cytosol then there would be competition between the two enzymes for the substrate P5C. The same situation occurs in mitochondria as P5C produced by PO must not be converted to glutamate by P5CDh in order for the P5C/proline cycle to function in a manner that does not require continual replenishing of substrates. It would appear that our finding that P5CDh is located solely in mitochondria in rat liver supports the possiblity of a

P5C/proline cycle in liver; however the problem of removal of P5C by P5CDh in the mitochondrial matrix has not been addressed, nor is anything known about the control of P5C transport out of mitochondria.

It has been suggested, by Hagedon and Phang (27) that P5CDh in the mitochondrial matrix could be subject to inhibition. The presence or identity of an inhibitor, however, has not been described. The effect of the proposed P5C/proline cycle would be to increase the concentration of $NADP^+$ in the cytosol and therefore stimulate ribose-5-phosphate synthesis. However P5C reductase isolated from liver appears to prefer NADH to NADPH, exhibiting 10-fold lower activity with the later cofactor (35). While the preference of hepatic P5C reductase for NADH would not abolish the activity of a P5C/proline cycle it could tend to decrease its effectiveness in generating $NADP^+$ which could lead to less nucleotide synthesis.

Chapter 4: Proline metabolism in rats receiving a diet containing adequate or excess protein

The protein content and hence availability of amino acids in a diet is of great importance if an organism is to thrive. The complement of amino acids in the diet, especially dietary essential amino acids must be sufficient to support protein synthesis and for the other functions of amino acids as previously mentioned. In addition, excess amino acids must be catabolized since there is no storage form of amino acids. The need for catabolism of excess amino acids is unique when considered against two other major dietary components, carbohydrate and fatty acids which can be stored as glycogen and triacylglycerol respectively.

Excess protein in a diet would therefore be expected to lead to an increase in amino acid catabolism and this has indeed been observed in numerous studies which demonstrate increased activity of the urea cycle enzymes as well as other enzymes involved in amino acid catabolism (8;57;77;111). We have therefore studied the oxidation of proline in response to a diet high in protein. The content of protein in the diets that we employed was 15% in the case of the adequate protein diet which is in line with current recommendations and 45% which is excessive but not uncommon in a western diet (8;57;77;89).

4.1 Characteristics of rats fed 15% or 45% protein diets

Figure 4.1 shows that the animals in both the excess and adequate protein groups in our study gained weight at the same rate. There was no change in the liver weights of animals fed the 15% or 45% diets and all animals consumed approximately the same quantity of their respective diets (table 4.1).

4.2 Proline oxidation in mitochondria

Using actively respiring mitochondria isolated from these animals we measured the production of $^{14}CO_2$ from ^{14}C-proline. The production of $^{14}CO_2$ was increased by approximately 40% in mitochondria isolated from animals fed the 45% protein diet as compared with that observed in animals fed the 15% diet (figure 4.2 A). Increased oxidation of proline occurred at all levels of proline utilized in the study including the physiological concentration of 0.25 mM (figure 4.2 A). The apparent K_m for proline oxidation was not altered in animals fed the adequate protein diet versus the excess protein diet while a significant increase in the V_{max} for the oxidation of proline was observed in the animals fed a high protein diet. (table 4.2 B).

The kinetic parameters that we have calculated are for the pathway of proline oxidation in intact mitochondria and therefore relate to the entire pathway of proline oxidation including transport across the inner mitochondrial membrane. Although an increase in V_{max} without an increase in the apparent K_m suggests that there is an increase in the absolute capacity of one or more of the proline

Figure 4.1: Growth curves for animals fed a modified synthetic AIN-93G diet.

Animals were fed either an adequate (15% protein) or an excessive (45%) protein diet for 7 days. Animals were weighed each morning at 10:00 AM.

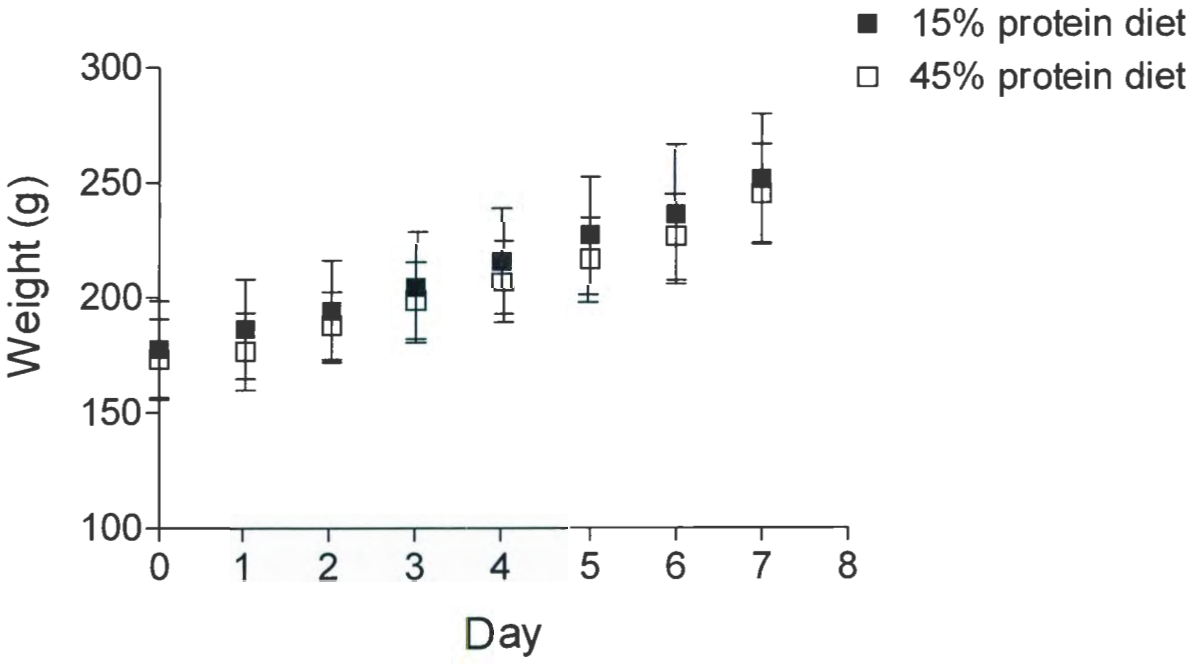

15% protein diet
45% protein diet
Weight (g)
300
250
200
150
100
0
1
2
3
4
5
6
7
8
Day

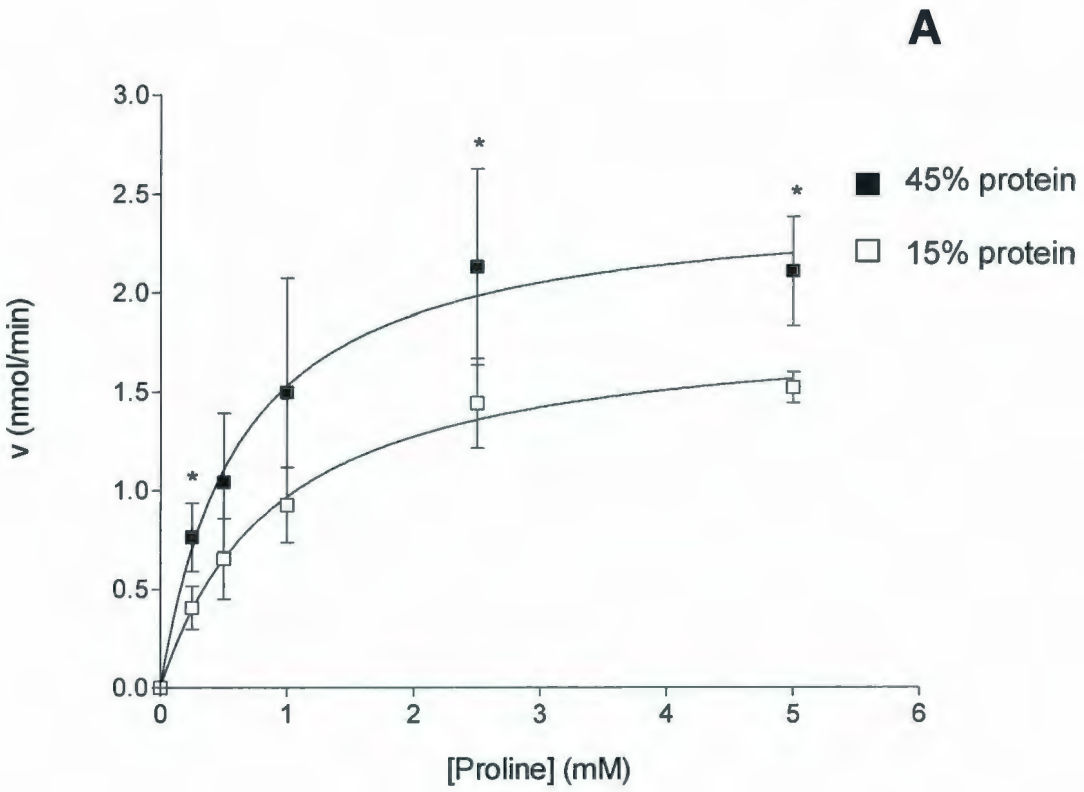
Table 4.1: Food intake and liver weight for rats fed 15% and 45% protein diets

	Food intake (g/day/rat)	Wet liver weight (g)
15% protein diet	25.6	9.8
45% protein diet	24.2	9.6

Animals were fed either an adequate (15% protein) or an excessive (45%) protein diet for 7 days. Animals were weighed and food intake calculated each morning at 10:00 AM. Livers were weighed immediately after removal. Animals were sacrificed at 10:00 AM on the last day of the study. Data presented are means, n=4.

Figure 4.2: Proline oxidation in mitochondria

Oxidation of [U]-^{14}C-proline by freshly isolated, actively respiring mitochondria. Mitochondria were isolated from the livers of animals that had been fed a control (15%) or high (45%) protein diet for 7 days. Animals were sacrificed at 10:00 AM on the last day of the study. Incubations were conducted with 0.25 mg/ml of mitochondrial protein for 10 minutes at 25°C at the concentrations of proline indicated. A) Michaelis-Menten plot B) Lineweaver-Burk plot. Data presented are means ± SD, n=4, * denotes statistical significance versus control, $p \leq 0.05$, Students t-test.


A
3.0
2.5
2.0
1.5
1.0
0.5
0.0
v (nmol/min)
0
1
2
3
4
5
6
[Proline] (mM)
*
*
*
45% protein
15% protein

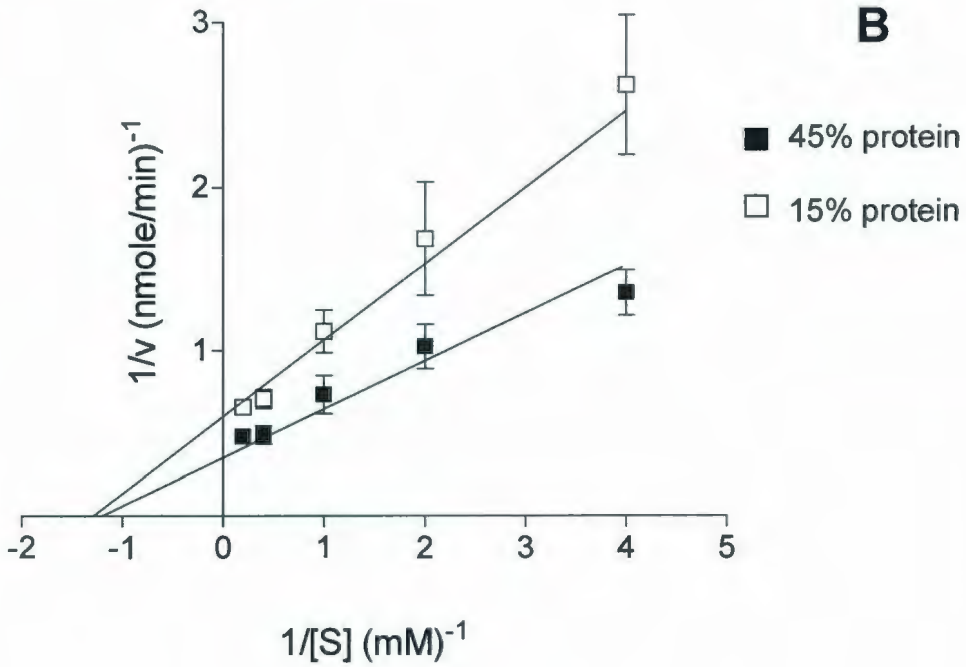

B
3
2
1
1/v (nmole/min)$^{-1}$
-2
-1
0
1
2
3
4
5
1/[S] (mM)$^{-1}$
45% protein
15% protein

Table 4.2: Kinetic data obtained for proline oxidation in isolated mitochondria

	Apparent K_m (mM)	V_{max} (nmoles/min)
Control	0.90±0.23	1.78±0.36
Treated	0.61±0.1	2.47±0.27*

Data were obtained using freshly isolated, actively respiring mitochondria. Mitochondria were isolated from the livers of rats that had been fed a control (15%) or high (45%) protein diet for 7 days. Rats were sacrificed at 10:00 AM on the last day of the study Incubations were conducted with 0.25 mg/ml of mitochondrial protein for 10 minutes at 25°C at the concentrations of proline indicated in figure 4.2. Data presented are means, n=4, * denotes statistical significance versus control, $p \leq 0.05$, Students t-test.

catabolic enzymes, it does not provide information as to exactly which step may be increased.

4.3 Proline catabolic enzymes

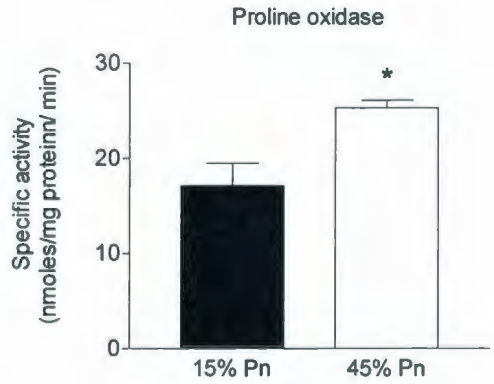
The increase in proline oxidation suggests that enzymes involved in proline catabolism may exhibit increased activity. We therefore studied the activities of the proline catabolic enzymes in animals fed diets as previously described (figure 4.3). The activity of PO was increased by approximately 50% while the activity of P5CDh increased by 40%. The activity of GDH was not increased in response to a diet high in protein.

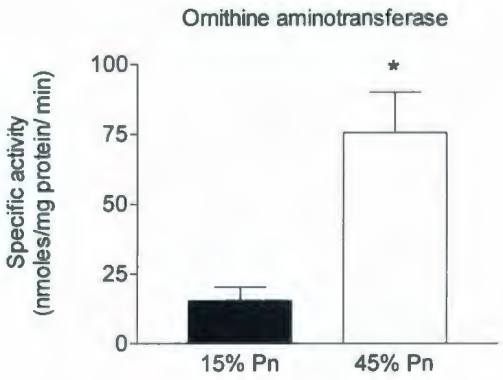
As previously mentioned Kawabata et al., (44) reported that the activity of proline oxidase could be increased in response to a diet high in protein. They observed that rats fed a diet containing 60% protein exhibited approximately a 2-fold increase in PO activity versus control animals fed a diet containing 25% protein. This study however did not consider possible changes in the other enzymes that are involved in proline catabolism and the 25% control diet is considered to be in excess of that required for animals to thrive (89).

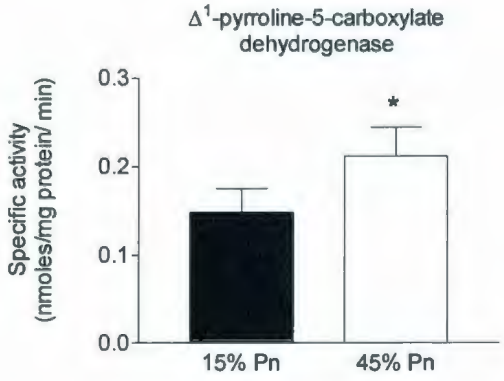
It has also been observed that the activity of P5CDh can be increased in response to diet (57). Once again however the protein diets in this study were not physiologically relevant. The low protein diet contained 5% protein while the high protein diet contained 70% protein and the study design did not contain a control group of animals. The animals in this study did not thrive and the animals

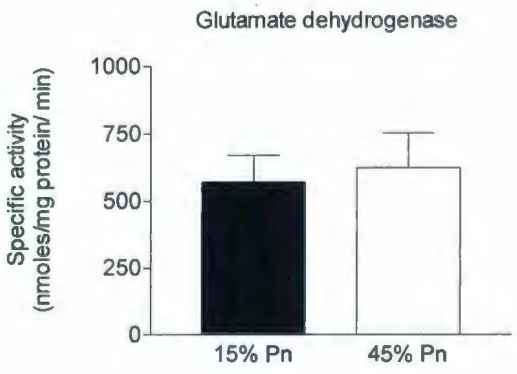
Figure 4.3: Enzyme activities in animals fed high protein diets.

Animals were fed a modified synthetic AIN-93G diet that contained either 15% protein (control) or a 45% protein (high) for 7 days. Animals were sacrificed at 10:00 AM on the last day of the study. All enzyme assays were performed on freshly isolated mitochondria which were subjected to 3 cycles of freezing and thawing to disrupt the mitochondrial membranes and were conducted at 37°C. Data presented are means ± SD, n=4, * denotes $p \leq 0.05$, Students t-test


Proline oxidase
Specific activity
(nmoles/mg proteinn/ min)
30
20
10
0
*
15% Pn
45% Pn


Ornithine aminotransferase
Specific activity
(nmoles/mg protein/ min)
100
75
50
25
0
*
15% Pn
45% Pn


Δ1-pyrroline-5-carboxylate
dehydrogenase
Specific activity
(nmoles/mg protein/ min)
0.3
0.2
0.1
0.0
*
15% Pn
45% Pn


Glutamate dehydrogenase
Specific activity
(nmoles/mg protein/ min)
1000
750
500
250
0
15% Pn
45% Pn

in the low protein group had to be enticed to eat by the addition of cheese and sardines to their food. In regard to growth the animals in the high protein group gained twice as much weight during the study period.

The greatest increase in activity occurred in OAT which displayed approximately a 500% increase in activity. The increase observed in the activity of OAT is quite substantial and is most likely important in the catabolism of excess arginine contained in a diet high in protein (table 2.2). An increase in OAT activity such as we observed in response to a diet high in protein has been observed by other investigators (54,57).

As mentioned above the increase in activity of OAT is probably important for the catabolism of dietary arginine. It could however be argued that the increase in OAT activity supplies substrates for the urea cycle which would be required to remove excess nitrogen during times of increased protein ingestion. This would not appear to be the case in liver since OAT has been shown to be non-reversible (ie. functions only in degradation) in both rat liver mitochondria (43) and hepatocytes (87).

Chapter 5: Proline metabolism in glucagon treated rats

In chapter 4 we saw that a diet containing excess protein would cause an increase in the liver's capacity to oxidize proline. It is well accepted that one of the physiological changes that occurs with a high protein diet is an increase in the plasma level of the hormone glucagon. Thus it is possible that the effect of a high protein diet is due, at least in part, to glucagon.

The regulatory effects of glucagon on amino acid metabolism have been well documented. It increases the catabolism of a variety of amino acids (e.g. glycine, glutamine, arginine, and phenylalanine), it increases gluconeogenesis from amino acids, and it increases the rate of ureagenesis (14;55;64;77;103;113). Despite the abundance of proline present both in plasma and intracellularly there are few data on the effect of glucagon on proline catabolism. With this in mind, an investigation of the specific effects of glucagon on proline catabolism is certainly warranted.

5.1 Characteristics of control and glucagon treated rats

Table 5.1 gives information on weight gain, food intake, liver weight and plasma levels of glucagon, insulin and glucose in our glucagon treated and control rats. Plasma glucose was increased by 50% following glucagon treatment. Plasma glucagon was increased approximately 30-fold versus control rats, while plasma insulin was unaltered. In our study glucagon administration did

Table 5.1: Body weight, food intake, plasma glucose, insulin and glucagon concentrations in control and glucagon-treated rats.

	Control	Glucagon
Weight gain (g/day)	4.57 ± 1.02	3.71 ± 2.32
Food Intake (g/day)	26.3 ± 4.5	27.5 ± 3.7
Wet liver Weight (g)	11.3 ± 0.6	12.1 ± 0.4
Plasma Glucagon (ηg /ml)	79.5 ± 12.1	2630 ± 1040 *
Plasma Insulin (ηg/ml)	8.9 ± 1.7	7.1 ± 2.6
Plasma Glucose (mM)	8.0 ± 0.8	14.1 ± 1.3*

Rats were administered glucagon (4mg/kg body weight/24 hrs for two days, subcutaneously) while control rats received the vehicle (diluting solution provided by Eli-Lily). Food intake and body weight were measured daily. Blood samples were taken at 10:00 AM on the last day of the study from the abdominal aorta and centrifuged for plasma separation. Data shown are means ± SD, n=4, * denotes a significant difference versus control rats, $P<0.05$, Students t-test.

not change weight gain, food intake, or wet liver weight in accordance with an earlier study (40).

Plasma proline levels were decreased by 82% by glucagon treatment (Table 5.2). Table 5.2 also shows the effects of glucagon on glutamate, glutamine and ornithine. The plasma concentration of each of these amino acids was decreased, but not to the extent of that observed with proline. Although the plasma concentration of each of the amino acids studied was reduced, proline was the only amino acid that displayed an increase in the liver concentration and a substantial increase in the liver:plasma ratio. The approximately 12-fold increase in the liver:plasma ratio of proline may suggests that there is an active transport of proline from plasma.

5.2 Proline oxidation by perfused liver

In order to determine flux through the entire pathway of proline oxidation we measured $^{14}CO_2$ production from ^{14}C-proline in the isolated non-recirculating perfused rat liver. The amount of $^{14}CO_2$ formed is a direct measure of hepatic proline oxidation. The production of $^{14}CO_2$ was constant during the 30-minute perfusion procedure representing a steady state of proline oxidation (figure 5.1 A). Statistical analysis via two-way anova revealed that there was no difference between time points in either the control or treated animals, but there was a statistically significant effect of glucagon. Since there was no difference with respect to time it was possible to combine the values at all time points and

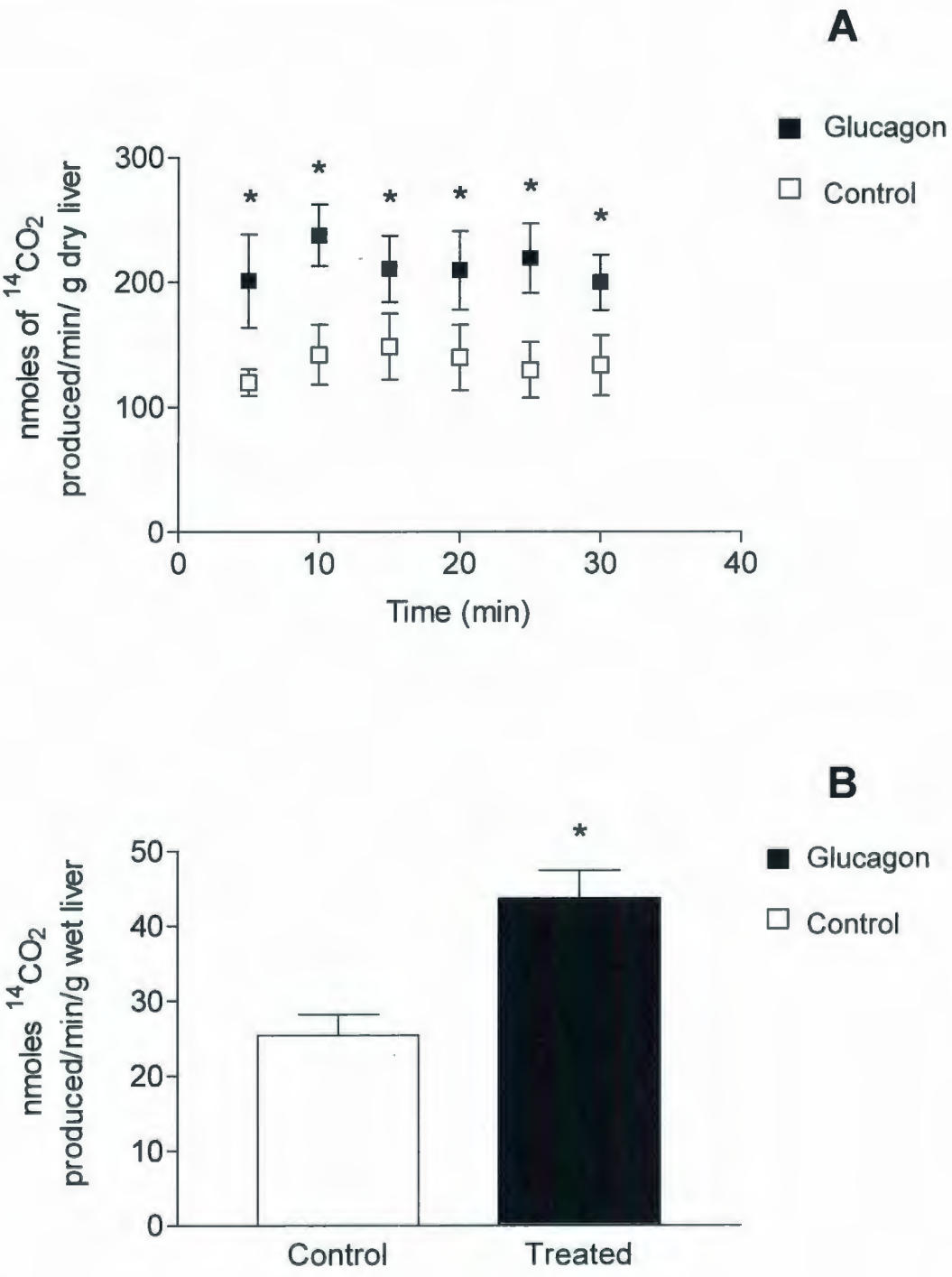
Table 5.2: Amino acids in plasma of control and glucagon treated animals

	Control		Ratio	Glucagon		Ratio
Amino acid	**[Plasma] (uM)**	**[Liver] (uM)**	**Liver:Plasma**	**[plasma] (uM)**	**[Liver] (uM)**	**Liver:Plasma**
Proline	196 ± 6	107 ± 20	0.54	35 ± 4 *	232 ± 76 *	6.70
Glutamate	144 ± 23	3252 ± 857	22.50	55 ± 14*	2233 ± 432	40.00
Glutamine	667 ± 71	10572 ± 2000	15.80	147 ± 11*	663 ± 117*	4.50
Ornithine	63 ± 5	340 ± 38	5.4	38 ± 10*	375 ± 95	10

Rats were administered glucagon (4mg/kg body weight/24 hrs for two days, subcutaneously) while control rats received the vehicle (diluting solution provided by Eli-Lily). Food intake and body weight were measured daily. Blood samples were taken at 10:00 AM on the last day of the study from the abdominal aorta and centrifuged for plasma separation, liver samples were removed from the rats and immediately freeze-clamped in liquid nitrogen. Data shown are means ± SD, n=4, * denotes statistical significance versus control, $p \leq 0.05$, Students t-test.

Figure 5.1: Proline oxidation during a non-recirculating perfusion of rat liver

Proline oxidation in rats given exogenous glucagon (4mg/kg body weight/24 hrs for two days, subcutaneously). Control animals received the vehicle (diluting solution provided by Eli-Lily). Flow-through assays were performed at 37°C, at a flow rate of 40 ml/min, with 0.50 mM proline. Data presented are means ± SD, n=6, * denotes $p \leq 0.05$, two-way anova. A) proline oxidation as a function of time B) comparison of proline oxidation in control and treated animals at a concentration of 0.50 mM proline expressed as nmoles of $^{14}CO_2$ formed/min/ g wet liver. Values in B are means ± SD of all time points for the corresponding group.


A
Glucagon
Control
nmoles of $^{14}CO_2$ produced/min/ g dry liver
300
200
100
0
0
10
20
30
40
Time (min)
*
B
Glucagon
Control
nmoles $^{14}CO_2$ produced/min/g wet liver
50
40
30
20
10
0
Control
Treated
*

analyze them as a group (figure 5.1 B). At approximately physiological concentrations (0.50 mM), proline oxidation was elevated 60% in the glucagon-treated rat (figure 5.1 B).

5.3 Proline transport

Our calculations show that the hepatic intracellular concentration of proline was elevated in the glucagon-treated rat (Table 5.2). Of particular interest is the fact that the ratio of liver to plasma proline was 12-fold greater in the glucagon-treated rat. This could be explained by an effect of glucagon on proline transport. Proline transport rates were, therefore, measured from hepatocytes isolated from control and glucagon-treated rats. Proline transport was found to be linear with time for up to 60 seconds (figure 2.2). Glucagon treatment resulted in an approximate 45% increase in proline uptake at concentrations of proline ranging from 0.50 to 9.0 mM in a concentration and time dependent manner (Figure 5.2). Visual examination of the data presented in figure 5.2 suggested the potential of two distinct transport systems. We therefore analyzed the data between the intervals of 0.50-4.0 mM and 5.0-9.0 mM by fitting lines via linear regression (figure 5.3, table 5.3). The results of this analysis show that over the interval of 0.50-4.0 mM the slopes of lines describing proline transport in hepatocytes isolated from control and glucagon treated rats are significantly different and that glucagon increases proline uptake. Over the interval 5.0-9.0 mM the slopes of the lines are not significantly different from each

Figure 5.2: Proline transport in isolated hepatocytes.

The transport of proline into isolated hepatocytes was conducted at final concentrations of [U]-^{14}C-proline from 0.0-9.0mM. Incubations were for 30 seconds with ~5mg of cells (dry weight) and were completed at 37°C. Data presented are means ± SD, n=4, Data were analyzed via a two-way anova $p \leq 0.05$, which determined that there is a significant difference in proline transport in both groups with respect to proline concentration and between the transport of proline in glucagon injected animals and control animals.

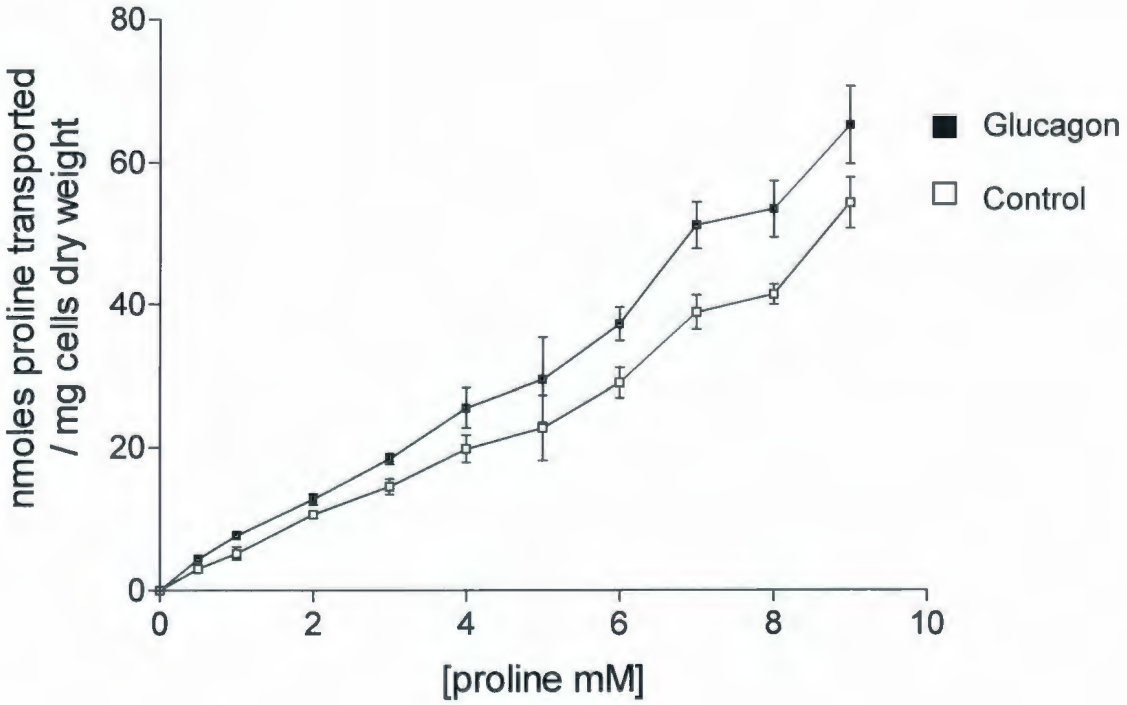

nmoles proline transported
/ mg cells dry weight
80
60
40
20
0
0
2
4
6
8
10
[proline mM]
Glucagon
Control

Figure 5.3: Proline transport in isolated hepatocytes.

The transport of proline into isolated hepatocytes was conducted at final concentrations of [U]-^{14}C-proline from 0.0-9.0mM. Incubations were for 30 seconds with ~5mg of cells (dry weight) and were completed at 37°C. Data presented are means ± SD, n=4, Data were analyzed via linear regression over the intervals of 0-4 mM and 5-9 mM.

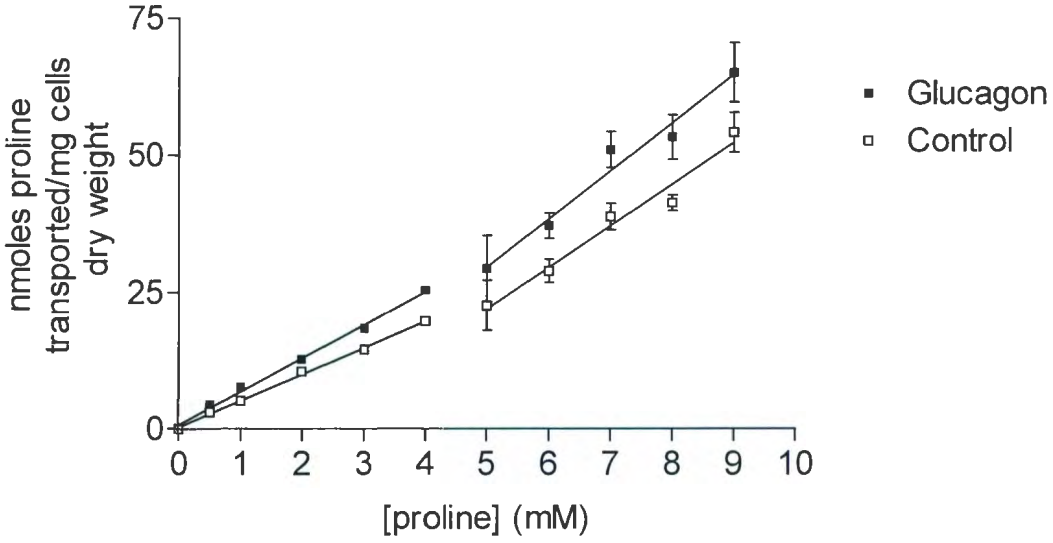

75
50
25
0
nmoles proline transported/mg cells dry weight
0 1 2 3 4 5 6 7 8 9 10
[proline] (mM)
Glucagon
Control

Table 5.3: Slope of lines describing proline transport in rat liver

Interval	0.0-4.0 mM	5.0-9.0 mM
Control	4.85±0.12	7.55±0.92
Treated	6.10±0.21*	8.77±1.29

The transport of proline into isolated hepatocytes was conducted at final concentrations of [U]-^{14}C-proline from 0.0-9.0mM. Incubations were for 30 seconds with ~5mg of cells (dry weight) and were completed at 37°C. Data presented are means ± SD, n=4, Data were analyzed via linear regression over the intervals of 0-4 mM and 5-9 mM. Data shown are means ± SD, n=4, * denotes statistical significance versus control, $p \leq 0.05$, Students t-test.

other with respect to control and glucagon treated rates; however the rate of proline uptake when analyzed as control versus control, and treated versus treated, over the intervals 0.50-4.0 mM and 5.0-9.0 mM, shows that proline uptake is significantly increased in both cases.

Thus our data may suggest that there is indeed more than one transporter for proline present in rat liver. One transport system appears to function over the interval of proline concentrations ranging between approximately 0.0-4.0 mM and is responsive to glucagon. The second transport system functions at least over the interval 5.0-9.0 mM, may have an increased V_{max} as compared to the transport system functioning at lower concentrations and may or may not be responsive to glucagon.

Relatively little specific information is available on mechanisms of proline transport in hepatocytes. It is generally felt that proline transport in hepatocytes occurs mainly by the system A transport system. The system A transporter, ATA2, which is present in hepatocytes and is known to transport proline is felt to be a weakly accumulating transporter due to the fact that this transporter exhibits trans-inhibition (30;96). As such it is possible that the increase in intra-hepatic proline concentration that we have observed may not be due solely to an increase in the activity of ATA2. Given that numerous amino acids are transported via ATA2 (30) it would also be unlikely that transport of one amino acid would predominate to the extent we noted for proline at the expense of all others. The 12-fold increase in the liver to plasma ratio for proline may therefore

imply that another more specific transporter for proline is active in hepatocytes in the presence of exogenous glucagon.

Recently it has been shown that there may indeed be another transporter present in liver that is capable of transporting proline (15). It is thought that this transporter may be the imino transporter that has been known to exist in the small intestine for some time (15). Amino acid transporters are generally present in limited amounts in plasma membranes and it is possible that the imino transporter had not been detected in liver for this reason. That is, the imino transporter may be present in the plasma membrane of hepatocytes at a barely detectable level under basal conditions and in response to the correct stimuli (eg. hormonal via glucagon) is induced or recruited from an intracellular pool to increase the uptake of proline from plasma.

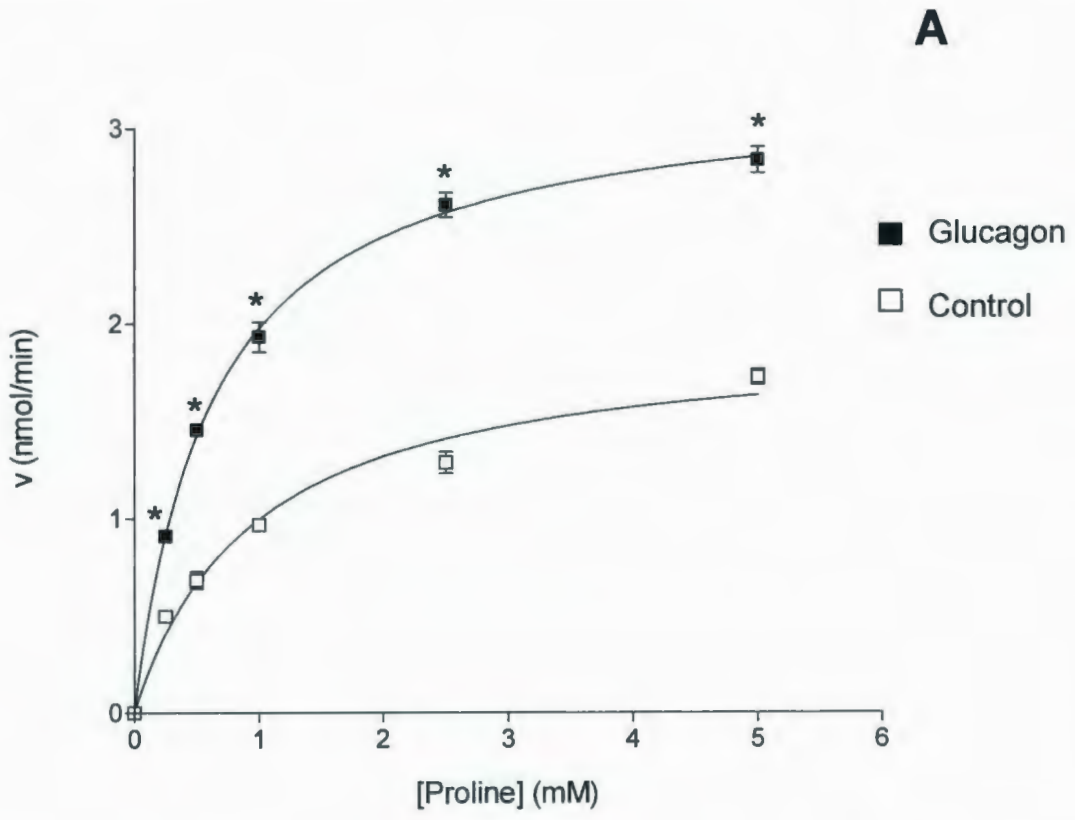
5.4 Proline oxidation in mitochondria

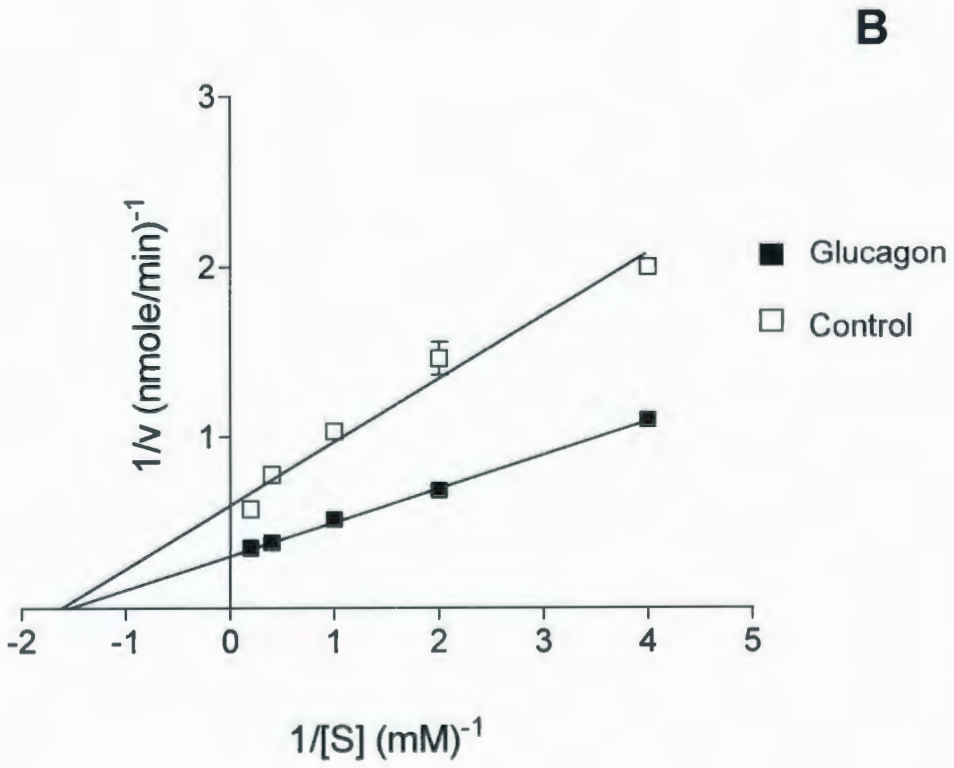
The enzymes involved in proline oxidation are all known to be located in mitochondria, so isolated actively respiring mitochondria are a viable model for studying proline oxidation. We observed a 2-fold increase in the production of $^{14}CO_2$ from ^{14}C-proline at virtually all concentrations of proline tested in mitochondria from glucagon treated rats, including the physiological concentration of proline of 0.25 mM (figure 5.4 A).

Kinetic parameters obtained from a Lineweaver-Burk plot (figure 5.4 B) of the data obtained for the oxidation of proline in actively respiring mitochondria indicated that the apparent K_m for oxidation was not altered by glucagon

Figure 5.4: Proline oxidation in mitochondria

Oxidation of [U]-^{14}C-proline in freshly isolated, actively respiring mitochondria. Mitochondria were isolated from the livers of animals that had received exogenous glucagon (4mg/kg body weight/24 hrs for two days, subcutaneously). Control animals received the vehicle (diluting solution provided by Eli-Lily). Incubations were conducted with 0.25 mg/ml of mitochondrial protein for 10 minutes at 25°C at the concentrations of proline indicated. A) Michaelis-Menten plot, B) Lineweaver-Burk plot. Data presented are means ± SD, n=4, * denotes statistical significance versus control, $p \leq 0.05$, Students t-test


A
v (nmol/min)
[Proline] (mM)
Glucagon
Control
0
1
2
3
4
5
6
*


B
1/v (nmole/min)$^{-1}$
1/[S] (mM)$^{-1}$
Glucagon
Control
-2
-1
0
1
2
3
4
5

treatment but the V_{max} of proline oxidation did show a significant increase in the treated animals (table 5.4). The increase in V_{max} for proline oxidation of approximately 2-fold coupled with no change in the apparent K_m values may suggest that an increase in the quantity of certain enzymes involved in the oxidation of proline has occurred.

In order to compare the oxidation of ^{14}C-proline in perfused liver and in isolated mitochondria we calculated values for proline oxidation based on wet liver weights. The calculation involved determining the protein content of a total homogenate as well as isolated mitochondria in control and glucagon treated animals. The values obtained for the content of mitochondrial protein (mg)/g of wet liver and the fact that we used 0.25 mg of mitochondrial protein in our kinetic assays allowed us to determine the mitochondrial activity with respect to grams of wet liver. The data obtained also confirmed that there was no change in the content of protein in a homogenate of liver or in mitochondria as a result of glucagon injections (figure 5.5 A/B).

The actual rates of $^{14}CO_2$ production in mitochondria versus that observed during non-recirculating perfusions are very similar when expressed as nmoles $^{14}CO_2$ produced/min/g wet liver in regard to the maximum production of $^{14}CO_2$ in both treated and control animals (table 5.5). The similar 60% increase in proline oxidation in intact liver and isolated mitochondria may therefore suggest that although transport may play a vital role in the increase in oxidation of proline in situations of increased plasma glucagon it may not be the rate-limiting step.

Table 5.4: Kinetic data obtained from proline oxidation in rat liver mitochondria

	Apparent K_m (mM)	V_{max} (nmoles/min)
Control	0.63	1.61
Treated	0.58	3.23*

Data were obtained using freshly isolated, actively respiring mitochondria. Mitochondria were isolated from the livers of animals that had received exogenous glucagon (4mg/kg body weight/24 hrs for two days, subcutaneously). Control animals received the vehicle (diluting solution provided by Eli-Lily). Incubations were conducted with 0.25 mg/ml of mitochondrial protein for 10 minutes at 25°C at the concentrations of proline indicated in figure 5.3. Data presented are means, n=4, * denotes statistical significance versus control, $p \leq 0.05$, Students t-test

Figure 5.5: Protein content of control and glucagon treated rats

Protein content /gram of wet liver was determined in a total homogenate of liver and in mitochondria which were isolated from the livers of control animals and livers of animals that had received exogenous glucagon (4mg/kg body weight/24 hrs for two days, subcutaneously). Control animals received the vehicle (diluting solution provided by Eli-Lily). Animals were sacrificed at 10:00 AM on the last day of study. A) Total homogenate, B) Mitochondria. Data presented are means ± SD, n=4, * denotes $p \leq 0.05$, Students t-test.

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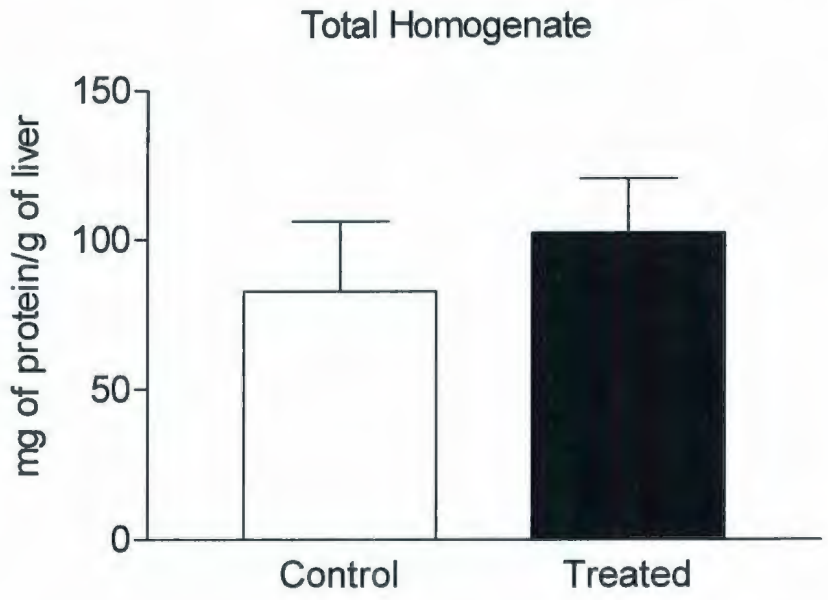
A


Total Homogenate
mg of protein/g of liver
150
100
50
0
Control
Treated

B

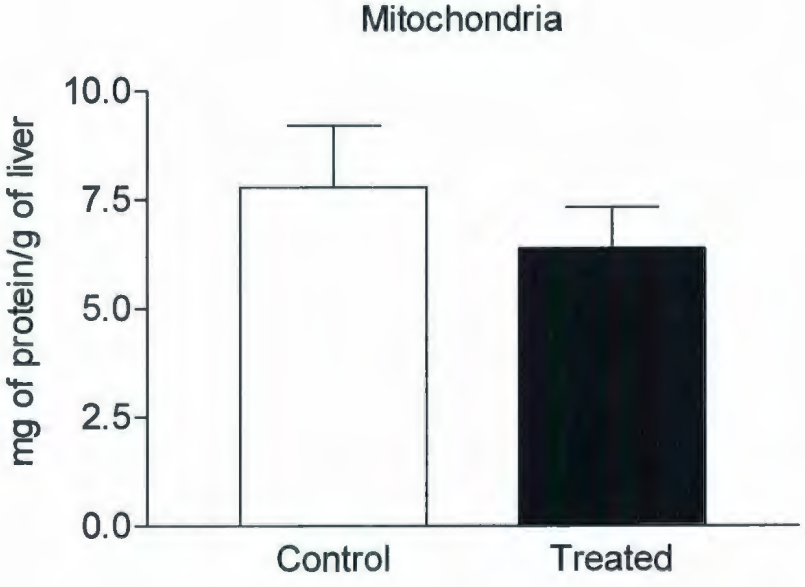

Mitochondria
mg of protein/g of liver
10.0
7.5
5.0
2.5
0.0
Control
Treated

Table 5.5: Comparison of $^{14}CO_2$ production during perfusion and in isolated mitochondria

	$^{14}CO_2$ production (nmoles/min/g wet liver)	
	Control	Glucagon treated
Isolated mitochondria	21.37	37.06
Perfused liver	25.41	43.72

Values for $^{14}CO_2$ production were calculated from perfusions of rat liver with a constant concentration of proline (0.50 mM) and from mitochondria incubated with 0.50 mM proline; mitochondrial assays were carried out at 25°C while liver perfusion were conducted at 37°C. Data presented in figure 5.3 were not obtained from the same rats as those used in the perfusion and mitochondrial incubations therefore no statistical analysis could be prepared but the data in figure 5.3 were integral to the calculation of activity per gram of wet liver. Data presented are means, n=6 for liver perfusions and n=4 for isolated mitochondria.

It is worthy of note that as described in chapter 2, assays with isolated mitochondria were conducted at 25°C since isolated mitochondria are not viable at 37°C, while the liver perfusions were carried out at 37°C. This difference in temperature for the two assays that we compare in table 5.4 could possibly lead us to conclude that there is no difference in the rate of proline oxidation in isolated mitochondria and during liver perfusions. However if the actual rate of proline oxidation in isolated mitochondria is being underestimated due to the lower assay temperature then there may indeed be a difference in the rates of proline oxidation in isolated mitochondria and the perfused liver. If the rate of proline oxidation in isolated mitochondria is indeed significantly higher than for the perfused liver then proline uptake by hepatocytes might potentially be a rate - controlling step.

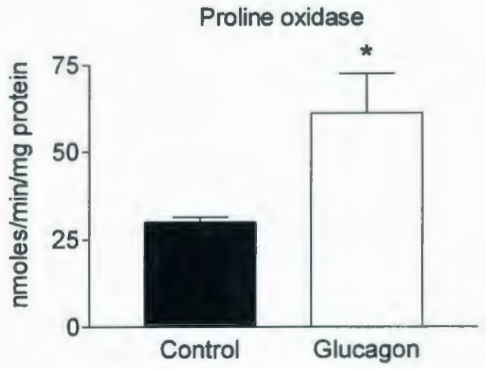
5.5 Proline catabolic enzymes

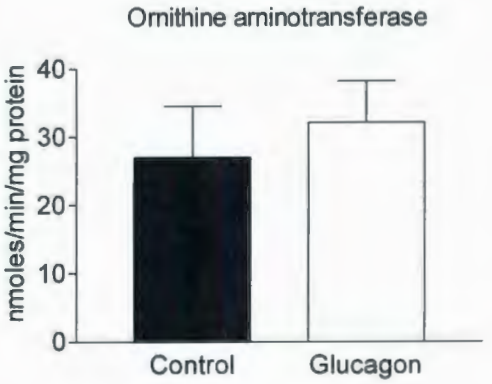
Such an increase in hepatic proline oxidation, coupled with altered levels of plasma and intracellular proline, suggests an appreciably altered metabolism. Therefore, we assayed enzymes related to catabolism of proline. The activities of P5CDh and GDH were elevated by 25% whereas proline oxidase was increased 2-fold by glucagon treatment (figure 5.6).

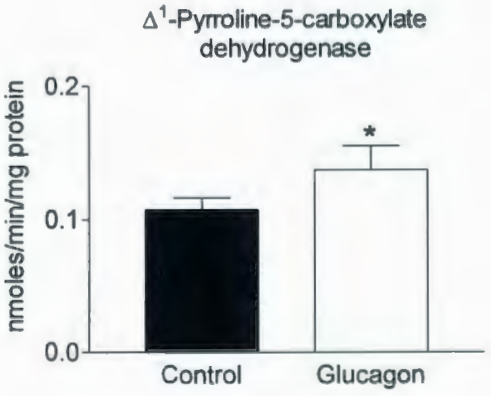
Glucagon, as previously described increases the production of glucose from proline (104). It was stated by these authors that the increase in glucose production that was observed in isolated hepatocytes could have been due to an increase in the

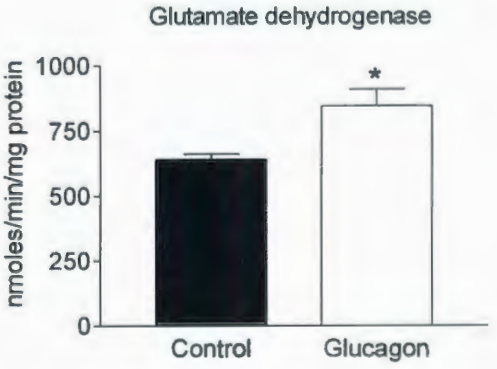
Figure 5.6: Enzyme activities in glucagon treated and control rats

Activity of hepatic enzymes metabolizing P5C in rats given exogenous glucagon (4mg/kg body weight/24 hrs for two days, subcutaneously). Control animals received the vehicle (diluting solution provided by Eli-Lily). All enzyme assays were performed on freshly isolated mitochondria which were subjected to 3 cycles of freezing and thawing to disrupt the mitochondrial membranes and were conducted at 37°C. Data presented are means ± SD, n=4, * denotes $p \leq 0.05$, Students t-test.


Proline oxidase
nmoles/min/mg protein
75
50
25
0
*
Control
Glucagon


Ornithine aminotransferase
nmoles/min/mg protein
40
30
20
10
0
Control
Glucagon


Δ1-Pyrroline-5-carboxylate dehydrogenase
nmoles/min/mg protein
0.2
0.1
0.0
*
Control
Glucagon


Glutamate dehydrogenase
nmoles/min/mg protein
1000
750
500
250
0
*
Control
Glucagon

activity of GDH. An increase in the activity of GDH certainly could account for increased production of α-ketoglutarate and subsequent production of oxaloacetate and finally glucose. However it is also true that in this situation that GDH would have to be the rate-limiting step in gluconeogenesis from proline. The maximal activity of GDH in control animals is approximately 30 fold greater than PO and 2000 fold greater than P5CDh. Given the substantially higher basal activity of GDH it is likely that the 25% increase in activity of GDH that we observed may not be of great significance under normal physiological conditions. Staddon and McGivan (104) did not investigate enzymes catalysing the intermediate steps between proline and glutamate/glucose and simply theorized that GDH could be responsible for increased glucose production since this enzyme connects proline catabolism with other pathways involved in glucose production.

As previously mentioned the activity of PO is increased 2-fold and the activity of P5CDh is increased by 25% versus control animals in response to glucagon injections. An increase in the activities of PO and/or P5CDh mediated by glucagon has not been previously described and may play a role in catabolism of the increased content of intracellular proline that we reported in table 5.2. The increased activity of PO may also be important as a first step in the generation of ATP or the production of glutamate from proline via PO and P5CDh could provide substrate for glutamine synthesis to aid in the removal of excess NH_4^+ generated from increased amino acid catabolism.

There was no change observed in the activity of OAT with increased levels of plasma glucagon (figure 5.6). This was somewhat surprising as it has long been reported that OAT is responsive to glucagon (88). However upon closer examination of data in the earlier studies, it became apparent that OAT only responds to glucagon when the diet is very low in protein so that the activity of OAT is very low (54). For example the increase in OAT activity observed in the presence of glucagon did not occur in animals fed a diet containing approximately 20-30% protein (or higher). While animals consuming a diet containing 60% protein did exhibit an increase in OAT activity, relative to animals on a 30% protein diet, OAT was not responsive to further increases in activity in the presence of glucagon (54).

Chapter 6: Discussion

6.1 Subcellular localization of P5CDh

Our experiments to determine the subcellular location of P5CDh involved the development of a reliable, timely and cost-effective assay for P5CDh activity. Small and Jones (98) stated that the spectrophotometric assay for P5CDh developed by Strecker (105) is not valid since it is not linear with time and protein. Small and Jones (98) developed a radiochemical assay for P5CDh. The assay developed by Small and Jones is certainly a valid assay, but drawbacks such as working with radioactivity, considerable time to complete the assay and cost make their assay less than desirable.

The assay developed by Strecker (105) relies on the reduction of NAD^+ and as such is susceptible to under-estimation should re-oxidation of NADH occur. With this in mind we added rotenone to the assay cocktail. The result of the addition of rotenone has been presented in chapter 3 and it is obvious that this modification to the original assay as employed by Strecker (105) has resulted in an assay that is linear with both time and protein. In addition our assay is reliable, cost effective and can be completed on numerous samples in a short time period (20 minutes) utilizing 96 well plates. The results we obtained with this assay are comparable to those obtained with the radioactive assay by Kowaloff et al. (47). The development of this assay is an invaluable asset to the continuing study of proline catabolism.

The second development of our experiments to locate P5CDh is that we have determined that P5CDh is located strictly in mitochondria in rat liver (chapter 3). While it may seem that this is the answer to a relatively old question, any attempt to study regulation of enzyme activity must begin with knowledge of the location of the enzyme in question. The subcellular location of an enzyme has implications for regulation and function. For example, P5CDh located in the cytosol would not have direct access to P5C produced in the mitochondrial matrix from either proline or arginine catabolism and would require P5C to be transported from the mitochondrial matrix or taken up from plasma. P5CDh in the cytosol would therefore be in direct competition with P5C reductase for P5C. The mitochondrial location of P5CDh places one enzyme utilizing P5C in the cytosol (P5C reductase) and one in the mitochondrial matrix (P5CDh) since OAT does not appear to function in the direction of ornithine production in liver (87).

Finally the increased interest in proline oxidase as a potential partner in a cycle to increase the production of nucleotides (114) or as a mediator of apoptosis (18) will require complete knowledge of the location within liver and regulation of the proline catabolic pathway in order to determine what role if any proline oxidation may play in these processes.

6.2 Regulation of amino acid catabolism

Following the subcellular localization of P5CDh, the main focus of the work presented here is in regard to the regulation of proline catabolism. However we have not addressed one simple question; why regulate proline catabolism at all? That is, why not simply allow proline catabolism to proceed at a rate sufficient to oxidize the total amount of ingested proline? In the absence of regulation, homeostasis would not be served and there would be large fluctuations in the plasma and intracellular concentrations of proline between the fed and fasted state. In addition, the inability to increase the activity of catabolic enzymes in response to a high protein diet could potentially increase the concentration of proline to the point where a negative effect (seizures due to CNS perturbations) would be observed. The opposite situation also applies during times of dietary restriction when proline levels could decrease such that inhibition of growth and development occurs or during injury/growth when there is increased demand for protein synthesis, especially collagen. The fact that there is no storage form of amino acids contributes to the need to closely regulate amino acid catabolism since there is no ready supply of amino acids should demand exceed the dietary supply and excess amino acids cannot be stored and therefore must be catabolized.

6.3 Proline metabolism in response to dietary protein

The increase in activity of PO, OAT and P5CDh and the increased flux through the proline degradative pathway (albeit under optimal conditions) that we have observed in response to a diet containing excess protein may play a role in removing surplus proline and arginine present in such a diet. An increase in the activity of amino acid catabolic enzymes has been reported by various investigators for amino acids such as threonine, glycine and arginine in response to a diet containing a moderately high protein content (8). We are now adding proline to this list and extending the studies of O'Sullivan et al. (77).

The use of a diet containing adequate (15%) or a moderate excess of protein (45%) in our study has allowed us to study proline catabolism under conditions that relate to those that are physiologically possible. The reports by Kawabata et al., (44) and Matsuzawa et al. (57), in regard to increases in the activity of PO and P5CDh respectively, while showing increased activity of these two enzymes did not utilize physiologically relevant conditions. The use of a diet containing an extremely low (5%), or extremely high (65%) protein content, as in these studies, does not provide clinically useful information. Our study provides information on the proline catabolic enzymes that has not previously been reported and our study design suggests that changes in proline catabolism may occur during normal fluctuations in dietary protein.

As previously mentioned the production of glutamate from proline could potentially play a role in a proposed scavenger system for the removal of NH_4^+

generated by the catabolism of amino acids. The increased catabolism of amino acids that must occur in response to a diet high in protein would increase the production of NH_4^+ and, as we have shown, the same diet also increases flux through the proline catabolic pathway. Increased flux through the proline catabolic pathway could increase the intracellular concentration of glutamate which could then be converted to glutamine via GS.

Glutamine production from proline may in fact be an important outcome of proline catabolism since it has been shown that intravenous injections of ^{14}C-proline in rats resulted in a considerable amount of ^{14}C becoming localized to the kidney in the form of ^{14}C-glutamate (26). It is quite possible that a portion of the ^{14}C-glutamate observed in the kidney was a result of ^{14}C-glutamine production in the liver and subsequent release to plasma. ^{14}C-glutamine could subsequently be taken up by the kidney where renal glutaminase could release NH_4^+ for excretion with the resulting production of ^{14}C-glutamate. Renal activity of P5CDh is only a fraction of that observed in liver which may make it difficult for kidney to produce a large amount of labelled glutamate from proline (57).

6.4 Glucagon treatment

The dose of glucagon used in this study was indeed pharmacological. We observed a substantial increase in plasma glucagon using this dosage (table 5.1), well above circulating levels in control animals. Although these levels are not observed in a healthy organism, patients with glucagon-producing tumours have been reported to have a 60-fold increase in plasma glucagon (4). Therefore

our model is a good reflection of human glucagonoma. This view is supported by the similar decreases in total and specific plasma amino acids in both the glucagon-treated rat and the human glucagonoma patient (4). The decrease in amino acids in glucagonoma patients has been linked to increased clearance from the plasma, likely by the liver, rather than to decreased export from the muscles (4).

We observed a 60% increase in the oxidation of proline during both non-recirculating liver perfusions (figure 5.1B) and incubations with intact mitochondria (figure 5.3) with the absolute rate of $^{14}CO_2$ production from ^{14}C-proline approximating 23 nmoles/min/g wet liver in control animals and 40 nmoles/min/g wet liver in glucagon treated animals. The fact that there is no change in the rate of proline oxidation in the intact perfused liver where transport across the plasma membrane would be a vital step and in intact mitochondria where transport across the plasma membrane is not an issue would suggest that the transport of proline across the plasma membrane may not be a rate-limiting step in the oxidation of proline. However the conditions used during the mitochondrial incubations are optimal conditions for the assay while the liver perfusion is a more physiologically correct situation. Specifically, the differences in assay temperatures as discussed in chapter 5 (25°C in isolated mitochondria versus 37°C during perfusions) could also lead to incorrect conclusions regarding the rate of proline oxidation in mitochondria and the importance of proline uptake.

Our results show that there is an increase in proline transport in glucagon treated animals versus control animals at concentrations of proline from 0.50 to 9.0 mM (figure 5.2). Using data from Berry et al. (7), we have calculated the rate of proline transport as a function of grams wet liver. At the physiologically relevant concentration of 0.50 mM our data for the transport of proline shows that the rate of proline transport was 22.34 and 32.83 μmoles/min/ g wet liver for control and glucagon treated animals respectively. The transport of proline across the plasma membrane would therefore appear to occur at a rate that is approximately 1000 fold in excess of the rates observed for proline oxidation in both perfused liver and isolated mitochondria. Once again these results must be considered as suggestive since the data were collected under ideal conditions and in vivo the situation may vary.

The 12-fold increase in the liver:plasma ratio coupled with an approximately 1000 fold higher rate of transport may suggest that the rate limiting step in proline oxidation occurs beyond transport across the plasma membrane. If this were not the case, then the hepatic concentration of proline should not increase to such an extent. That is, it would appear from our data that the transport of proline into hepatocytes is occurring at a rate in excess of that displayed by the proline catabolic pathway.

It is apparent from our data that there must be an activation event that occurs in response to glucagon that signals hepatocytes to actively transport proline from plasma. The approximately 12-fold increase in the intracellular

concentration of proline could be due to induction or activation of the system A transporter ATA2. However, ATA2 is described as a weakly accumulating transporter which transports numerous small neutral amino acids and as such may not be responsible for the very specific increase in intra-hepatic proline concentration (108). Proline transport in response to glucagon may therefore be due to the induction or activation of a transporter with the ability to concentrate proline beyond what may be possible via the system A transporter ATA2.

Our data suggest that there may be more than one transporter for proline present in hepatocytes. This suggestion is also supported by data obtained in our lab that show that Δ^1-pyrroline-2-carboxylate (P2C) a competitive inhibitor of proline oxidase is able to decrease the amount of proline transported by isolated hepatocytes by no more than 50% regardless of the ratio of inhibitor to proline concentration used in the assay (19). It has been shown that P2C does not affect the transport of the model substrate for system A, methyl-aminoisobutyric acid (MeAIB) (68). Given our data and that P2C can inhibit the transport of proline by 50% while not affecting the transport of MeAIB it would appear that there is indeed a previously uncharacterized transporter for proline present in hepatocytes.

In regard to activities of the proline catabolic enzymes the lack of an increase in the activity of OAT in response to glucagon while being previously described has not been satisfactorily explained to date (54). It has been postulated that OAT activity is directly related to plasma glucagon levels and that

the endogenous supply of glucagon that is released in response to protein in the diet is sufficient to produce a maximal response under the conditions at that time (54). Given that there is an increase in OAT activity in animals fed a diet containing 60% protein versus those fed a 30% protein diet, it would seem logical that animals fed a 30% protein diet and administered exogenous glucagon should exhibit OAT activity that approaches or exceeds that observed in animals fed a 60% protein diet. This is not the case.

It appears that the administration of glucagon results in an increase in hepatic OAT activity only when the protein content of the diet is low but has no effect on OAT activity when the protein content of the diet is in the normal range . However, ingestion of a diet containing excess protein can further increase the activity of OAT. The fact that OAT activity does not increase in a direct relationship with glucagon may suggest that the induction of OAT by glucagon or amino acids is not as straightforward as originally thought.

We have observed that the activity of P5CDh under optimal conditions is somewhat lower than that observed for the other enzymes involved in proline catabolism (figure 4.3/5.6). The flux through the proline catabolic pathway under ideal conditions in both perfused rat liver and isolated mitochondria is considerably higher than the activity of P5CDh in broken mitochondria. This would suggest that in vivo the activity of P5CDh must be increased since the maximal rate of P5CDh that we have observed (which agrees with other investigators (47;105)) cannot account for the rate of $^{14}CO_2$ we have observed

during perfusions and with isolated mitochondria. An increase in the activity of P5CDh in vivo could potentially occur if the proline catabolic enzymes constitute a metabolon. For example, P5CDh could be associated with PO in the mitochondrial inner membrane and/or with the unknown transporter that is required to mediate the entrance of proline into the mitochondrion, such that the product of one step has direct access to the active site of the next enzyme, for which it is a substrate.

6.5 Conclusions

Subcellular localization

a) The spectrophotometric assay is valid and provides a quick, easy and inexpensive method for assays of P5CDh

b) P5CDh is located strictly in the mitochondrial matrix

High protein diet increases:

a) flux through the proline catabolic pathway in mitochondria resulting in the production of CO_2

b) activity of PO, P5CDh and OAT in rat liver mitochondria

Glucagon increases:

a) proline transfer from plasma to hepatocytes

b) oxidation of proline by perfused liver

c) flux through the proline catabolic pathway resulting in the production of CO_2

d) activity of PO, P5CDh and GDH in rat liver mitochondria

References

1. Alfieri, R. R., P. G. Petronini, M. A. Bonelli, A. E. Caccamo, A. Cavazzoni, A. F. Borghetti, and K. P. Wheeler. Osmotic regulation of ATA2 mRNA expression and amino acid transport System A Activity. *Biochem. Biophys. Res Comm.* 283: 174-178, 2001.

2. Atkinson, D. E. and E. Bourke. Metabolic aspects of the regulation of systemic pH. *Am.J.Physiol* 236: F947-F956, 1986.

3. Ault, B., C. M. Wang, and B. C. Yawn. L-Proline depolarizes rat spinal motorneurons by an excitatory amino acid antagonist-sensitive mechanism. *Br.J.Pharmacol.* 92: 319-326, 1987.

4. Barazzoni, R., M. Zanetti, A. Tiengo, and P. Tessarri. Protein metabolism in glucagonoma. *Diabetologia* 42: 326-329, 1999.

5. Barber, E. F., M. E. Handlogten, and M. S. Kilberg. Induction of amino acid transport System A in rat hepatocytes is blocked by tunicamycin. *J.Biol.Chem.* 258: 11851-11855, 1983.

6. Bergmeyer, H. U., E. Bernt, F. Schmidt, and H. Stork. D-glucose:determination with hexokinase and glucose-6-phosphate

dehydrogenase. In Bergmeyer, H. U., ed. Methods of Enzymatic Analysis. DeerfieldBeach,FL, Verlag Chemie International. 1981, 1196-1201.

7. Berry, M. N., A. M. Edwards, and G. J. Barritt. High yield preparation of isolated hepatocytes from rat liver. In Burdon, R. H. and P. H. Van Knippenberg, eds. Laboratory Techniques in Biochemistry and Molecular Biology. The Netherlands, Elsevier. 1991, 15-58.

8. Boon, L., W. J. C. Geerts, A. Jonker, W. H. Lamers, and C. J. F. Van Noorden. High protein diet induces pericentral glutamate dehydrogenase and ornithine aminotransferase to provide sufficient glutamate for pericentral detoxification of ammonia in rat liver lobules. *Histochem Cell Biol* 111: 445-452, 1999.

9. Broer, S. Adaptation of plasma membrane amino acid transport mechanisms to physiological demands. *Pflugers Arch-Eur.J.Physiol* 444: 457-466, 2002.

10. Brosemer, R. W. and P. S. Veerabhadrappa. Pathway of proline oxidation in insect flight muscle. *Biochim Biophys.Acta.* 110: 102-112, 1965.

11. Brosnan, J. T. Glutamate, at the interface between amino acid and carbohydrate metabolism. *J.Nutr.* 130: 988S-990S, 2000.

12. Brunner, G. and W. Neupert. Localization of proline oxidase and delta-pyrroline-5-carboxylic acid dehydrogenase in rat liver. *FEBS Lett.* 3: 283-286, 1969.

13. Burton, K. A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem.J.* 62: 315-322, 1955.

14. Carr, F. P. and C. I. Pogson. Phenylalanine metabolism in isolated rat liver cells: Effects of glucagon and diabetes. *Biochem.J.* 198: 655-660, 1981.

15. Chen, Z., Y. J. Fei, C. M. H. Anderson, K. A. Wake, S. Miyauchi, W. Huang, D. T. Thwaites, and V. Ganapathy. Structure, function and immunolocalization of a proton-coupled amino acid transporter (hPAT1) in the human intestinal cell line Caco-2. *J Physiol* 546.2: 349-361, 2003.

16. deDuve, C. Exploring cells with a centrifuge. *Science* 189: 186-193, 1975.

17. deDuve, C., B. C. Pressman, R. Gianetto, R. Wattiaux, and F. Appelmans. Tissue fractionation studies. *Biochem.J.* 60: 604-617, 1955.

18. Donald, S. P., X. Sun, C. A. Hu, J. Yu, J. M. Mei, D. Valle, and J. M. Phang. Proline oxidase, encoded by p53-induced gene-6, catalyzes the

generation of proline-dependent reactive oxygen species. *Cancer Research* 61: 1810-1815, 2001.

19. Duffet, L. D. M. Proline Transport in Rat Hepatocytes. 2003. Memorial University of Newfounland. Bsc. Honors thesis.

20. Edmondson, J. W. and L. Lumeng. Biphasic stimulation of amino acid uptake by glucagon In Hepatocytes. *Biochem. Biophys. Res. Comm.* 96: 61-68, 1980.

21. Eggleston, L. V. and H. A. Krebs. Regulation of the pentose phosphate cycle. *Biochem.J.* 138: 425-435, 1974.

22. Fleming, G. A., A. Granger, Q. R. Rogers, M. Prosser, D. B. Ford, and J. M. Phang. Fluctuations in plasma pyrroline-5-carboxylate concentrations during feeding and fasting. *J.Clin.Endocrinol.Metab* 69: 448-452, 1989.

23. Forte-McRobbie, C. M. and R. Pietruszko. Purification and characterization of human liver "high Km" aldehyde dehydrogenase and its identification as glutamic gamma-semialdehyde dehydrogenase. *J.Biol.Chem.* 261: 2154-2163, 1986.

24. Geraghty, M. T., D. Vaughn, A. J. Nicholson, WW. Lin, G. Jimenez-Sanchez, C. Obie, M. P. Flynn, D. Valle, and C. Hu. Mutations in the Δ^1–pyrroline 5-carboxylate dehydrogenase gene cause type II hyperprolinemia. *Human Molecular Genetics* 7: 1411-1415, 1998.

25. Gornall, A. G., C. J. Bardawill, and M. M. David. Determination of serum proteins by means of the Biuet reaction. *J.Biol.Chem.* 177: 751-766, 1949.

26. Greth, W. E., S. O. Thier, and S. Segal. The transport and metabolism of L-Proline-^{14}C in the rat In vivo. *Metabolism* 27: 975-985, 1978.

27. Hagedorn, C. H. and J. M. Phang. Transfer of reducing equivalents into mitochondria by the interconversions of proline and delta 1-pyrroline-5-carboxylate. *Arch.Biochem.Biophys.* 225: 95-101, 1983.

28. Halling, P. J., M. D. Brand, and J. B. Chappel. Transport of neutral amino acids across mitochondrial membranes. *FEBS Lett.* 34: 169-171, 1973.

29. Handlogten, M. E. and M. S. Kilberg. Induction and decay of amino acid transport in the liver. Turnover of transport activity in isolated hepatocytes after stimulation by diabetes or glucagon. *J.Biol.Chem.* 259: 3519-3525, 1984.

30. Hatanaka, T., W. Huang, H. Wang, M. Sugawara, P. D. Prasad, F. H. Leibach, and M. E. Ganapathy. Primary structure, functional characteristics and tissue expression pattern of human ATA2, a subtype of amino acid transport system A. *Biochim Biophys.Acta.* 1467: 1-6, 2000.

31. Hatanaka, T., W. Huang, R. Ling, P. D. Prasad, M. Sugawara, F. H. Leibach, and V. Ganapathy. Evidence for the transport of neutral as well as cationic amino acids by ATA3, a novel and liver-specific subtype of amino acid transport system A. *Biochim. Biophys Acta* 1510: 10-17, 2001.

32. Haussinger, D. Nitrogen metabolism in liver: structural and functional organization and physiological relevance. *Biochem.J.* 267: 281-290, 1990.

33. Haussinger, D., S. Soboll, A. J. Meijer, W. Gerok, J. M. Tager, and S. Helmut. Role of plasma membrane transport in hepatic glutamine metabolism. *Eur J Biochem* 152: 597-603, 1985.

34. Herzfeld, A. and W. E. Knox. The properties, developmental formation, and estrogen induction of ornithine aminotransferase in rat tissues. *J.Biol.Chem.* 243: 3327-3332, 1968.

35. Herzfeld, A., V. A. Mezl, and W. E. Knox. Enzymes metabolizing delta1-pyrroline-5-carboxylate in rat tissues. *Biochem.J.* 166: 95-103, 1977.

36. House, J. D., B. N. Hall, and J. T. Brosnan. Threonine metabolism in isolated rat hepatocytes. *Am.J.Physiol* 281: E1300-E1307, 2001.

37. Hu, C. A., W. W. Lin, and D. Valle. Cloning, characterization, and expression of cDNAs encoding human delta 1-pyrroline-5-carboxylate dehydrogenase. *J.Biol.Chem.* 271: 9795-9800, 1996.

38. Humbertclaude, V., F. Rivier, A. Roubertie, B. Echenne, H. Bellet, C. Vallat, and D. Morin. Is hyperprolinemia type I actually a benign trait? Report of a case with severe neurologic involvement and vigabatrin intolerance. *Journal of Child Neurology* 16: 622-623, 2001.

39. Jacobs, E. E., J. Jacob, D. R. Sanadi, and L. B. Bradley. Uncoupling of oxidative phosphorylation by cadmium ion. *J.Biol.Chem.* 223: 147-156, 1956.

40. Jacobs, R. L., L. M. Stead, M. E. Brosnan, and J. T. Brosnan. Hyperglucagonemia in rats results in decreased plasma homocysteine and increased flux through the transsulfuration pathway in liver. *J.Biol.Chem.* 276: 43740-43748, 2001.

41. Johnson, A. B. and H. J. Strecker. The interconversion of glutamic acid and proline IV. The oxidation of proline by rat liver mitochondria. *J.Biol.Chem.* 237: 1876-1882, 1962.

42. Jois, M., B. Hall, K. Fewer, and J. T. Brosnan. Regulation of hepatic glycine catabolism by glucagon. *Journal of Biological Chemistry* 264: 3347-3351, 1989.

43. Jones, M. E. Conversion of glutamate to ornithine and proline: pyrroline-5-carboxylate, a possible modulator of arginine requirements. *J.Nutr.* 115: 509-515, 1985.

44. Kawabata, Y., N. Katunuma, and Y. Sanada. Characteristics of proline oxidase in rat liver. *J Biochem* 88: 281-283, 1980.

45. Kelly, A. and C. A. Stanley. Disorders of glutamate metabolism. *Mental Retardation and Developmental Disabilities Research Reviews* 7: 287-295, 2001.

46. Kilberg, M. S. and D. Haussinger. Mammalian Amino Acid Transport Mechanisms and Control. New York, Plenum Press. 1992.

47. Kowaloff, E. M., A. S. Granger, and J. M. Phang. Alterations in proline metabolic enzymes with mammalian development. *Metabolism* 25: 1087-1094, 1976.

48. Kowaloff, E. M., A. S. Granger, and J. M. Phang. Glucocorticoid control of hepatic proline oxidase. *Metabolism* 26: 893-901, 1977.

49. Kowaloff, E. M., J. M. Phang, A. S. Granger, and S. J. Downing. Glucocorticoid induction of proline oxidase in LLC-RK1 cells. *J.Cell Physiol* 97: 153-159, 1978.

50. Lacey, J. H., N. M. Bradford, S. K. Joseph, and J. D. McGivan. Increased activity of phosphate-dependent glutaminase in liver mitochondria as a result of glucagon treatment of rats. *Biochem.J.* 194: 29-33, 1981.

51. LeBlanc, J., J. Soucy, and A. Nadeau. Early insulin and glucagon response to different food items. *Horm.Metab.Res.* 28: 276-279, 1996.

52. LeCam, A. and P. Freychet. Glucagon stimulates the System A for neutral amino acid transport in isolated hepatocytes of adult rat. *Biochem. Biophys. Res. Comm.* 72: 893-901, 1976.

53. Linn, T., B. Santosa, D. Granemeyer, S. Aygen, N. Scholz, M. Busch, and R. G. Bretzel. Effect of long-term dietary protein intake on glucose metabolism in humans. *Diabetologia* 43: 1257-1265, 2000.

54. Lyons, R. T. and H. C. Pitot. The Regulation of ornithine aminotransferase synthesis by glucagon in the rat. *Arch.Biochem.Biophys.* 174: 262-272, 1976.

55. Mabrouk, G. M., M. Jois, and J. T. Brosnan. Cell signalling and the hormonal stimulation of the hepatic glycine cleavage system by glucagon. *Biochem.J.* 330: 759-763, 1998.

56. Mallette.L.E., J. H. Exton, and C. R. Park. Effects of glucagon on amino acid transport and utilization in perfused rat liver. *J.Biol.Chem.* 244: 5724-5728, 1969.

57. Matsuzawa, T., T. Kobayashi, K. Tashiro, and M. Kasahara. Changes in ornithine metabolic enzymes induced by dietary protein in small intestine and liver: Intestine-liver relationship in ornithine supply to liver. *J Biochem* 116: 721-727, 1994.

58. Maxwell, S. A. and G. E. Davis. Differential gene expression in p53-mediated apoptosis-resistant vs. apoptosis-sensitive tumor cell lines. *Proc. NAtl. Acad. Sci.* 97: 13009-13014, 2000.

59. Maxwell, S. A. and A. Rivera. Proline oxidase induces apoptosis in tumor cells, and its expression is frequently absent or reduced in renal carcinomas. *J.Biol.Chem.* 278: 9784-9789, 2003.

60. McGivan, J. D., N. M. Bradford, and A. D. Beavis. Factors influencing the activity of ornithine aminotransferase in isolated rat liver mitochondria. *Biochem.J.* 162: 147-156, 1977.

61. McGivan, J. D., J. H. Lacey, and S. K. Joseph. Localization and some properties of phosphate dependant glutaminase in disrupted rat liver mitochondria. *Biochem.J.* 192: 537-542, 1980.

62. McGivan, J. D. and M. Pastor-Anglada. Regulatory and molecular aspects of mammalian amino acid transport. *Biochem.J.* 299 (Pt 2): 321-334, 1994.

63. McKnight, J. A. and F. J. Hird. The oxidation of proline by mitochondrial preparations. *Comp Biochem.Physiol* 85: 289-294, 1986.

64. Meijer, A. J., W. H. Lamers, and R. A. Chamuleau. Nitrogen metabolism and ornithine cycle function. *Physiol Rev.* 70: 701-748, 1990.

65. Merrill, M. J., G. C. Yeh, and J. M. Phang. Purified human erythrocyte pyrroline-5-carboxylate reductase. *J.Biol.Chem.* 264: 9352-9358, 1989.

66. Meyer, J. Proline transport in rat liver mitochondria. *Arch.Biochem.Biophys.* 178: 387-395, 1977.

67. Mezl, V. A. and W. E. Knox. Properties and analysis of a stable derivative of pyrroline-5- carboxylic acid for use in metabolic studies. *Anal.Biochem.* 74: 430-440, 1976.

68. Mixson, A. J. and J. M. Phang. Structural analogues of pyrroline 5-carboxylate specifically inhibit its uptake into cells. *J.Membr.Biol.* 121: 269-277, 1991.

69. Morris, S. M. Jr. and D. Kepka-Lenhart. Hormonal induction of hepatic mitochondrial ornithine/citrulline transporter mRNA. *Biochem Biophys.Res.Commun.* 294: 749-752, 2002.

70. Morrison, G. R., F. E. Brock, D. T. Sobral, and R. E. Shank. Cold-acclimatization and intermediary metabolism of carbohydrates. *Arch.Biochem.Biophys.* 114: 494-501, 1966.

71. Muller, W. A., G. R. Faloona, and R. H. Unger. The Influence of the antecedant diet upon glucagon and insulin secretion. *The New England Journal of Medicine* 285: 1450-1454, 1971.

72. Nadler, J. V., A. Wang, and A. Hakim. Toxicity of L-proline toward rat hippocampal neurons. *Brain Res.* 456: 168-72, 1988.

73. Nakatani, Y., M. Schneider, C. Banner, and E. Freese. Complete nucleotide sequence of human glutamate dehydrogenase cDNA. *Nucleic Acids Res.* 16: 6237-6241, 1988.

74. Newsholme, P., J. Procopio, M. M. R. Lima, T. C. Pithon-Curi, and R. Curi. Glutamine and glutamate--their central role in cell metabolism and function. *Cell Biochem Funct* 21: 1-9, 2003.

75. Nickolson, V. J. "On" and "off" responses of K^+-induced synaptosomal proline release: involvement of the sodium pump. *Neurochem* 38: 289-292, 1982.

76. O'Sullivan, D., J. T. Brosnan, and M. E. Brosnan. Hepatic zonation of the catabolism of arginine and ornithine in the perfused rat liver. *Biochem.J.* 330 (Pt 2): 627-632, 1998.

77. O'Sullivan, D., J. T. Brosnan, and M. E. Brosnan. Catabolism of arginine and ornithine in the perfused rat liver: effect of dietary protein and of glucagon. *Am.J.Physiol Endocrinol.Metab* 278: E516-E521, 2000.

78. Peret, J., S. Foustock, M. Chanez, B. Bois-Joyeux, and R. Assan. Plasma glucagon and insulin concentrations and hepatic phosphoenolpyruvate carboxykinase and pyruvate kinase activities during and upon adaptation of rats to a high protein diet. *J Nutr.* 111: 1173-1184, 1981.

79. Phang, J. M. The regulatory functions of proline and pyrroline-5-carboxylic acid. *Current Topics In Cellular Regulation* 25: 91-132, 1985.

80. Phang, J. M., S. J. Downing, and G. C. Yeh. Linkage of the HMP pathway to ATP generation by the proline cycle. *Biochem.Biophys.Res.Commun.* 93: 462-470, 1980.

81. Phang, J. M., S. J. Downing, G. C. Yeh, R. J. Smith, J. A. Williams, and C. H. Hagedorn. Stimulation of the hexosemonophosphate-pentose pathway

by pyrroline-5- carboxylate in cultured cells. *J.Cell Physiol* 110: 255-261, 1982.

82. Phang, J. M., C.A. Hu, and D. Valle. Disorders of proline and hydroxyproline metabolism in , The Metabolic and Molecular Basis of Inherited Disease. New York, McGraw Hill. 2001, Vol II, 1821-1838.

83. Phang, J. M., D. Valle, and E. M. Kowaloff. Proline biosynthesis and degradation in mammalian cells and tissue. *Ann.Clin.Lab Sci.* 5: 298-302, 1975.

84. Phang, J. M., G. C. Yeh, and C. H. Hagedorn. The intercellular proline cycle. *Life Sci.* 28: 53-58, 1981.

85. Phang, J. M., G. C. Yeh, and C. R. Scriver. Disorders of Proline and Hydroxyproline Metabolism in, The Metabolic and Molecular Basis of Inherited Disease. New York, McGraw-Hill Book Company. 1983, Vol I 1125-1146.

86. Piez, K. A., F. Irreverre, and H. L. Wolff. The Separation and determination of cyclic amino acids. *J.Biol.Chem.* 223: 687-697, 1956.

87. Pink, D. Hepatic Zonation of Proline Metabolism. 2000. Memorial University of Newfoundland. Masters thesis dissertation

88. Pitot, H. C. and C. Peraino. Studies on the Induction and Repression of Enzymes in Rat Liver. I. Induction of threonine dehydrogenase and ornithine transaminase by oral intubation of casein hydrolysate. *Journal of Biological Chemistry* 239: 1783-1788, 1964.

89. Reeves, P. G., F. H. Nielsen, and G. C. Fahey. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition Ad Hoc Writing Committee on the reformulation of the AIN-76 rodent diet. *J.Nutr.* 123: 1939-1951, 1993.

90. Roth, E., F. Muhlbacher, J. Karner, G. Hamilton, and J. Funovics. Free amino acid levels in muscle and liver of a patient with Glucagonoma syndrome. *Metabolism* 36: 7-13, 1987.

91. Ruiz-Montasell, B., M. Gomez-Angelats, F. J. Casado, A. Felipe, J. D. McGivan, and M. Pastor-Anglada. Evidence for a regulatory protein involved in the increased activity of System A for neutral amino acid transport in osmotically stressed mammalian cells. *Proc. Natl. Acad. Sci.* 91: 9569-9573, 1994.

92. Salter, M., R. G. Knowles, and C. I. Pogson. Transport of the aromatic amino acids into isolated rat liver cells: properties of uptake by two distinct systems. *Biochem.J.* 233: 499-506, 1986.

93. Schneider, W. C. Phosphorus compounds in animal tissue extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. *J.Biol.Chem.* 223: 293-303, 1945.

94. Schulze-Osthoff, K., A. C. Bakker, B. Vanhaesebroeck, R. Beyaert, W. A. Jacob, and W. Fiers. Cytotoxic activity of tumor necrosis factor is mediated by early damage of mitochondrial functions. Evidence for the involvement of mitochondrial radical generation. *J.Biol.Chem.* 267: 5317-5323, 1992.

95. Scriver, C. R. Disorders of Proline and Hydroxyproline Metabolism in, The Metabolic and Molecular Basis of Inherited Disease. New York, McGraw-Hill Book Company. 1978, 336-361.

96. Shotwell, M. A., M. S. Kilberg, and D. L. Oxender. The regulation of neutral amino acid transport in mammalian cells. *Biochem. Biophys.iActa* 737: 267-284, 1983.

97. Sips, H. J., A. K. Groen, and J. M. Tager. Plasma-membrane transport of alanine is rate-limiting for its metabolism in rat-liver parenchymal cells. *FEBS Lett.* 119: 271-274, 1980.

98. Small, C. and M. E. Jones. A specific radiochemical assay for pyrroline-5-carboxylate dehydrogenase. *Anal.Biochem.* 161: 380-386, 1987.

99. Small, W. C. and M. E. Jones. Pyrroline 5-carboxylate dehydrogenase of the mitochondrial matrix of rat liver. Purification, physical and kinetic characteristics. *J. Biol.Chem.* 265: 18668-18672, 1990.

100. Snodgrass, P. J., R. C. Lin, W. A. Muller, and T. T. Aoki. Induction of urea cycle enzymes of rat liver by glucagon. *J.Biol.Chem.* 253: 2748-2753, 1978.

101. Sochor, M., P. McLean, J. Brown, and A. L. Greenbaum. Regulation of pathways of ornithine metabolism. *Enzyme* 26: 15-23, 1981.

102. Sottocassa, G. L., B. Kuylenstierna, L. Ernster, and A. Bergstrand. An electron-transport system associated with the outer membrane of liver mitochondria. *J. Cell Biol.* 32: 415-438, 1967.

103. Squires, S. A., H. S. Ewart, C. McCarthy, M. E. Brosnan, and J. T. Brosnan. Regulation of hepatic glutaminase in the Streptozotocin-induced diabetic rat. *Diabetes* 46: 1945-1949, 1997.

104. Staddon, J. M. and J. D. McGivan. Distinct effects of glucagon and vasopressin on proline metabolism in isolated hepatocytes. The role of oxoglutarate dehydrogenase. *Biochem.J.* 217: 477-483, 1984.

105. Strecker, H. J. The interconversion of glutamic acid and proline. *J. Biol. Chem.* 235: 3218-3223, 1960.

106. Stryer, L. Biochemistry. New York, W.H. Freeman and Company. 1995.

107. Sugawara, M., T. Nakanishi, Y. J. Fei, R. G. Martindale, M. E. Ganapathy, F. H. Leibach, and V. Ganapathy. Structure and function of ATA3, a new subtype of amino acid transport system A, primarily expressed in the liver and skeletal muscle. *Biochem Biophys.Acta.* 1509: 7-13, 2000.

108. Sugawara, M., T. Nakanishi, Y. J. Fei, W. Huang, M. E. Ganapathy, F. H. Leibach, and V. Ganapathy. Cloning of an amino acid transporter with functional characteristics and tissue expression pattern identical to that of System A. *J. Biol. Chem.* 275: 16473-16477, 2000.

109. Szent-Gyorgyi, A. G. and C. Cohen. Role of proline in polypeptide chain configuration of proteins. *Science* 13: 697-698, 1957.

110. Tapiero, H., G. Mathe, P. Couvreur, and K. D. Tew. Glutamine and glutamate. *Biomed Pharmacother* 56: 446-457, 2002.

111. Ulbright, C. and P. J. Snodgrass. Coordinate induction of the urea cycle enzymes by glucagon and dexamethasone is accomplished by three different mechanisms. *Arch.Biochem.Biophys.* 301: 237-243, 1993.

112. Wakabayashi, Y., R. Yamada, and A. Iwashima. Factors stabilizing pyrroline-5-carboxylate synthase of rat intestine mucosa at a physiological temperature. *Arch.Biochem.Biophys.* 238: 464-468, 1985.

113. Wasserman, D. H., J. A. Spalding, D. B. Lacy, C. A. Colburn, R. E. Goldstein, and A. D. Cherrington. Glucagon is a primary controller of glycogenolysis and gluconeogenesis during muscular work. *Am.J.Physiol* 257: E108-E117, 1989.

114. Yeh, G. C., E. F. Roth, Jr., J. M. Phang, S. C. Harris, R. L. Nagel, and A. Rinaldi. The effect of pyrroline-5-carboxylic acid on nucleotide metabolism in erythrocytes from normal and glucose-6-phosphate dehydrogenase-deficient subjects. *J. Biol. Chem.* 259: 5454-5458, 1984.