HEPATIC ZONATION OF Δ^1-PYRROLINE-5-CARBOXYLATE METABOLISM

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by

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The Plight of Prometheus

Prometheus was the son of Iapetus who was one of the Titans. He tricked the gods into eating bare bones instead of good meat. He stole the sacred fire from Zeus and the gods. Prometheus did not tell Zeus the prophecy that one of Zeus's sons will overthrow him. In punishment, Zeus commanded that Prometheus be chained for eternity to Mount Caucasus. Every day an eagle swooped on him and devoured the lobes of his liver, which grew by night as much as the eagle had devoured during the day. But when Hercules came to Caucasus he shot the eagle and released Prometheus.

Abstract

Metabolic zonation in the liver is characterized by a heterogeneous distribution of enzymes along the sinusoids of the liver acinus. Periportal hepatocytes are enriched in enzymes associated with gluconeogenesis, amino acid degradation and urea synthesis. Xenobiotic metabolism, lipogenesis and glutamine synthetase activity occur predominantly in the perivenous cells. The localization of proline oxidase activity has been examined to investigate the zonal implications of P5C metabolism.

Administration of the hepatotoxins, carbon tetrachloride and bromobenzene, was used to induce damage (*in vivo*) to the perivenous zone. Allyl alcohol administration was used to damage the periportal zone of the acinus. Alternatively, controlled antegrade and retrograde perfusion of rat liver with digitonin was used to selectively permeabilize cells in the periportal or perivenous zone of the acinus. These techniques were combined with the collagenase method for hepatocyte isolation to provide enriched suspensions of specific hepatocyte populations. The activities of perivenous zone-specific marker enzymes, glutamine synthetase and ornithine aminotransferase, as well as the periportal zone-specific ornithine transcarbamylase were used in conjunction with histological analysis to ascertain the extent of damage.

Administration of bromobenzene provided strong evidence to suggest a perivenous enrichment of proline oxidase. This result was further substantiated by retrograde digitonin-perfusion. In both instances, cells in the perivenous zone

were destroyed and remaining cells demonstrated significantly decreased activities of perivenous zone markers and of proline oxidase.

The results of the toxin and digitonin experiments have also demonstrated that the (perivenous) zone of proline oxidase enrichment is not as discrete as glutamine synthetase. The zone of positive proline oxidase activity was similar to the zone of positive ornithine aminotransferase activity.

Proline production from P5C was used to assess the activity of P5C reductase in isolated hepatocytes following either toxin or digitonin treatment. The results indicated that as more of the perivenous region was damaged, proline production increased. Specifically, proline production was highest following bromobenzene intoxication, followed by carbon tetrachloride and allyl alcohol intoxication. These results suggest a periportal enrichment of P5C reductase. Retrograde digitonin-perfusion however, resulted in a significant decrease in proline production, indicative of a perivenous enrichment.

The utilization of DL-P5C as a substrate for amino acid production was compared in hepatocytes from fed and fasted animals. Following a 48 hour fast, P5C was converted to proline and glutamate equally. In the fed state, P5C was preferentially converted to proline with marginal conversion to glutamate. The production of ornithine from P5C was not observed in either nutritional state.

Acknowledgements

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List of Abbreviations and Symbols

AA	allyl alcohol
ADH	alcohol dehydrogenase
AZCA	L-azetidine-2-carboxylic acid
BB	bromobenzene
BSA	bovine serum albumin
CAT	chloramphenicol acetyltransferase
CCl_4	carbon tetrachloride
CHO cells	Chinese hamster ovary cells
CPS	carbamoylphosphate synthetase
CV	central vein (terminal hepatic venule)
CYP	cytochrome P450
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether) *N,N,N,',N'* –tetra-acetic acid
GDH	glutamate dehydrogenase
GS	glutamine synthetase
GSH	reduced glutathione
LDH	lactate dehydrogenase
MEOS	microsomal ethanol oxidizing system
M_r	molecular weight
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
OTC	ornithine transcarbamylase
P5C	(D,L) Δ^1-pyrroline-5-carboxylate/carboxylic acid
P5CDh	Δ^1-pyrroline-5-carboxylate dehydrogenase
P5CR	Δ^1-pyrroline-5-carboxylate reductase
PCA	perchloric acid
PEPCK	phosphoenolpyruvate carboxykinase
PO	proline oxidase
PP	periportal
PV	perivenous (centrilobular)
TCA	trichloroacetic acid
TEA	triethanolamine
THV	terminal hepatic venule (central vein)

1 Introduction

1.1 Hepatic Zonation

1.1.1 Historical Account of Liver Zonation

More than a century has passed since Beale first described the heterogeneous contribution of different hepatocytes to bile secretion and to the deposition of "oil" [43]; since that time, researchers have investigated many facets of liver microstructure in hopes of understanding the interactions between structure and function of the liver. In a 1955 paper on liver morphology, Hans Elias[36], describes his efforts to construct a filmstrip illustrating liver architecture. Through the discussion, which spans approximately 50 years of research, the history of our understanding of liver morphology unfolds. The reader is introduced to the observations of Stöhr-Schultze (opposing cells within a "*liver cord*" were arranged on equal levels), Braus (opposing cells could be arranged on alternate levels), Zimmerman (Kupffer cells may bridge sinusoids by long processes) and the many other researchers who helped shape our knowledge of liver structure. Morphological and enzymological studies continued throughout the 1950s and 1960s[149], and proceeded to demonstrate that the liver was not a homogeneous mass but rather composed of cells which, to the untrained observer, were similar but actually were morphologically, biochemically and functionally (with respect to transport and metabolism) diverse[2]. Using Rappaport's acinar model as the basis of their research, Jungermann, Katz and Sasse[83] further defined the metabolic heterogeneity of the liver architecture and

the functional significance of this heterogeneity (for complete reviews see references [43;81;82;87]).

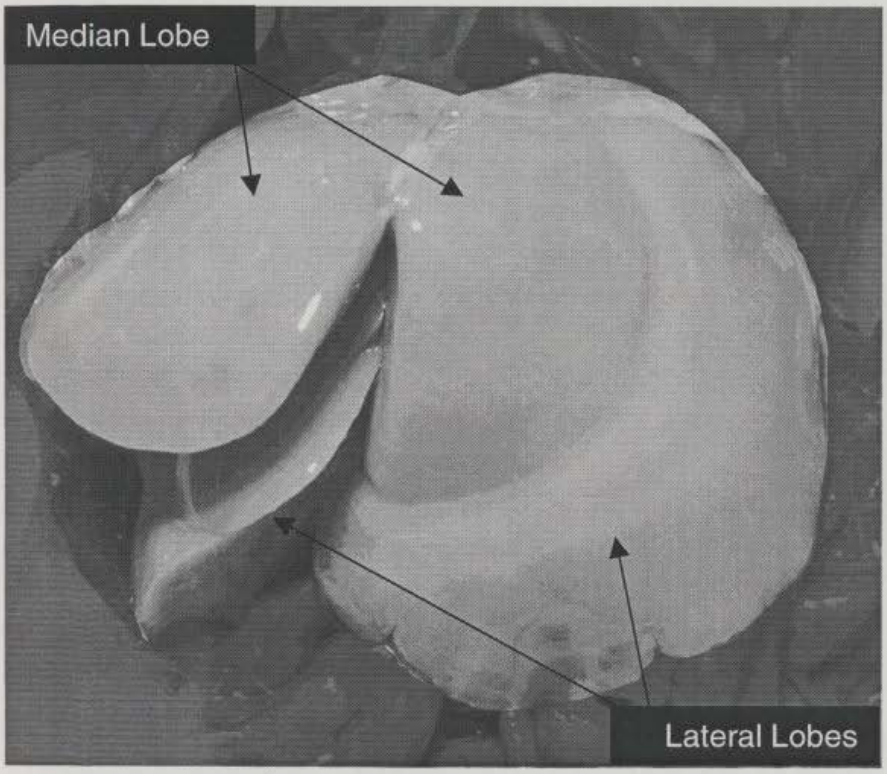
1.1.1.1 Ultrastructure of the Liver

Today, a clearer picture of the cellular composition of the liver and the submicroscopic structures of the various cell types has been achieved. The largest solid organ of the body, the liver constitutes approximately 2% to 5% of human adult body weight and 5% in the neonate. The (rat) liver is composed of four lobes (Figure 1.1), the median lobe, right and left lateral lobes and the caudate lobe. The majority of the liver (~ 70%) is comprised of the median and left lateral lobes and a partial hepatectomy refers to their removal. The lobes are bound together by peritoneal folds which aid in the suspension of the liver in the abdominal cavity[183]. The hepatic artery (about 20% of flow) and portal vein (about 80% of flow)[82] provide a dual afferent blood supply to the liver, respectively providing nutrition and facilitating exposure of absorbed nutrients and toxins to liver cells. Efferent blood is collected in three major hepatic veins (right, middle and left) and eventually empties into the inferior vena cava to then return to the systemic circulation[2].

The immense vasculature system of the liver endows it with a superior capacity to transport a large variety of endobiotics and xenobiotics, including amino acids, carbohydrates, lipids and vitamins, as well as toxins and other components of the afferent blood supply; the liver then, plays a central role in the maintenance of whole body homeostasis. Following transport, the liver is

Figure 1.1 Gross Ultrastructure of the Rat Liver.

Shown are the median and lateral lobes; the caudate lobe is hidden underneath the lateral lobes.


Median Lobe
Lateral Lobes

challenged with storage, metabolic conversion, biotransformation, and the subsequent release of these components into blood and bile.

1.1.1.2 Microstructure and Cellular Composition of the Liver

The liver is composed primarily of "parenchymal cells" otherwise known as hepatocytes (Table 1-1) which constitute 60-65% of the total cells in the liver, and approximately 80% of the organ volume[12;43]. Hepatocyte morphology is typically not dissimilar to other eukaryotic cells. In three dimensions, hepatocytes are arranged in plates that anastomose with another. Parenchymal cells are polygonal in shape and may be in contact with the sinusoids (sinusoidal face) or neighbouring hepatocytes (lateral faces). Bile canaliculi arise from modified sections of the lateral face. The sinusoidal face has microvilli which may project sparsely into the bile canaliculi.

Large, usually spherical nuclei of varying sizes are easily seen under the light microscope. Parenchymal cells of the fetus or newborn are chiefly mononucleate and diploid, while adults may exhibit both polyploid nuclei and multinucleate cells[160]. The cytosol contains soluble proteins, RNA, and enzymes that comprise about 25% of the total cell protein[2]. Mitochondria, which constitute ~20% of the cytoplasmic volume, vary in size and shape. Periportal hepatocytes in the rat have mitochondria which are shorter and larger, and thus have a greater mean volume compared to mitochondria in the perivenous hepatocytes[160].

Four main types of nonparenchymal cells (Endothelial cells, Kupffer cells, Ito and Pit cells) complement the hepatocyte populations of the liver in its capacity as a major metabolic, endocrine, exocrine, and defensive organ of the body.

Endothelial cells having long processes known as fenestrae, yet lacking a basement membrane, form the basis of the sinusoidal walls. The fenestrae form a physical barrier, allowing macromolecules such as plasma proteins (in the space of Disse*), to gain direct access to hepatocytes, but not permitting large macromolecular components, such as blood cells, large chylomicra, viruses and bacteria to contact the parenchymal cells[2:2:82].

Resident macrophages, known as Kupffer cells, are anchored to the endothelial cells (on the luminal surface) via long cytoplasmic processes. Highly variable in their shape, but mainly stellate, Kupffer cells account for about 10% of the liver cell population but about 80% to 90% of the total population of fixed tissue macrophages in the entire body[2]. Kupffer cells are found in all regions of the liver, but appear to be slightly larger and more concentrated in the periportal region, a characteristic which may be explained by greater concentrations of potential immunogenic material in the region of afferent blood flow.

Ito cells are known by several names including fat-storing cells, perisinusoidal cells, parasinusoidal cells, stellate cells and lipocytes. Situated in

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* The space of Disse refers to the small space between the endothelial cells and the hepatocytes. Endothelial cells and Kupffer cells are in close contact with the bloodstream, but the parenchymal cells are in contact with the plasma only *via* the space of Disse.[82]

Table 1.1 Cellular Composition and Heterogeneity Along the Sinusoid

Cell Type	Cell Number (%)	Parenchymal Volume (%)	Lobular Heterogeneity
Hepatocytes	60-65	77.8	—
Endothelial Cells	15-20	2.8	PV: larger with increasing porosity
Kupffer Cells	8-12	2.1	PP: more numerous PV: increased phagocytic activity
Ito cells	3-8	1.4	PV: slightly predominant
Pit Cells	< 2	—	Not known
Intercellular spaces	- - -	15.9	- - -

Adapted from Blouin et al.[15].

the space of Disse, behind the endothelial cells, these cells are long-lived with low proliferative activity in a normal, healthy liver. Conversely, in a chronically diseased liver, the quiescent phenotype is no longer present, rather the cells proliferate actively and they acquire the features of myofibroblasts[2;82]. Ito cells have several roles including retinoid turnover and extracellular matrix development.

The Pit cells are large granular lymphocytes, which exhibit natural killer activity. Located on the endothelial lining, Pit cells are ideally located to play a defensive role against viral infection and tumor metastasis[2].

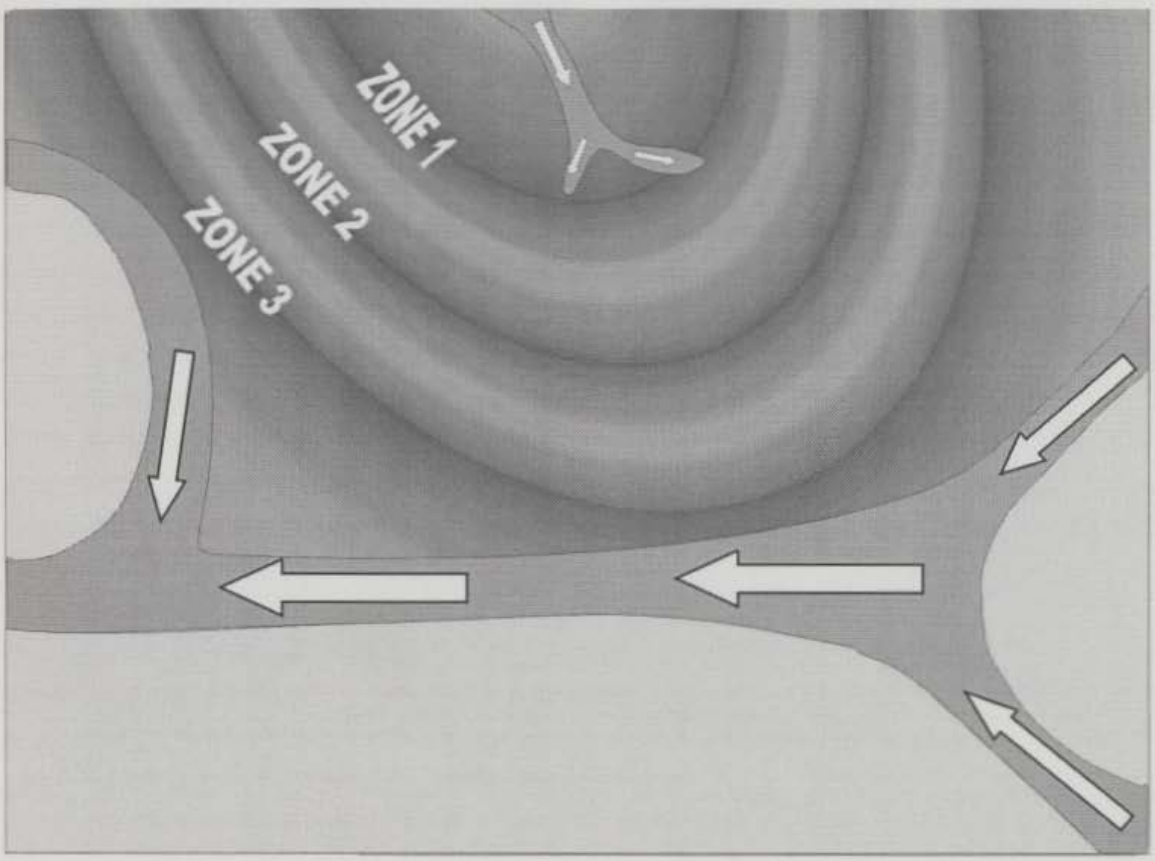
1.2 The Structural and Functional Unit of the Liver – The Acinus

The *hepatic acinus* (Figure 1.2), as described by Rappaport, is comprised of a three dimensional arrangement of a small group (~ 20 – 25) of liver cells nourished by a vascular system including a terminal portal venule and a hepatic arteriole. These vascular components and the bile ductule comprise the portal triad. Liver cells are grouped around sinusoids that drain into terminal hepatic venules. Rappaport's model holds that blood flows (in a pulsatile fashion) from the portal triad at the centre of the functional unit, to the peripheral cells around the central vein[149].

Beginning at a centralized portal triad, portal venous and arterial blood flows in a unidirectional fashion to the peripheral central vein. This unidirectional flow initiates the formation of absorptive and metabolic gradients for most

Figure 1.2 Rappaport's Hepatic Acinus.

Shown are the three zones arranged from the afferent vessels (portal vein and hepatic arteriole (Zone 1) to the terminal hepatic venules (Zone 3), which collect blood from adjacent acini. Adapted from Rappaport et al. (1966)[149].


ZONE 1
ZONE 2
ZONE 3

solutes. Cells in Rappaport's *Zone 1* receive blood with the highest concentration of oxygen and solutes. Zone 3 hepatocytes receive blood that has been modified by *Zone 1* hepatocyte absorption and secretion. Although these zones are referred to as distinct entities, there are no clear-cut boundaries between zones[2]. One contemporary trend describes populations of hepatocytes as either proximal or distal to a particular fixed point of origin, such as the terminal hepatic venule[82].

1.3 Metabolic Zonation

Extensive research over the last century has revealed that the liver parenchyma demonstrate a heterogeneous distribution of enzyme activities[43]. Jungermann and Sasse[83] understood that the sum of relatively small quantitative differences, resulted in the metabolic specialization of cells within the various zones of the liver. Enzymes of opposing metabolic processes, like gluconeogenesis and glycolysis, may be active across the entire acinus, but may be enriched in a particular zone. Certain regions of the acinus are thus better equipped to perform particular functions. Periportal hepatocytes are functionally better suited for glucose release, whereas the perivenous hepatocytes are better equipped for glucose uptake[84]. The maintenance of glucose homeostasis then, is aided in part by the zonation of the processes involved in glucose metabolism.

For the purposes of this thesis, I would like to present brief accounts of zonation pertaining to gluconeogenesis from amino acids, metabolism of the

glutamate family of amino acids and xenobiotic metabolism. For more details on particular aspects of these and other specific types of metabolic zonation, there are many reviews available (e.g., Arias et al.[2],Jungermann and Katz[81], Haussinger[53], Gebhardt[43], Katz[87], Haussinger, Lamers and Moorman[66], and Oinonen and Lindros[133]).

1.3.1 Amino Acid and Ammonia Metabolism

1.3.1.1 Zonation of Gluconeogenesis from Amino Acids

There is no underlying rule when it comes to the zonal characteristics of amino acid metabolism and in fact, relatively little information is available regarding the actual acinar heterogeneity of amino acid metabolism. It appears that the periportal zone is characterized by a high capacity for uptake and catabolism of many amino acids[66:83] with the exception of glutamate[64,21] arginine[132] and proline (this thesis). Gluconeogenesis from amino acids is supported in the periportal zone by the action of several aminotransferases e.g. alanine aminotransferase, aspartate aminotransferase (EC 2.6.1.1) and tyrosine aminotransferase (EC 2.6.1.5). Ornithine aminotransferase (EC 2.6.1.13) on the other hand has been localized to a discrete perivenous zone[95] using *in situ* hybridization. The activities of the gluconeogenic enzymes such as glucose-6-phosphatase(EC 3.1.3.9)[17], fructose 1,6-bisphosphatase(EC 4.1.2.13)[99], and

phosphoenolpyruvate carboxykinase(4.1.1.49)[116] have been localized to the periportal zone.

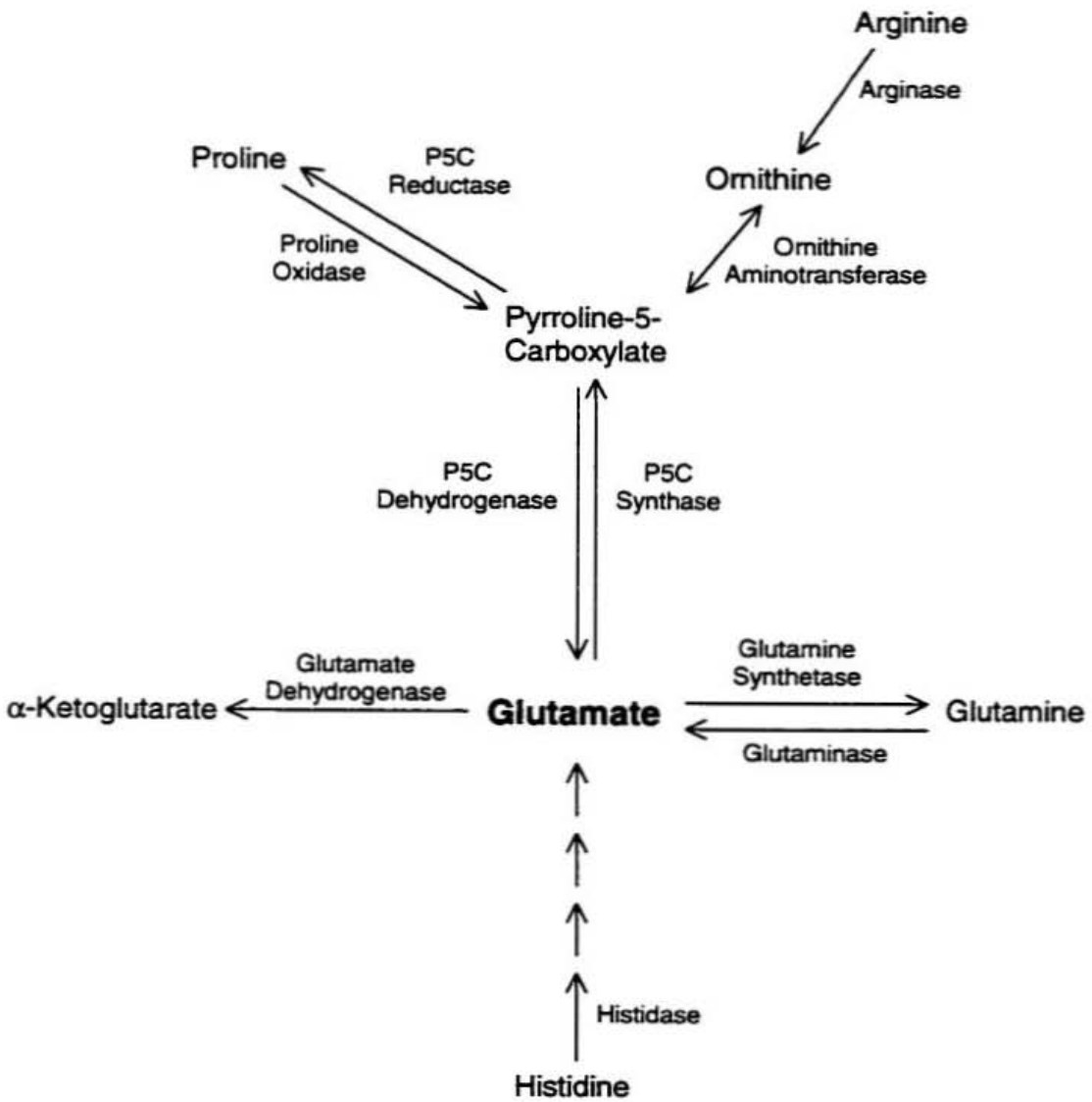
1.3.1.2 Zonation of Metabolism of the Glutamate Family of Amino Acids

The glutamate family of amino acids includes proline, arginine (ornithine), histidine, glutamine and of course glutamate (Figure 1.3). The carbon skeletons of these five amino acids enter the citric acid cycle as α-ketoglutarate, thus they share a common catabolic end product. The catabolism of this family of amino acids in the liver is not localized to one acinar zone. The degradation of arginine and ornithine is catalyzed by the perivenous localized enzyme ornithine aminotransferase[95]. Glutamate dehydrogenase activity has been shown histochemically to increase toward the perivenous zone[110]; the enzyme protein demonstrated a u-shaped gradient by immunohistochemical techniques[97]. Glutaminase activity is found exclusively in the periportal zone[62;182]. Histidine ammonia-lyase (histidase) catalyzes the first step in the oxidative degradation of histidine to glutamate. This enzyme was shown to be enriched in the periportal zone of the acinus using an anti-(rat histidase)[158].

1.3.1.2.1 Amino Acid Transport

The uptake and release of amino acids by the liver is of great importance with respect to hepatic metabolism and maintenance of general metabolic

Figure 1.3 The Glutamate Family of Amino Acids.


Arginine
Arginase
Proline
P5C
Reductase
Ornithine
Proline
Oxidase
Ornithine
Aminotransferase
Pyrroline-5-
Carboxylate
P5C
Dehydrogenase
P5C
Synthase
Glutamate
Dehydrogenase
Glutamine
Synthetase
α-Ketoglutarate
Glutamate
Glutamine
Glutaminase
Histidase
Histidine

homeostasis in the entire animal. For example, amino acid supply may be a direct regulatory process controlling amino acid-driven gluconeogenesis. Transport of physiological concentrations of alanine (0.2 to 0.5 mM) into isolated rat hepatocytes has been reported to be the rate limiting step in alanine metabolism, during both fed and fasted metabolic states[43]. Kilberg[88]describes in detail, characteristics of the various amino acid transport systems seen in isolated rat hepatocytes. These include System A (most neutral amino acids), System ASC (neutral amino acids with small side chains, especially those with an –OH or –SH group), Anionic (2 systems; dicarboxylic amino acids with net –1 charge), System Gly (glycine and sarcosine), System L1 and L2 (neutral amino acids with large branched, or aromatic side chains), and System N ($(Na^+$-dependent) histidine, glutamine, and asparagine). Moseley (1996)[129] further describes the Na^+-independent transport of glutamine in hepatocytes, designated system n. Kilberg's discussion[88] however, does not incorporate any zonal characteristics of the various transporters. The full implication of any regulatory aspect, described in conjunction with a specific transport system then, can not be appreciated in the context of transport effects on metabolic zonation.

Perivenous hepatocytes, isolated by digitonin-collagenase perfusion, were shown to be enriched in transport system A, only after induction with glucagon and dexamethasone. No difference was seen under control conditions, or after treatment with dexamethasone only[21]. A low K_m transport system for cysteine was localized to the perivenous zone of the hepatic acinus following

administration of [^{35}S]cysteine via either single-pass antegrade or retrograde perfusion[156]. The predominant perivenous uptake of cysteine, seen at physiological concentrations (≤10 μM) decreased to uniform distribution at supraphysiologic high cysteine concentrations (≥1000 μM). These results suggest that a low affinity, high capacity transport system is homogeneously distributed in the acinus and most likely represents the ASC system. Heterogeneous hepatic transport of the other amino acids has not been extensively studied, with the notable exception of glutamine and glutamate.

1.3.1.2.2 Glutamine and Glutamate Transport

Heterogeneous transport characteristics of glutamine and glutamate are more pronounced. Indeed, a strong linear correlation[21] ($r = 0.88$; $p < 0.001$) with glutamate uptake and the relative glutamine synthetase activity links transport capacity with the heterogeneity of ammonia metabolism. Three important transporters are involved in the uptake and release of glutamine and glutamate across the liver acinus.

System G^- is a Na^+ -dependent, high affinity transport process for L-glutamate[21;129]. Glutamate transport by this system appears to be preferentially localized to the canalicular membrane[129], but has been localized to the sinusoids when induced by streptozotocin-induced diabetes[107] and dexamethasone[108].

glutamate uptake. The transport of glutamate in perfused rat liver is significantly decreased following CCl_4-induced necrosis of perivenous hepatocytes[64]. Haussinger[64] demonstrated that after destruction of the perivenous region, $^{14}CO_2$ production from [1-^{14}C]glutamate was decreased by 70%, in agreement with a perivenous glutamate uptake. Increased glutamate uptake due to dexamethasone induction was selectively diminished in primary cultures of hepatocytes from CCl_4-treated rats[44]. Finally, enriched sub-populations of perivenous cells isolated using digitonin-collagenase perfusion demonstrate greater Na^+ -dependent glutamate uptake than periportal sub-populations[21]. At least three glutamate transporter subtypes have been isolated, but only the EAAC1 subtype has been localized (at the mRNA level) to liver[85]. This high affinity glutamate transporter appears to transport one glutamate ion into the cell coupled to the co-transport of two Na^+ ions, the counter-transport of one K^+ ion, and either the cotransport of one H^+ or the counter-transport of an OH^- [86]. The EAAC1 transporter and system G* are thought to represent the same transport system[129].

Glutamine, histidine and asparagine are taken up by hepatocytes via the Na^+ - dependent transport system N. Gebhardt and Kleeman[48] demonstrated that system N activity could be induced by insulin, glucagon and glucocorticoids in primary cultures of hepatocytes. Sodium-dependent transport of glutamine,

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* System G* is more correctly designated System X^-_{AG} as it represents the transport process for DL-aspartate and L-glutamate.

histidine and asparagine via system N revealed no detectable heterogeneity, even after hormonal induction with glucagon and dexamethasone[21].

Glutamine efflux from hepatocytes has been linked to a Na^+ -independent transport system, designated system n[129]. Using isolated hepatocytes, Fafournoux et al.[38]described a sodium-independent transport system characterized by saturable kinetic parameters suggestive of a facilitated diffusion process. Pacitti and colleagues[135] utilized hepatic (sinusoidal) membrane vesicles to further characterize the sodium-independent transport of glutamine in rat liver. Their results corroborated the findings of Fafournoux et al.[38] suggesting that the Na^+ -independent system n had a high K_m and low V_{max} appropriate for release of glutamine from the cell. No description of the zonal characteristics for system n have been described.

Systems G^-, N, and n are thus involved in the intercellular cycling of glutamine and glutamate (Figure 1.3). Glutamine is taken up by the periportal cells via the high affinity, Na^+ -dependent system N and converted to ammonia by glutaminase. Glutamate is selectively taken up in the perivenous region by the Na^+ -dependent system G^-. Glutamine synthetase combines the incorporated glutamate with scavenged ammonia to form glutamine. The glutamine can then be released back into systemic circulation via the Na^+ -independent system n.

1.3.1.2.3 Glutamine and Ammonia Metabolism

Afferent (portal) blood flow delivers large amounts of glutamine and ammonia to the liver. Glutaminase (EC 3.5.1.2) activity, predominant in the early

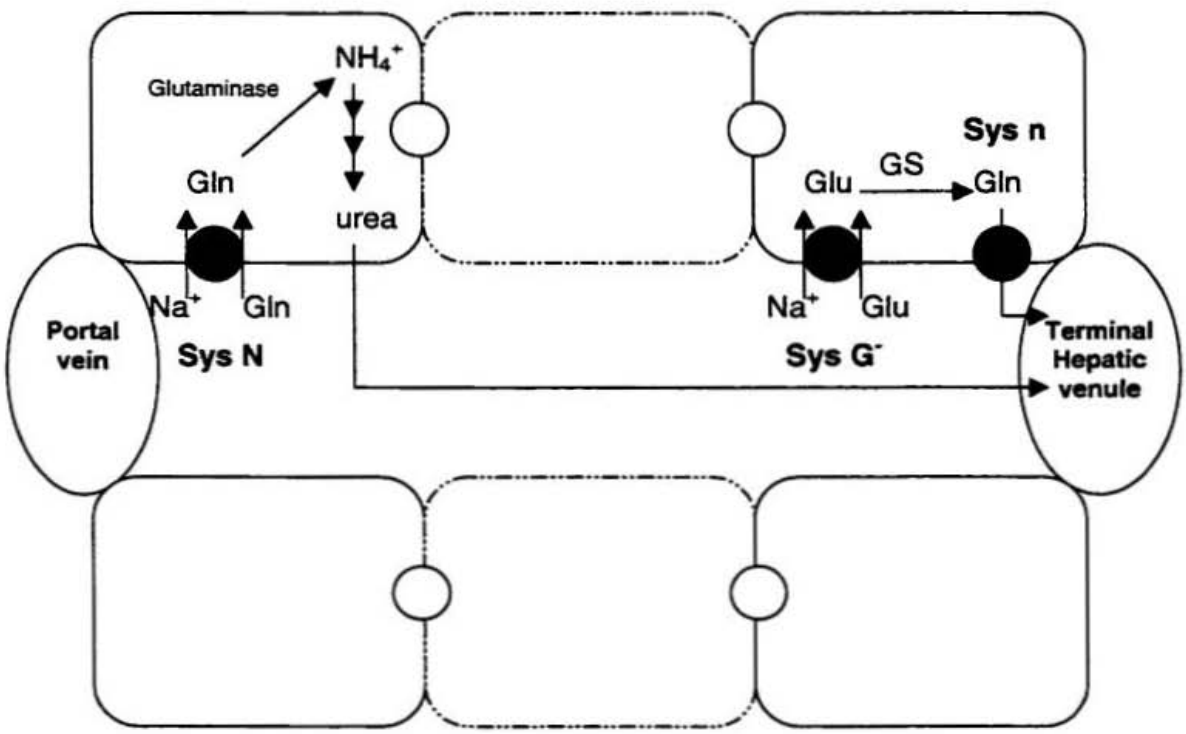
periportal region, as demonstrated by Haussinger[62] using $^{14}CO_2$ production from [1-^{14}C]glutamine and later confirmed by Watford[182] using digitonin-collagenase perfusion, provides substrates for gluconeogenesis and urea synthesis. Co-localization of the activities of glutaminase (which is activated by ammonia and hence stimulated at times of ammonia excess), some amino acid catabolizing enzymes and urea cycle enzymes, means that the periportal hepatocytes utilize some of the glutamine and most of the ammonia[181]present in the afferent blood flow in the periportal region. Thus the glutamine and ammonia are metabolized to urea by a high capacity/low affinity system. The relatively high K_m of carbamoylphosphate synthetase I (CPS I) for ammonia permits some ammonia to reach the perivenous cells. Glutamine synthetase, localized exclusively in those hepatocytes immediately surrounding the central vein[65], scavenges this ammonia and combines it with glutamate to generate glutamine. Potential source(s) of glutamate include intracellular proteolysis, synthesis from glucose or lactate [181], uptake form circulation, and synthesis from proline or arginine[132]. These sources can not account for the quantity of glutamate utilized and so, determination of alternate (or adequate) sources of glutamate remains an active area of research.

1.3.2 Xenobiotic Metabolism

The heterogeneous pattern of liver damage due to toxic insult has been recognized for more than a century. The underlying mechanisms which cause

Figure 1.4 Intercellular Glutamine Cycle.

Glutamine (Gln) is taken up by the periportal cells via the high affinity, Na^+ -dependent system N and converted to ammonia by glutaminase. Glutamate (Glu) is selectively taken up in the perivenous region by the Na^+ -dependent system G^-. Glutamine synthetase (GS) combines the incorporated glutamate with scavenged ammonia to form glutamine. The glutamine can then be released back into systemic circulation via the Na^+ -independent system n.


Glutaminase
NH_4^+
Gln
urea
Sys n
Glu
GS
Gln
Na^+
Gln
Sys N
Na^+
Glu
Sys G$^-$
Portal vein
Terminal Hepatic venule

this damage are not completely understood, but major advances have been made by studies on zonation of xenobiotic metabolism.

1.3.2.1 Hepatotoxins and Regiospecificity of Hepatotoxic Damage

Hepatotoxins are ubiquitous in nature and exposure can be encountered under a variety of conditions. Similarly, the nature of hepatotoxic agents and the types of hepatic injury derived from exposure are very diverse. The most important factor that determines the susceptibility of the liver to damage is the liver's role in toxin metabolism. It is often the case, that metabolism of an inert, foreign compound generates metabolites which are toxic and exert damage to the liver[43;174], i.e. biotransformation is often required to produce a toxic event.

Intrinsic hepatotoxins, those which generate predictable hepatotoxicity characterized by high incidence, dose-dependence, experimental reproducibility, and short latency period, typically induce toxicity within specific zones of the liver. Toxicity may be manifested as steatosis (disruption of normal processes of triglyceride synthesis and transfer) or necrosis (disruption of normal cellular processes resulting in cell death) of hepatocytes. Necrosis derived from particular hepatotoxins may be zonal, massive or diffuse[174].

The regiospecific toxicity of hepatotoxins is most likely a result of zonation of the steps involved in their metabolism, uptake, activation, conjugation, and excretion. It is probable that upsetting the balance of these various processes would lead to an accumulation of reactive intermediates in a particular region of

the acinus[43]. These accumulated intermediates may very well initiate necrotic events.

1.3.2.2 Detoxification, Conjugation and Protection

The CYP (cytochrome P-450) superfamily of enzymes metabolizes a wide variety of structurally different xenobiotics including drugs, alcohols, and aromatic organic compounds. These enzymes also contribute to the modification and elimination of endogenous steroids, bile acids, fatty acids, leukotrienes, prostaglandins and biogenic amines, most likely the role which nature had intended. A little more than 20 years ago, Gooding et al.[50] noted that parenchymal cells in the perivenous region demonstrated a two-fold enrichment of the red CYP protein. Total cytochrome P450 content in the liver shows an increasing periportal – perivenous gradient described by several researchers[43]. Many of the enzymes associated with various CYP isozymes are also localized to the perivenous region: the enzymes responsible for electron transfer in the mono-oxygenase system, NADPH:CYP reductase and cytochrome b_5[133;173], and also 7-ethoxycoumarin-*O*-demethylase, 7-ethoxyresorufin-*O*-deethylase, aniline-*p*-hydroxylase, benzphetamine-*N*-demethylase and microsomal ethanol oxidation (MEOS)[43]. While such enzymes are predominantly perivenous, induction by a variety of chemicals and hormones can elicit an increase in the mid-zonal and periportal activities. It is also important to note that the activities of the NADPH-generating cytosolic enzymes, glucose-6-phosphate dehydrogenase, 6-

phosphogluconate dehydrogenase, malic enzyme and isocitrate dehydrogenase are also higher in the perivenous region[81]. The enzymes of the monooxygenase system (mentioned above) are thus supplied with cofactor necessary for their continued activity. While total cytochrome P450 content is predominantly perivenous, different isozymes of cytochrome P450 show different zonal patterns (this point is made very clear by the summary of research provided in Table 3 of Oinonen and Lindros[133]).

Glucuronidation and sulfation (the capacity of xenobiotic conjugation with glucuronic acid and sulfuric acid, respectively) processes appear to be oppositely zoned in the liver[81;173]. Total UDP-glucuronyl transferase (EC 2.4.1.17) activity was higher in perivenous cells while the activity of sulfotransferase (EC 2.8.2.1) was enriched in the periportal zone[43]. Glutathione is present across the entire acinus; some researchers contend intracellular glutathione concentration is significantly higher in periportal hepatocytes[43], enabling these cells to be more effective in the protective pathways of the liver. The activities of glutathione dependent enzymes are heterogeneously localized. Glutathione-S-transferase (EC 2.5.1.18) is the catalyst responsible for many conjugation reactions in the perivenous region, while glutathione-peroxidase (EC 1.11.1.9) is responsible for reduction of many peroxides formed during the detoxification of many foreign compounds in the periportal zone.

As blood, carrying a foreign compound, traverses the acinus, enzymes of the CYP family become activated. Biotransformation of the compound into a

variety of intermediates, both harmless and toxic, activates the enzymes of the protective metabolic pathway. Conjugation of the toxic intermediates with glutathione, where possible, prevents cellular damage and hence disruption to normal metabolic function. Parenchymal cells in the periportal region have a significantly lower concentration of enzymes of the cytochrome P450 family, and a higher concentration of glutathione. Thus the periportal zone is more aptly suited to produce low quantities of reactive electrophiles, and yet able to protect against greater quantities of these toxic intermediates than are cells in the perivenous region which possess a higher capacity to produce electrophiles (due to the higher concentration of CYP enzymes), but decreased capacity to effectively deal with them. Perivenous hepatocytes are thus more susceptible to hepatotoxic damage, and this is quite possibly why more hepatotoxins appear to exert a selective toxicity in this zone. It is not the nature of the hepatotoxin, but rather the inherent metabolic environment of the hepatocyte population which determines the site of damage exerted by a toxin or toxic intermediate.

1.3.3 Molecular Basis of Zonation

Zonation can not be described in terms of a consistent pattern for all enzymes in the liver. Instead, different types of zonation occur for particular enzymes. The factors which determine the type of zonation a particular gene or enzyme shows are diverse and change with development and metabolic status of the liver. The heterogeneous nature of parenchymal cells adds a complicating

factor when addressing the question of regulation of gene expression. Exactly what facet of the dynamic environment of the hepatocyte directs expression of a particular phenotype is unclear.

The heterogeneous expression of the hepatocyte genome is most likely affected by physiological factors associated with the microstructure of the liver itself. Periportal to perivenous gradients of oxygen, substrates, hormones and different sympathetic and parasympathetic innervation quite possibly direct development of the heterogeneous liver unit. However, one would presume specific controls permit particular genes to be expressed in either periportal or perivenous cells, rather than expression being directed solely by environmental conditions of the cell.

1.3.3.1 Zonal Patterns of Enzyme Activity

Metabolic activity across the liver acinus is characterized by two "types" of zonation: "gradient-type" and "strict-" or "compartment-type". The best known example of strict- or compartment-type zonation is that of glutamine synthesis. Glutamine synthetase (EC 6.3.1.2) activity is restricted to the most distal hepatocytes surrounding the terminal hepatic venules, a region which is physically one to three cells thick immediately circumscribing the terminal hepatic venule. The activity of hydroxymethylglutaryl-CoA reductase[166] and of glutaminase[126] is also described as compartment-type; approximately 20% of the tissue around the terminal portal vein contains activity while the remaining cells extending to the hepatic venule show no activity. Most metabolic activity in the

liver however, can be characterized as gradient-type: metabolic activity is expressed in all hepatocytes, but predominates in the distal periportal region, decreasing from the portal vein to the central vein (e.g. tyrosine aminotransferase[184]), or in the reciprocal fashion, predominating in the perivenous region, but decreasing toward the periportal region (e.g. glucokinase[99]). Other enzymes such as glucagon-activated adenylate cyclase[188] and cGMP-activated cAMP phosphodiesterase[155] show no differences in activity across the zones.

Gradient-type zonation can be either dynamic or stable; by definition compartment-type zonation is stable. *Dynamic* expression of mRNA zonation is characterized by adaptive changes in the gradient expression of the gene. For example, if a relative increase in enzymic activity in one zone is noted in response to changes in the metabolic status of the environment, the gradient will become more steep. The expression of PEPCK[16] has been shown to increase as the protein content of the liver increases, thus demonstrating dynamic gene expression. Stable-type zonation of gene expression does not show these adaptive changes; an increase in enzyme activity may be noted but still only in the same zone. OAT[16], CPS I[123] and GS[47] are examples of the stable-type of zonation.

1.3.3.2 Environmental Influences on Zonation

The multitude of blood-borne signals which travel through the liver present a logical means of directing gene expression. These signals should form gradients as they move across the acinus and are acted upon by the different hepatocyte populations. The concentration of a particular substance may decrease as it moves from the periportal zone to the perivenous zone, thus hepatocytes in the different zones are exposed to sometimes very different concentrations of the substance. Substances such as oxygen, hormones, neurotransmitters, amino acids, fatty acids, as well as intermediate metabolites and secreted proteins demonstrate variable concentrations across the acinus and do act as signaling molecules[28]. Gupta et al. (1999) demonstrated that the microenvironment of transplanted dipeptidyl peptidase IV-positive (DPPIV+) hepatocytes in regenerating livers of DPPIV- deficient F344 rats directed gene expression of dipeptidyl peptidase IV[55].

One of strongest demonstrations of environmental influences on zonation occurs during the development of the liver. Prior to birth, the developing liver is supplied with well oxygenated blood from the placenta, but postpartum, the major blood flow to the liver is the less oxygenated splanchnic blood supply. The mother supplies a high-carbohydrate diet to the neonate, which is changed to a low-carbohydrate, high lipid diet as processed by the liver, via the gut, from a milk supply after birth[144]. Morphology of the neonatal rat liver does not indicate acinar units, but rather appears uniform. Contrasting the actual formation of acinar zones (with respect to metabolism) which arise 4-5 days postpartum[100],

the definition of the acinar zones appears to be programmed in utero. Burns et al[22] showed that the livers of offspring of rat dams fed a low protein diet demonstrated decreased glucose (produced from lactate in the periportal zone) uptake and glucokinase activity in the perivenous zone. As well, the livers of these animals showed structural differences from controls, including increased lobular volume, but decreased overall number of lobules (functional units).

1.3.4 Differentiation as a Determinant of Zonation

Zonal expression patterns of mRNA may be due to different rates of transcription, mRNA degradation, mRNA translation or protein synthesis / degradation. Localization of equivalent amounts of both protein and mRNA in the same zone indicates either a pretranslational regulation of mRNA formation or degradation[82] (e.g. α-fetoprotein[43], glutamine synthetase[46;95] and carbamoylphosphate synthetase[125]). Translational or posttranslational regulation of enzyme synthesis explains unequal distribution of protein and signal; i.e. a uniform distribution of mRNA across the acinus but a zonal pattern of protein localization[82](e.g. pyruvate kinase L and α1-antitrypsin demonstrate a perivenous enrichment of protein but uniform acinar mRNA distribution[43]).

The "differentiation" hypothesis[55] proposes that cells in the periportal zone are merely less differentiated progenitor cells followed by the highly differentiated perivenous cells demonstrating specialized functions. Periportal hepatocytes in

culture can be induced to express perivenous glutamine synthetase mRNA if exposed to appropriate stimuli[43]. Thus while proponents of this theory may state that perivenous cells are more highly differentiated, the less highly differentiated cells of the periportal zone are still capable of the same metabolic functions of cells in the perivenous zone.

The "streaming liver"[144] theory proposes that the stem cells of hepatocytes reside in the periportal region and upon mitosis they migrate away from the periportal region. Thus cells in the perivenous zone are older and more highly diferentiated[101]. Injection of ^{3}H-thymidine followed by autoradiographic analysis demonstrated that the label was most highly concentrated in the periportal zone and gradually decreased towards the perivenous. This indicated that the entire hepatocyte plate migrated towards the terminal hepatic venule, where individual cells eventually die[144]. However, when dividing cells are stably marked with a reporter gene, continued streaming toward the central vein was not observed, even in long running experiments.

Our understanding of the regulation of position-specific expression of liver enzymes is increasing as knowledge about the structures of these genes increases. Many of the genes have been cloned and characterized (glutamine synthetase[177] and glutaminase[29]). Lie-Venema et al. (1995)[104] have described spatio-temporal control elements in the 5'-enhancer region of the glutamine synthetase gene. Using two classes of transgenic mice and a chloramphenicol acetyltransferase (CAT) reporter gene construct, these authors demonstrated

that the sequences within the upstream regulatory region of the GS gene direct its expression to the hepatocytes surrounding the central vein. A more detailed analysis of the regulatory capacity of this upstream element has been performed. Using the CAT reporter system under the control of the upstream regulatory region of the GS gene, ribonuclease-protection assays and in situ hybridization, Lie-Venema[103] was able to demonstrate that the upstream regulatory region was involved in the level and topography of GS gene expression in certain tissues (liver, and skeletal muscle for example), but that another element in this region appears to direct expression in other organs (e.g. a silencer element in the regulatory region controls GS expression in brain[5]).

The pattern of gene expression is strongly affected by the concentration gradient of components in the hepatic blood flow and transcriptional elements inherent in the gene construct. However, as techniques for the investigation of hepatocyte heterogeneity become increasingly sensitive and diverse, the apparent contribution of those factors which have become discounted, hepatocyte age, nervous innervation, interaction of hepatocytes with other cells and matrix components, etc., may need to be re-evaluated.

1.4 Investigating Heterogeneity across the Acinus

1.4.1 Introduction

Appreciation of the heterogeneous nature of liver cells, and the zonal patterns of distribution of these cells throughout the liver, has generated the

necessity for specialized techniques of investigation. Each technique attempts to draw upon a particular characteristic inherent to a specific population of hepatocytes, but, with any specialized technique there are strengths and weaknesses which must be considered when proposing generalized conclusions from the experimental results.

The next few sections offer some brief remarks which highlight some of the specific advantages and drawbacks of the various methods. In particular, the methods employed in the present studies will be discussed in greater depth.

1.4.2 Histology, Histochemistry and Immunohistochemistry

A variety of histological, histochemical and immunohistochemical techniques are available to investigate liver cell heterogeneity. Although it is not easy to discern differences in hepatocytes using standard light microscopical techniques, morphometric analysis of electron micrographs has been utilized to describe structural differences, including mitochondria and smooth endoplasmic reticulum of parenchymal cells located in either perivenous or periportal zones[43].

Histochemistry has proven to be an important tool in the study of metabolic zonation[30;35;72;73;98]. For example, glucose-6-phosphatase activity has been detected primarily in the periportal zone using a cerium-diaminobenzidine method[98].

Immunohistochemical techniques permit the detection of minute quantities of protein in samples. Immunofluorescence and immunoenzymatic techniques

have permitted the detection and localization of a variety of enzymes, e.g. glutamine synthetase in the perivenous zone[46] and carbamoylphosphate synthetase in periportal cells[124]. In fact, the major urea-cycle enzymes have been visualized in rat liver using immunohistochemical techniques[121].The common alkaline phosphatase or peroxidase color reactions are used for detection and thus localization of bound antibodies. In situ hybridization has been used to detect mRNA for a variety of proteins, e.g. mRNA for the HNF4 transcription factor was localized primarily to the perivenous zone[106].

1.4.3 Selective Zonal Necrosis

Over the years an abundance of literature has surfaced concerning a variety of toxins applicable to the study of zone specific liver necrosis. Three particular hepatotoxins, allyl alcohol, bromobenzene and carbon tetrachloride, have been subjected to more intense focus in the last number of years[78]. Leading to circumscribed zonal damage, these particular toxins have been employed as probes for liver cell heterogeneity. Much debate has occurred regarding the actual mechanistic features which lead to the necrotic event for each of these toxins. Lipid peroxidation, superoxide anion generation, mitochondrial dysfunction leading to oxidative stress, covalent bonding of toxic intermediates to macromolecules, and interrupted calcium homeostasis leading to impaired ATP synthesis are some of the mechanistic possibilities proposed to explain hepatocyte necrosis by one or another of these three toxins[78].

1.4.3.1 Carbon Tetrachloride

Carbon tetrachloride (CCl_4) was initially recognized as a hepatotoxin during the mid 1850's[150] when a related chemical, chloroform, was recognized for its own toxic attributes. Both substances were often utilized for their anesthetic properties until the realization of their toxicity became generally appreciated. Subsequently, the proliferation of CCl_4 as an industrial poison became apparent to the medical personnel of the late 19th and early 20th centuries.

Poisoning of the liver with CCl_4 results in two major manifestations: fatty liver and necrosis[44;45;128;150;170]. The impairment of hepatic secretion of triglycerides is thought to be a primary cause of fatty liver after CCl_4 poisoning. Hepatic necrosis due to CCl_4 is primarily observed in the perivenous zone causing temporary alterations in the microcirculation and normal metabolic zonation[42;162]; necrotic areas are visualized as pale discolourings on the liver surface. The perivenous localization of cytochrome P450 dependent enzymes[105] involved in the biotransformation of CCl_4 to a $CCl_3^{\bullet}$ free radical is the most probable reason for the selective zonal necrosis due to this toxin.

1.4.3.2 Bromobenzene

One of the first investigators to employ bromobenzene as a hepatotoxin was Koch-Weser[91]. He was investigating the relationship between depletion of amino acids, especially methionine and cysteine, and hepatic injury. It was

already known that bromobenzene coupled with cysteine to form bromobenzylcysteine, which is then acetylated and excreted as mercapturic acid in the urine. Bromobenzene was therefore used to facilitate the depletion of sulfur amino acids from the body. The perivenous zone in rats subjected to bromobenzene revealed necrosis, while those animals that had had diets supplemented with methionine or cysteine, showed suppressed levels of necrosis when exposed to bromobenzene. Presumably, the greater quantities of methionine or cysteine are able to couple to the bromobenzene, and less bromobenzene is available to be biotransformed to toxic intermediates.

The mechanism(s) behind the necrotic effects of bromobenzene may be attributed to its biotransformation to a number of relatively dangerous epoxides and hepatotoxic aromatic hydrocarbons[18] (see Appendix 6.1 for bromobenzene biotransformation detail). These, and other, authors suggest that the formation of glutathione conjugates corresponds to an increase in lipid peroxidation[18;24]. Other authors have attributed damage to a partial uncoupling of oxidative phosphorylation in mitochondria; while the effect was not irreversible, the onset of these anomalies during the first 3-4 hours after the toxic insult may provide a contributing role in the overall process of cellular necrosis[109]. Necrosis localized to the perivenous region, induced by bromobenzene intoxication, is thought to be "site-directed" due to the zonal characteristics of a variety of drug metabolizing enzymes within the liver unit[18;78].

1.4.3.3 Allyl Alcohol

Derivatives of allyl alcohol have a widespread natural distribution in vegetable components of the human diet and chemically synthesized esters of the alcohol have been used as food flavorings[96]. Human injury by allyl alcohol is a rare event, with only a few industrial toxic effects being reported; especially noted is a severe irritation of mucous membranes about the eyes[3]. Yet, allyl formate and its metabolite, allyl alcohol were shown to cause extensive periportal necrosis almost a century ago[152]. Conversion of allyl alcohol by alcohol dehydrogenase (ADH) (EC 1.1.1.1) to the aldehyde, acrolein (prop-2-enal) (see Appendix 6.2 for allyl alcohol biotransformation detail) had been deemed the most significant contributing factor to the hepatotoxicity of allyl alcohol by many researchers. It was thought that ADH activity was primarily periportal, thus lending support to the notion of zone-specific hepatotoxicity of allyl alcohol being due to the location of the enzyme responsible for its biotransformation to the toxic metabolite. However, Belinsky et al.[9] employed microlight detection of reduced nicotinamide adenine dinucleotide (NADH) fluorescence to demonstrate that allyl alcohol metabolism occurs at a slightly higher rate in the perivenous region. Other reports have shown that ADH is primarily localized to the perivenous, and not the periportal region of the liver acinus[6]. Alternative mechanisms to explain hepatocyte necrosis following allyl alcohol intoxication include depletion of cellular glutathione (the highly reactive α-β-unsaturated aldehyde is an excellent substrate for glutathione-S-transferases)[8;136], alterations in cellular Ca^{2+}

homeostasis caused by loss of soluble and protein bound thiols (secondary to the GSH depletion), and an increase in lipid peroxidation[6;7] (this point is debated in the literature centering on the techniques used). O_2 tension[6] may also be contributing to hepatocyte necrosis following exposure to allyl alcohol.

1.4.4 Perfusion Studies

Liver perfusion offers an extremely powerful means of investigating metabolic heterogeneity and flux across the liver acinus. The unidirectional flow in the liver and a relatively simple surgery (described in Section 2.4.1) allow for easy comparison between antegrade (portal vein – vena cava) and retrograde (vena cava – portal vein) dynamics. For example, Haussinger[64] using $^{14}CO_2$ production from [1-^{14}C]glutamate, demonstrated that while the perfused liver has the ability to simultaneously take up and release glutamate, approximately 60% or more of total glutamate uptake can be accounted for by the glutamine-synthesizing hepatocytes in the perivenous zone.

The perfused liver model retains both structural and functional characteristics closest to *in vivo* conditions. As well this model offers greater control of effects of humoral or neural signals which may interfere and generate secondary effects. The monitoring of O_2 uptake, pH, pressure, concentrations of electrolytes (Ca^{2+}, K^+), and marker enzyme release are all relatively easy to accomplish[134]. There are disadvantages with perfusion studies including the duration of the viable organ and the cost of the experiments. The perfused liver is viable for only 2-4 hours depending upon conditions. The cost of the chemicals

and equipment involved per liver may be considerable, especially when one relates one data point to one liver.

1.4.5 Isolation of Liver Cells using the Two-Step Collagenase Procedure

The isolated hepatocyte procedure was introduced by Howard et al.[75], and modified by Berry and Friend[13] to produce high yields of viable cells. Procedures for isolating organelles were well established before the isolation of intact cells from solid organs was even considered feasible. Ignorance of the biochemical properties of those components involved in cellular adhesion was the main stumbling block to achieving a successful protocol for the separation and isolation of hepatocytes.

Histological assessment of the microstructure of the liver indicated the intense permeation of the entire organ by fibroconnective tissue composed chiefly of collagenous fibres[160]. This permeation is seen as a fine meshwork around the sinusoids and perisinusoidal spaces. The adhesive quality of cells was explained with the advent of the electron microscope. Cellular junctions have since been elucidated and the definition of junctional complexes include the tight junction (zonula occludens), the intermediate junction (zonula adherens), the desmosome and the gap (communicating) junction[12].

The importance of Ca^{2+} for cell adhesion was demonstrated by Ringer toward the end of the 19th century[153]. He showed that distilled water was able to loosen the "cement" that binds cells together, and that this effect was prevented

by the bicarbonate of lime. The role for calcium was shortly thereafter confirmed and it is now known that the function of cell adhesion molecules is dependent upon the presence of Ca^{2+} [49].

Initial attempts at preparing hepatocyte suspensions employed mechanical methods including forcing liver through cheesecloth, bolting silk, steel screens or even repeated passages through a narrow orifice. The realization that suspensions of cells prepared mechanically were thoroughly contaminated with damaged, non-viable cells led to enzymatic methods. Trypsin has been utilized to loosen cells from tissue for many years[12]. It remained the enzyme of choice for many years, until 1953, when collagenase was isolated and characterized from *Clostridium histolyticum* by Mandl[12]. Less than a decade later, Worthington Biochemical Corporation prepared collagenase on a commercial scale and in 1967, Howard and colleagues[75] reported the first successful isolation of intact hepatocytes from rat liver using collagenase perfusion. Many minor modifications have been added to this technique, but perhaps the only major alteration has been the introduction of the two-step collagenase procedure described by Seglen[163]. This, now widely employed method, starts with a Ca^{2+} free (and enzyme free) perfusion medium. The depletion of calcium brings about the irreversible cleavage of desmosomes, via invagination of the portion of plasma membrane containing the desmosome. These invaginations pinch off, forming endosomes, and in the process, desmosome fibres separate from their plaques beginning the process of hepatocyte separation. The Ca^{2+}-free perfusion medium

is changed to a Ca^{2+}-plus medium, and then collagenase is introduced to the now Ca^{2+}-enriched environment to facilitate separation of the parenchymal cells. Many research facilities employ this technique with slight modifications; our current procedure is described in detail in the Methods section. This procedure has been in application for more than 25 years and high yield preparations of cells are routinely prepared with acceptable viability set at 95% trypan blue exclusion.

It is interesting to note that the actual role of collagenase is not exactly certain. Purified preparations of collagenase are less effective in cell separation than are crude collagenase mixtures. That the enzyme may act in a synergistic fashion with other proteases is a possibility. Collagenase-free methods are used to isolate hepatocytes, albeit with increased contamination of damaged cells. Other enzymes such as pronase and lysozyme have been used in lieu of collagenase to facilitate cell separation. Pronase is typically used in the isolation of non-parenchymal cells, since this enzyme will selectively destroy hepatocytes and leave Kupffer cells intact[12;163].

Development of the two-step collagenase procedure allowed investigators to examine enriched populations of either perivenous or periportal hepatocytes following selective hepatic necrosis with a variety of zone-specific toxins. Following toxic insult, affected cells become necrotic and can be removed from the total sample through a series of centrifugation washes that is a normal part of the isolation procedure[12;163]. This generates samples of liver cell populations

which can then be assessed for their relative activities of specific zonal marker enzymes.

1.4.6 Selective Zonal Damage Using Digitonin Perfusion

The realization that digitonin selectively binds cholesterol moieties of the plasma membrane and thus renders it leaky[146] provided a novel means of isolating enriched populations of either periportal or perivenous cells. Quistorff[143] employed a quick perfusion with digitonin in either the antegrade or retrograde direction to selectively damage cells in the periportal or perivenous regions respectively. Following damage by digitonin perfusion, enriched populations of hepatocytes are usually prepared by the collagenase procedure described above. The technique was originally designed for the study of intracellular compartmentation of metabolites and has since been expanded as a means of studying the concentrations of enzymes and metabolites in the eluate from permeabilized perivenous or periportal cell populations. Perfusion with digitonin has also been successfully used to examine zonation of gluconeogenesis[143], amino acid transport[21], P450 isozymes[20] and a variety of mRNA[20;148;187].

The process of permeabilizing cells in the periportal or perivenous zone with digitonin is based upon the specific interaction of digitonin with cholesterol moieties in the plasma membrane[146] and the unidirectional microcirculation described by Rappaport[149]. The digitonin-cholesterol interaction resulting in the formation of insoluble complexes was first described by Windaus (1909)[186]. The

unexpected observation that lactate dehydrogenase and citrate synthase were released more slowly from primary cultures of isolated hepatocytes than from suspensions of freshly isolated hepatocytes subjected to digitonin treatment, led Quistorff to investigate the digitonin effect via perfusion of intact liver[146]. The realization that application of digitonin to the cell suspensions somehow enhanced the release of cytosolic proteins led Quistorff to develop* the now common technique of digitonin/collagenase perfusion[146].

1.5 Hepatic Metabolism of Δ^1-Pyrroline-5-Carboxylate

1.5.1 Introduction

The metabolic inter-conversions among proline, ornithine and glutamate involve pyrroline-5-carboxylic acid as a single common intermediate (see Figure 1.5 and Table 1.2). P5C may be formed from either of three precursors - glutamic acid, ornithine or proline. Ornithine aminotransferase catalyzes the conversion of ornithine to P5C†. Proline is oxidized to P5C by proline oxidase. Glutamate is converted to P5C by P5C synthase which has only been found in intestinal mucosa and marginally in thymus tissue[179;180], it is not thought to be in present in liver.

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* Quistorff is often cited in reference to techniques utilizing digitonin perfusion; however another group, Postius and Platt[142] had actually employed the digitonin technique 4 years earlier. The reference is in German which may hinder circulation of the report.

† P5C and glutamic γ-semialdehyde (GSA) are in spontaneous chemical equilibrium (not shown in Figure 1.4). The mixtures of P5C and GSA will be referred to as P5C in this thesis.

P5C can also be converted to each of these amino acids. The activity of OAT catalyzes the reversible inter-conversion of ornithine and P5C. P5C reductase converts P5C to proline. Glutamate production from P5C is catalyzed by P5C dehydrogenase. The next few sections will highlight these enzymes in more detail.

1.5.2 Proline Oxidase

The first enzyme involved in proline catabolism, proline oxidase (EC unassigned) is tightly bound to the inner side of the mitochondrial inner membrane and is limited to only a few tissues[141]. Early analyses[1] of the *proline oxidase system* suggested that the system was composed of a proline dehydrogenase which required NAD and was linked to the respiratory chain. These analyses were confounded by the use of crude mitochondrial and bacterial particulate preparations[1]. Today the existence of such a proline dehydrogenase has yet to be shown in an animal system. The only purified proline dehydrogenase was obtained from *Escherichia coli*. This near homogenous purification resulted in a 200,000 - 260,000 dalton protein[1].

Proline oxidase can be solubilized with detergents such as Triton X-100, but the mammalian enzyme has not yet been purified[92]. Characterization of the tissue distribution of the enzyme (in rat) by Herzfeld, Mezl and Knox[69] demonstrated activity in several tissues with adult liver specific activity ranking highest. The kidney enzyme activity was approximately 40% that of the liver and brain and heart activity followed with less than 10% of the liver activity. The

Figure 1.5 Metabolism of Δ^1-Pyrroline-5-Carboxylic Acid.

Enzymes are in red. Amino acids are in blue. P5C synthase activity is not found in liver. P5C is in spontaneous chemical equilibrium with GSA.

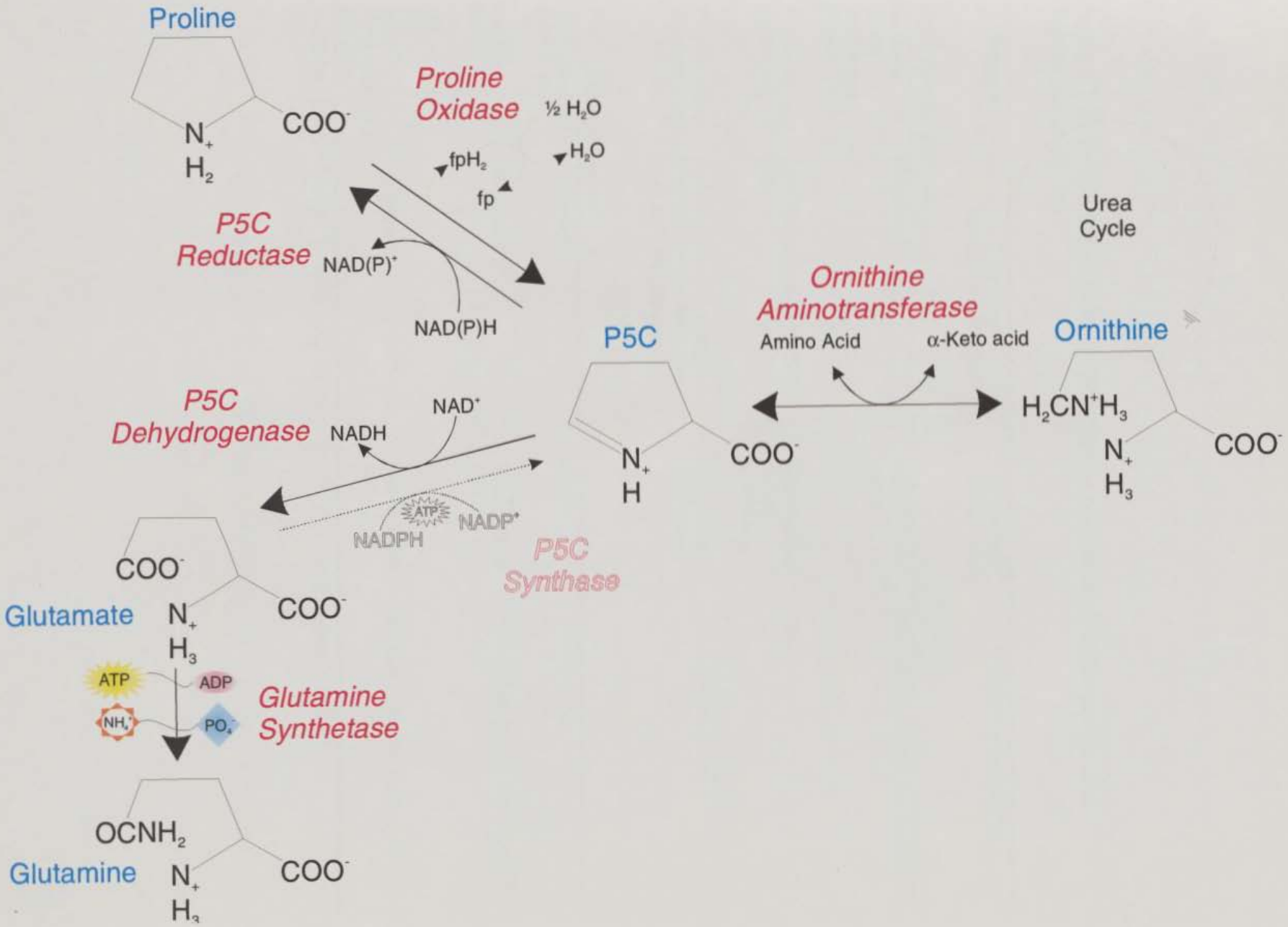

Proline
Proline Oxidase
½ H2O
fpH2
H2O
fp
P5C Reductase
NAD(P)+
NAD(P)H
Urea Cycle
Ornithine Aminotransferase
Amino Acid
α-Keto acid
Ornithine
P5C
P5C Dehydrogenase
NADH
NAD+
ATP
NADP+
NADPH
P5C Synthase
Glutamate
ATP
ADP
NH4+
PO4-
Glutamine Synthetase
Glutamine

Table 1.2 Enzymes Metabolizing P5C: Tissue Distribution, Subcellular and Acinar Localization

Enzyme	Tissue or cellular distribution	Subcellular localization	Acinar Localization
P5C - Forming Enzymes			
Ornithine amino transferase EC 2.6.1.13	Ubiquitous	Mitochondrial matrix	Perivenous; mRNA and protein[95]
Proline oxidase EC unassigned	Liver, kidney, heart, LLC-RK1 cells	Mitochondrial (inner) membrane	Unknown
P5C synthase EC unassigned	Intestinal mucosa, thymus, Chinese hamster lung cells, CHO cells (revertants)	Mitochondrial membranes	Not present in liver[80].
P5C - Utilizing Enzymes			
P5C reductase EC 1.5.1.2	Ubiquitous	Cytosol	Unknown
Ornithine amino transferase EC 2.6.1.13	Ubiquitous	Mitochondrial matrix	Perivenous; mRNA and protein[95]
P5C dehydrogenase EC 1.5.1.12	Ubiquitous	Mitochondria and cytosol	At least perivenous activity[132]

Adapted from Phang [140], with vertical column (Acinar Localization) added.

stoichiometric conversion of proline to P5C requires an obligatory participation of cytochrome c and the cytochrome-containing electron transport chain[79]. Confirmation of an FAD linked catalysis was provided using the electron chain inhibitors rotenone and Antimycin A[111].

1.5.3 Ornithine Aminotransferase

Ornithine aminotransferase (OAT, L-ornithine:2-oxoglutarate-5-aminotransferase) is a pyridoxal phosphate-dependent enzyme located in the mitochondrial matrix[157]. It catalyzes the reversible ω-transamination[1] of L-ornithine to an α-ketoacid, namely α-ketoglutarate, to form glutamate and glutamic-γ-semialdehyde which cyclizes to P5C. OAT is widely distributed in mammalian tissues, with the kidney, small intestine and liver all having high activity[140]. OAT from these three sources shows no significant differences when compared on a molecular level other than organ-specific behavior in response to dietary and hormonal control[157].

1.5.4 Δ^1-Pyrroline-5-Carboxylate Reductase

Δ^1-Pyrroline-5-carboxylate reductase (L-proline:NAD(P)$^+$-5-oxidoreductase EC 1.5.1.2) catalyzes the final step in the endogenous proline biosynthetic pathway by way of a NAD(P)H dependent reduction of P5C. The cytosolic enzyme[69] is typically found in all animal cells and has been purified from a variety

of organisms and tissues including *Escherichia coli* [140] and more recently from rat lens[165] and human erythrocytes[114]. However, the mammalian hepatic enzyme has not been purified to any great extent due to the apparent lability of the enzyme protein[165].

Herzfeld et al.[69] provide substantial evidence on the cold lability of hepatic P5C reductase activity. They have observed that the enzyme may be partially protected against cold inactivation by heat treatment of liver homogenates (eight minutes at 60°C) prior to analysis. Treatment with β-mercaptoethanol heightened enzyme activity at 25°C, but provided no protection against cold inactivation. The factor responsible for the temperature sensitivity was non-diffusible and was not equally distributed in all tissues (lending additional support to a tissue-specific variant hypothesis[114]). It appears that P5C reductase itself may not be cold labile, but rather is inactivated by an interaction with a second moiety apparent in the cold. Greengard and Herzfeld[53] describe differences in cold lability of human P5C reductase activity in both adult and fetal lung and liver tissue. They propose that the adult form is actually an isozyme of the fetal P5C reductase.

Inhibition of the enzyme, other than by cold inactivation has been seen with various heavy metals such as Hg^{2+} and Cu^{2+}, but not Cd^{2+}. Also the sulfhydryl-modifying reagent *p*-chloromercuribenzoate caused inhibition while iodoacetate did not[165]. Various tissue and cellular sources of P5C reductase are differentially sensitive to inhibition by proline[176], $NADP^+$ and adenine nucleotides[165]. In a system consisting of activated lymphocytes[175], a large

increase in proline-inhibitable activity accompanied the activation process: the enzyme activity in the control cells however was much less sensitive to proline inhibition, again suggesting variants of the enzyme exist. The hepatic and erythrocyte enzymes are proline insensitive but $NADP^+$ sensitive; adenine nucleotides inhibit the hepatic but not the erythrocyte form of P5C reductase (providing further evidence for the supposed isozymes of P5C reductase). The results presented by Shiono et al.[165] and Valle and colleagues[175] support the notion that the level of inhibition is also dependent on the cofactor utilized in the assay.

Debate continues as to which is the true cofactor for P5C reductase - NADH or NADPH? A more appropriate question asks which cofactor is more readily preferred in which tissues? Kinetic studies from various tissue sources have helped to answer this question.

P5C reductase from rat lens[165], human erythrocyte[114] and bovine retina[33] have a 20-60 fold lower K_m for NADPH *versus* NADH and have a 5-12 fold higher affinity for P5C with NADPH as cofactor. Cultured human fibroblasts and a lymphoblastoid cell line exhibit P5C reductase enzymes which show no preference for either cofactor, nor is the affinity for P5C dependent on the cofactor choice[33]. Purified P5C reductase from human erythrocytes preferentially and exclusively utilizes NADPH when both cofactors are present in assay medium[114]. P5C reductase from rat liver demonstrated a preference for NADH ; activity with NADPH at any concentration was less than one tenth that with

NADH[69]. These factors, especially temperature and cofactor considerations, combine to make determination of P5C reductase activity difficult.

1.5.5 .Δ^1-Pyrroline-5-Carboxylate Dehydrogenase

Δ^1-Pyrroline-5-carboxylate dehydrogenase (EC 1.5.1.12) catalyzes the NAD-dependent conversion of P5C to glutamic acid[41]. The protein has been located in the mitochondrial matrix[1;141] and in the cytosol[19]. A recent study by Haslett[61] has shown that the enzyme is primarily localized in the mitochondria (confirmed using succinate cytochrome c reductase as the positive marker enzyme for the mitochondrial fraction) with no enzyme (activity) in the cytosol. P5C dehydrogenase has been purified to relative homogeneity from human liver[41] and rat liver[167] mitochondria. The rat protein is thought to be an α_2 dimer with subunits of 59,000 daltons; the human protein, also an α_2 dimer may be slightly larger at 70,600 daltons[41]. The larger size of the human enzyme was challenged by Small and Jones[167] after polyclonal anti-rat P5C dehydrogenase immunoglobulins became available. Western blot analysis of tissue extracts from various rat and human tissues illustrated that antigens from both species gave heavily stained bands corresponding to a M_r of 59,000. Valle and coworkers[76] have cloned and characterized the human P5C dehydrogenase. Two full length P5C dehydrogenase cDNAs were generated; the structural gene appears to be a single copy (~ 20 Kb in size) mapping to chromosome 1. The gene encodes a protein of 563 residues with a predicted molecular mass of 62 kDa[76].

A variety of factors influence the unidirectional enzyme. Activity of the rat enzyme shows a sharp curve between pH 6.0 and 8.0 and a pH maximum between 8.0 and 8.5 . The human enzyme has a broad pH optimum (with NAD^+ as the cofactor) from pH 7.0 to pH 8.5[167]. $NADP^+$ may be utilized, but the preferred cofactor is NAD^+; the enzyme did not show preference for any other cofactors such as a flavin coenzyme[41:167].

1.6 Clinical Relevance of Hepatic Zonation

The basis of therapeutic intervention in the treatment of any disease state is comprehension of the normal state. Our understanding of the liver microstructure and its heterogeneous nature dictates the type and quality of treatment one may provide when liver function is impaired. There are many types of manifestations which may impair normal liver function. I hope to briefly highlight some examples which I feel are important in relation to hepatic zonation and metabolism.

1.6.1 Cirrhosis

What are the functional implications of hepatic cirrhosis? The development of *in vitro* models of cirrhosis have aided investigators in their understanding of the gross impairments of the microcirculation[149] and microvascular[14] perfusion, especially in the perivenous region. Glutamine synthetase, in the perivenous cells of the normal liver, scavenges ammonia which has escaped urea synthesis by enzymes in the periportal region[127]. Thus glutamine synthetase acts as the

fail-safe for ammonia detoxification. In the cirrhotic liver, the perivenous zone is generally compromised, showing marked decrease of glutamine synthetase activity[147]. The loss of glutamine synthetase activity can result in decreased synthesis of glutamine and ammonia removal. The disruption of glutamine synthetase activity in the cirrhotic liver is most likely only one indication that the entire metabolic capability of the perivenous region is compromised or at least altered. As we begin to understand the implications of altered physiology in the cirrhotic liver, we can begin to understand and appreciate the altered metabolism manifested in these situations[147].

1.6.2 Toxic Insult and Necrosis

Chemicals such CCl_4[65], bromobenzene[56], and allyl alcohol[159] can be used to generate specific zonal insults in the liver. Toxic insults which result in zone-localized necrosis provide a means of evaluating the effects of regional destruction. For example, glutamine is taken up and released by the liver however the glutamine transporters in different zones of the liver transport glutamine with different affinities. Haussinger and Gerok[64] used CCl_4 to destroy the perivenous zone and were thus able to demonstrate that the small population of hepatocytes surrounding the terminal hepatic venule (those cells capable of glutamine synthesis), had a 20-fold higher capacity for glutamate transport than other hepatocytes. Haussinger and Gerok[64] also demonstrated that the capability for glutamine synthesis is significantly decreased after CCl_4 administration. If the damage is substantial, glutamine may become a limiting

factor (for optimum growth early in life for example) and so glutamine supplementation may then be a consideration. Controlled damage to a particular zone of the liver then allows for a greater understanding of the functions of that zone and factors which must be considered following damage to that zone.

1.7 Problem of Investigation

Pyrroline-5-carboxylate is a common intermediate in the metabolism of arginine (ornithine), glutamate and proline (Figure 1.5). Proline catabolism begins with the action of proline oxidase; proline is converted to P5C. Glutamate formed by the action of P5C dehydrogenase, may then be converted to glutamine in the perivenous cells by glutamine synthetase[46]. The location of proline oxidase and P5C dehydrogenase is not known; different lines of research have suggested periportal[64] and perivenous[95] locations. The location of P5C reductase, which converts P5C to proline, has not been investigated.

Thus the purpose of this endeavor was to investigate the zonal metabolism of proline, synthesis and degradation, i.e. the zonal metabolism of P5C. Investigation of the zonal characteristics of any protein requires the use of several techniques. To date, a cDNA has not been described for proline oxidase. However, the zonal localization of mRNA may not accurately reflect the zonal activity of the enzyme protein. Ideally, comparisons of both enzyme activity and mRNA (or protein) localization of the enzyme being investigated to specific zonal marker activity and mRNA (or protein) is required to adequately describe the

zonation of a protein across the hepatic acinus. Thus, following selective zonal destruction using different hepatotoxins or by retrograde perfusion of digitonin, the zonal location of proline oxidase activity and of P5C reductase was investigated and compared to the zonal marker activities of glutamine synthetase, ornithine aminotransferase and ornithine transcarbamylase.

Glutamine synthetase and ornithine aminotransferase are located in the perivenous zone, with OAT having a slightly broader zone of positive activity than GS[95]. The activities of GS and OAT were used as positive markers of the perivenous zone. Ornithine transcarbamylase was employed as a positive marker of the periportal zone.

The capacity of isolated hepatocytes to interconvert proline, ornithine and glutamate, via P5C (i.e. flux through the pathways given in Figure 1.5) was also investigated using HPLC and amino acid analysis.

2 Materials and Methods

2.1 Chemicals

All chemicals were of suitable grade and quality with emphasis placed on purchasing high or "ultra" grade quality chemicals. Chemicals were purchased from Sigma Aldrich Ltd. (Oakville, Ont., Canada) unless otherwise stated in the text.

2.2 Animals

Male Sprague-Dawley rats (Memorial University of Newfoundland Vivarium), weighing 250-300 g, were used throughout all studies. Animals were housed in polycarbonate cages and allowed water and standard rat chow (ProLab Rat/Mouse/Hamster 3000, St. Louis, MO, USA) *ad libitum* unless otherwise stated. Environmental conditions were monitored daily and included an ambient temperature of 22°C, 30% relative humidity and a 12 hour light/dark cycle which commenced with lights on at 0800 hours. Experimental protocols were approved by the University Animal Care Committee and are in accordance with the guidelines of the Canadian Council on Animal Care.

2.3 Selective Zonal Destruction In Vivo

Several chemicals are available for the selective destruction of particular regions of the liver acinus. Carbon tetrachloride, bromobenzene and allyl alcohol

are three compounds often used to cause damage to particular regions in the liver acinus[78].

Experimental hepatic cirrhosis was induced with intragastric administration of either carbon tetrachloride (1.0 mmol/100 g body weight)[65], bromobenzene (3.8 mmol/kg body weight)[90] or allyl alcohol (1.0 mmol/kg body weight)[90]. All toxins were diluted in 1 mL of Mazola corn oil; control animals received corn oil only. Animals were fasted for 24 hours prior to and after intoxication to reduce variability of response among animals[139]. At 24 hours post-intoxication, animals were killed and portions of liver were taken for histological analysis. No mortalities due to carbon tetrachloride, bromobenzene or allyl alcohol injury occurred during the experiments.

2.4 Enzymatic Assays

Unless otherwise stated, all assays were performed in triplicate using broken hepatocytes. Isolated hepatocytes, utilized for the determination of zonal marker activity and proline oxidase, were freeze-thawed a minimum of three times, using liquid N_2 to facilitate a rapid freezing process and tap water to thaw the samples (samples did not reach ambient room temperature (22°C - 24°C)). This procedure was used to disrupt mitochondria membranes. Protein and DNA determinations for these samples were performed after the freeze-thaw procedure. P5C reductase activity was determined using liver homogenates whereas the production of proline from P5C was investigated using freshly isolated hepatocytes. The isolated hepatocyte model proved a much more

reliable model for the determination of proline production from P5C, although it includes transport of P5C into hepatocytes as well as P5C reductase activity.

2.4.1 Protein and DNA Determination

Protein was measured by the Biuret method[52] after solubilization with deoxycholate[77]. Bovine serum albumin was used as a standard. DNA was extracted by the method of Schneider[161], and was determined using the diphenylamine reagent described by Burton[23]. Calf thymus DNA was used as a standard. Typical standard curves for protein and DNA determinations are presented in Figures 2.1 and 2.2 respectively.

2.4.2 .Δ^1-Pyrroline-5-Carboxylate Reductase

Δ^1-pyrroline-5-carboxylate reductase activity was determined by assaying the P5C–dependent production of proline in cytosol[69]. Rats were killed by cervical dislocation. Liver was excised and placed in homogenization buffer (50 mM KH_2PO_4, 2mM β-mercaptoethanol, 0.25 M sucrose, pH 6.8)* at room temperature (~ 22°C - 24°C) using a 1:10 ($^w/_v$) dilution ratio. The liver was minced using scissors, homogenized with 8 – 10 passes of a Dounce homogenizer and then centrifuged for 30 minutes at 100 000 X g (30°C). The cytosolic fraction

* The assay of P5C reductase was tried in the presence and absence of a protease inhibitor cocktail (Sigma) with no difference in activity noted (data not shown); hence the cocktail was not used in later experiments.

Figure 2.1 Typical Standard Curve for the Determination of Protein

BSA protein standards (0 – 4 mg) were used to determine protein content in hepatocyte and homogenate samples using the Biuret method. Data are presented as the mean ± standard deviation of triplicate samples for a typical experiment.

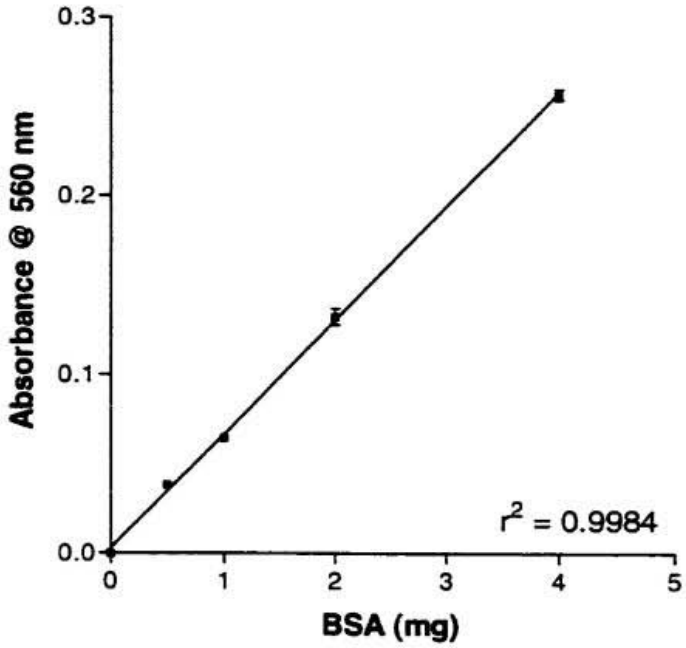
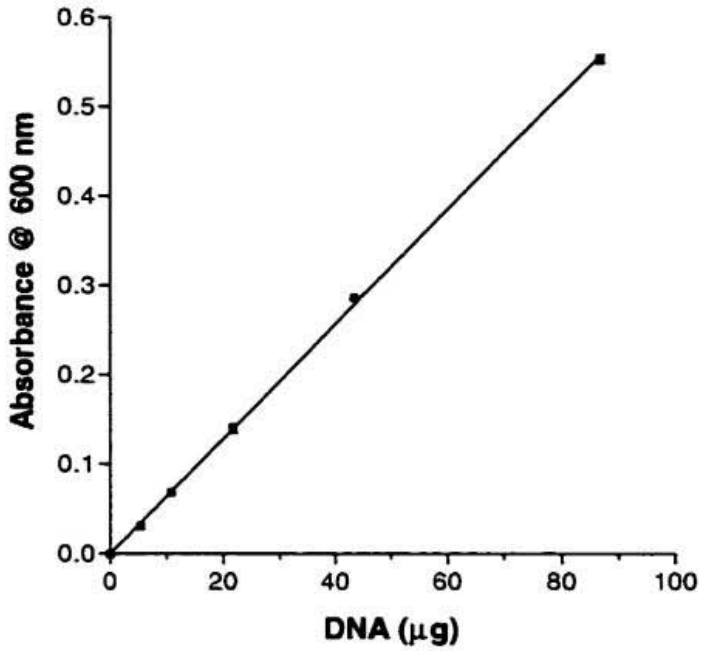

0.3
0.2
0.1
0.0
Absorbance @ 560 nm
r^2 = 0.9984
0
1
2
3
4
5
BSA (mg)

Figure 2.2 Typical Standard for the Determination of DNA

Calf thymus DNA standards (0 – 85 μg) were used to determine DNA content in hepatocyte samples using the Burton method. Data are presented as the mean ± standard deviation of triplicate samples for a typical experiment.


Absorbance @ 600 nm
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
20
40
60
80
100
DNA (µg)

was carefully removed and stored at room temperature until assayed. During temperature stability studies, the cytosol was heated to 50°C for five minutes and then allowed to cool to room temperature prior to assay.

The reaction mixture (total volume of 0.75 mL) contained 3 mM DL-P5C, 1.56 mM NADH, 30 mM KH_2PO_4 (pH 6.8) and cytosol (0.5 mg). The reaction mixture was equilibrated to 37°C for five minutes in 25 mL Erlenmeyer flasks. Cytosol was added to start the reaction which proceeded for five minutes at 37°C in a shaking water bath. The reaction was terminated with the addition of 250µL of ice cold 50% TCA. The samples were transferred to eppendorf tubes, incubated on ice for 20 minutes (to facilitate protein precipitation) and then centrifuged for 5 minutes at 15000 X g. The assay was determined to be linear with time and protein (Figure 2.3).

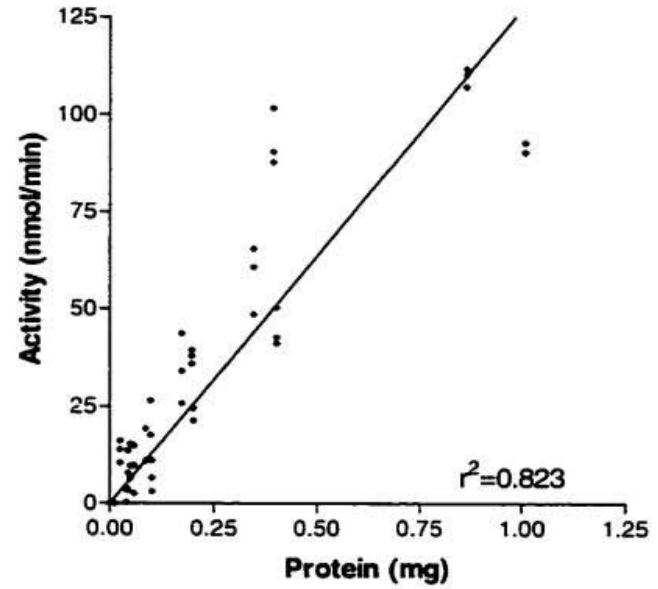
P5C reductase activity was confirmed to be prominent in the cytosol (Figure 2.4). Herzfeld, Mezl and Knox[69] described the cold sensitivity of P5C reductase. Cold sensitivity was noted in liver homogenates (Figure 2.4), but interestingly when P5C reductase was assayed (through the production of proline), it was absent in isolated hepatocyte preparations.

2.4.2.1 Acid-Ninhydrin Determination of Proline

The determination of proline in biological samples is often confounded by the imino ring structure of the amino acid. Assay of the amino acid using

Figure 2.3 Time and Protein Curves for P5C Reductase activity.

P5C reductase activity in cytosol obtained from rat liver homogenates. The data are presented as the individual points to demonstrate variability, n > 3.


125
100
75
50
25
0
Activity (nmol/min)
r^2=0.823
0.00
0.25
0.50
0.75
1.00
1.25
Protein (mg)

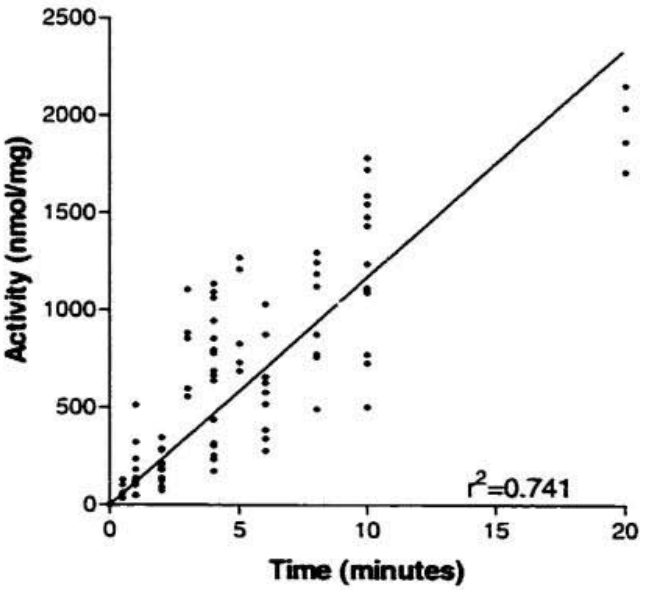
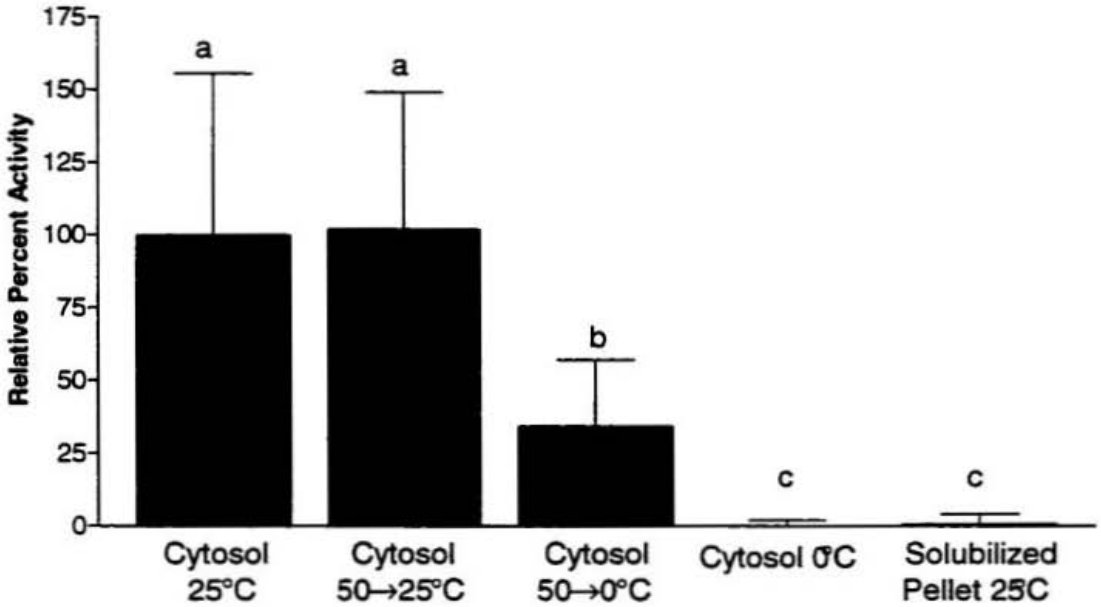

2500
2000
1500
1000
500
0
Activity (nmol/mg)
r^2=0.741
0
5
10
15
20
Time (minutes)

Figure 2.4 Subcellular Localization and Temperature Stability Characteristics of P5C Reductase

P5C reductase activity was determined in cytosolic fractions maintained at either 25°C, heated to 50°C for 5 minutes and cooled slowly to 25°C, heated to 50°C for 5 minutes and cooled slowly to 0°C, maintained at 0°C, and in combined non-cytosolic fractions (resuspended pellet following centrifugation to isolate cytosol). Data are presented as the mean ± standard deviation, n=3. Results were compared using Student's t-test and $p < 0.05$ set as statistical significance. Bars with different letter notation are significantly different.


Relative Percent Activity
175
150
125
100
75
50
25
0
a
a
b
c
c
Cytosol 25°C
Cytosol 50→25°C
Cytosol 50→0°C
Cytosol 0°C
Solubilized Pellet 25°C

conventional ninhydrin-based assays is complicated by contamination with other amino acids which give the classical Rhuemans purple associated with a positive interaction of ninhydrin and an amino acid. To counter this interference, Goodwin[51] used preliminary nitrosation followed by acid hydrolysis to destroy non-proline compounds. The red colored ninhydrin-proline complex is then stabilized by salting out[10]. This particular method is basically a modification of the Chinard[27] reaction and is described in detail below (see also Appendix 6.3).

The sequential reactions were carried out in a single, disposable glass tube. Deproteinized samples (250 µL) were combined with 250 µL of 2.5 M $NaNO_2$ and allowed to react for 10 minutes at room temperature. Concentrated hydrochloric acid (500µL) was added and the tubes were vortexed to mix the system. Following a 10 minute reaction at room temperature, 4.8 M ammonium chloride (150 µL) was added. The tubes were covered with marbles and then heated for 20 minutes in a boiling-water bath. After cooling, the tubes were neutralized with 400 µL of 10 M NaOH. Next, 1.5 mLs of 1.78 M NaH_2PO_4/H_3PO_4 were added, followed by 1.0 mL of 100 mM ninhydrin; ninhydrin is dissolved in the minimal amount of 95% ethanol prior to dissolution in distilled water. The covered tubes were then heated for 60 minutes in the boiling-water bath, at which time the proline-ninhydrin complex is visualized as a red precipitate. After cooling, 3.0 mL of benzene are added, and the tubes vortexed (2X) for 30 seconds. The benzene, containing the extracted proline-ninhydrin complex, was pipetted into a 13X100mm glass tube (using a Pasteur pipette) after the benzene

and aqueous layers separate. Absorbance was determined at 520 nm using a LKB Novaspec (4049) spectrophotometer (Biochrom).

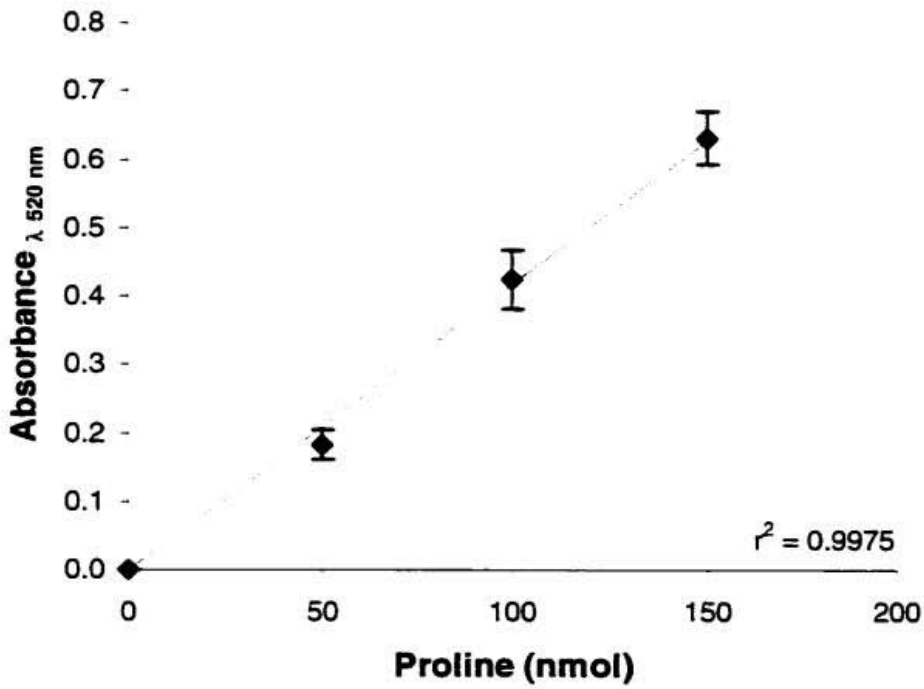
The concentration of proline in tissue and hepatocyte samples was obtained using a standard curve of L-proline (0 – 0.15μmol) (Figure 2.5). Standards were treated in the same manner as tissue and hepatocyte samples for the determination of proline and were processed at the same time. All samples and standards were processed in triplicate. Proline recovery was checked and determined to be ≥ 99%.

2.4.2.2 Preparation of D,L-Δ^1-Pyrroline-5-Carboxylate

Although specific radioisotopic assays have been developed to investigate the enzymes which synthesize and degrade P5C, pure L-P5C has remained difficult to obtain. A variety of enzymatic techniques have been employed to drive P5C production either from ornithine[169] or proline[185], but these techniques are relatively time consuming and labor intensive when compared with the actual amount of final product one obtains. Syntheses of a purely chemical nature have not yet resulted in a stable dry chemical form of the compound that is free of contaminants toxic to cell cultures. DL-P5C can, however, be synthesized[185] and stored as the hydrazone. DL-P5C may then be extracted in an acidic solution and utilized in metabolic studies[115].

Figure 2.5 Typical Proline Standard Curve.

L-proline standards 0 – 150 nmol were used to determine proline content in tissue and hepatocyte samples using the acid-ninhydrin method of proline determination. Data are presented as the mean ± standard deviation of triplicate samples for a typical experiment.


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Absorbance λ 520 nm
r² = 0.9975
0
50
100
150
200
Proline (nmol)

One of the major problems associated with P5C is a lack of stability once prepared. Williams and Frank[185] describe the lability of P5C due to an irreversible, concentration-dependent reaction with itself; destruction of the chemical is rapid at neutral pH. For this reason, Mezl and Knox[115] prepared the 2,4-dinitrophenylhydrazine derivative of P5C which is stable and can easily be converted to P5C as it is required.

2.4.2.2.1 Chemical Synthesis

D,L-Δ^1-Pyrroline-5-carboxylate was prepared from the 2,4-dinitrophenylhydrazone derivative of P5C, using a procedure described by Mezl and Knox[115]. Suspensions of up to 30 mM concentration were prepared in 0.5 M hydrochloric acid. To this suspension, acetophenone was added to a final volume of 0.2 mLs acetophenone / milligram solid hydrazone. This mixture was shaken for 30 minutes, after which time the acetophenone layer was carefully removed. An equal volume of toluene was added to the aqueous fraction to remove contaminating acetophenone and the mixture shaken for an additional 30 minutes. After shaking, the mixture was allowed to settle into two layers and the aqueous fraction, containing P5C, carefully removed. This 'toluene wash' was repeated two times to decrease the acetophenone to a tenth of its original contaminating concentration (~0.6mM).

2.4.2.2.2 Quantitative Assay of P5C with Ninhydrin

The following procedure[185] was only used to ascertain the concentration of P5C solutions derived from chemical preparations yielding 'pure' solutions of DL-P5C. The procedure was not used to ascertain the concentration of P5C in solutions or preparations, which may contain other amino acids such as glutamate or ornithine. Unfortunately, a simple and economical method for the precise determination of P5C in biological samples has not been developed and thus this hampers research in this field.

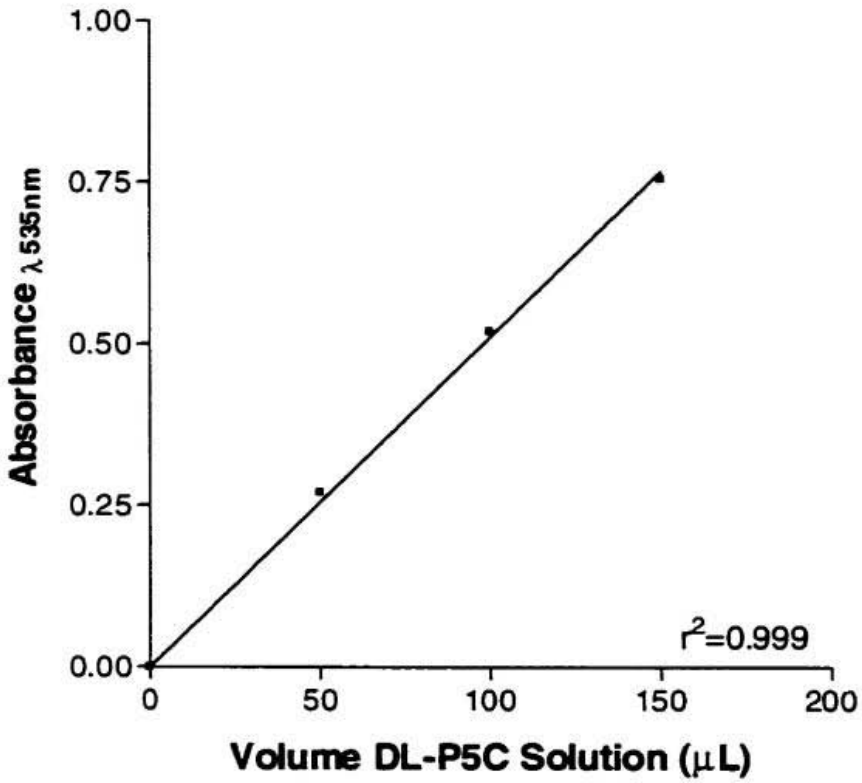
The aqueous stock solution of P5C was diluted 1:10 with deionized water. To 150 μL of appropriately diluted stock solution, 150 μL of 3M sodium acetate and 2.0 mL of 0.15% ninhydrin in glacial acetic acid were added. The glass tubes were mixed, capped with marbles and incubated at 50°C for 15 minutes at which time a rosy pink color had developed in P5C positive tubes. Absorbance was determined at 535 nm (determined to be λ_{OPT}) and concentration calculated using a molar extinction coefficient of 4600 $M^{-1}cm^{-1}$. The absorbance was a linear function of P5C concentration using these criteria (Figure 2.6).

2.4.3 Proline Oxidase

The determination of proline oxidase activity is based upon the formation of a P5C-*o* - aminobenzaldehyde complex in the presence of excess proline. The

Figure 2.6 Typical Standard Curve for P5C Concentration Determination.

Stock DL-P5C (~ 30-35 mM) was diluted 1:10 and assayed using a ninhydrin-based method. An $\varepsilon = 4600\ M^{-1}cm^{-1}$ was used to determine concentration. The data are presented as the mean ± standard deviation for triplicate samples of one solution of DL-P5C (30 mM).


1.00
0.75
0.50
0.25
0.00
Absorbance λ 535nm
r²=0.999
0
50
100
150
200
Volume DL-P5C Solution (μL)

method of Johnson and Strecker [79] as modified by Herzfeld, Mezl and Knoz[69] has been used to determine the activity of proline oxidase.

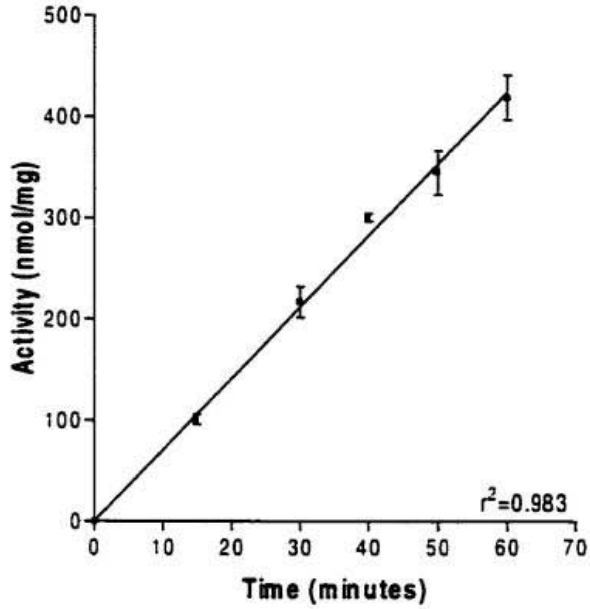
In 25 mL Erlenmeyer flasks, 53mM KH_2PO_4, pH 8.0, 1.6 µM cytochrome c and 158mM L-proline were combined with a maximum of 7 mg of protein, after equilibration to 37°C, in a final volume of 1.90 mLs. Samples were incubated for 30 minutes in a shaking water bath at 37°C. The reaction was terminated by the addition of 1.0 mL of 10% TCA. Then 100 µL of 0.2M *o* - aminobenzaldehyde (OAB) (aminobenzaldehyde is first dissolved in 95% ethanol (final 40% $^{v}/_{v}$) and then mixed with deionized water) was added and the system shaken for an additional 30 minutes at room temperature. The samples were decanted into disposable tubes and centrifuged in a clinical centrifuge for 15 minutes to pellet particulate matter. The supernatant was very carefully removed and the absorbance measured at 440 nm. Concentration of P5C formed was determined using the millimolar extinction coefficient of 2.59 described for the P5C-OAB complex[115]. Enzyme activity was linear with time and protein (Figure 2.7).

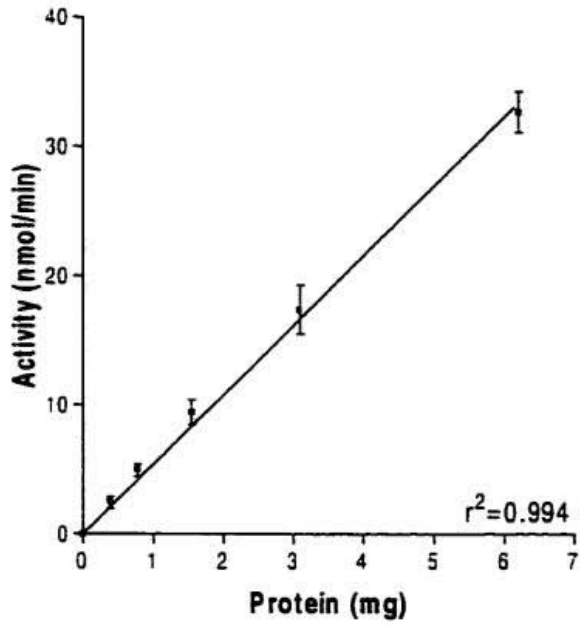
2.4.4 Ornithine Aminotransferase

Ornithine aminotransferase activity was based upon the colorimetric assay developed by Peraino and Pitot[138] in which *o*-aminobenzaldehyde reacts with P5C to form a yellow dihydroquinazolium compound. The spectrophotometric method was modified to include pyridoxal-5-phosphate[171]. In 25 mL Erlenmeyer

Figure 2.7 Time and Protein Linearity Curves for Proline Oxidase.

Proline oxidase activity was determined to be linear with time (using 2 mg protein) and protein content. Data are presented as mean ± standard deviation, n = 3.


500
400
300
200
100
0
Activity (nmol/mg)
r^2=0.983
0
10
20
30
40
50
60
70
Time (minutes)


40
30
20
10
0
Activity (nmol/min)
r^2=0.994
0
1
2
3
4
5
6
7
Protein (mg)

flasks, 125 mM KH_2PO_4 buffer, pH 7.6, was combined with 20 mM α-ketoglutarate, 50 μM pyridoxal-5-phosphate, 100 mM L-ornithine, and 10 mM o-aminobenzaldehyde. After equilibrating the system to 37°C in a shaking water bath, ~2 mg protein was added to start the reaction (final volume of 1.0 mL). After 30 minutes, the reaction was terminated by the addition of 2.0 mLs of 7.5% TCA. The samples were decanted into disposable tubes and centrifuged in a clinical centrifuge for 15 minutes to pellet particulate matter. The supernatant was very carefully removed and the absorbance measured at 440 nm. Concentration of the P5C-aminobenzaldehyde complex was determined using the millimolar extinction coefficient of 2.59[115].The assay was determined to be linear with both time and protein (Figure 2.8).

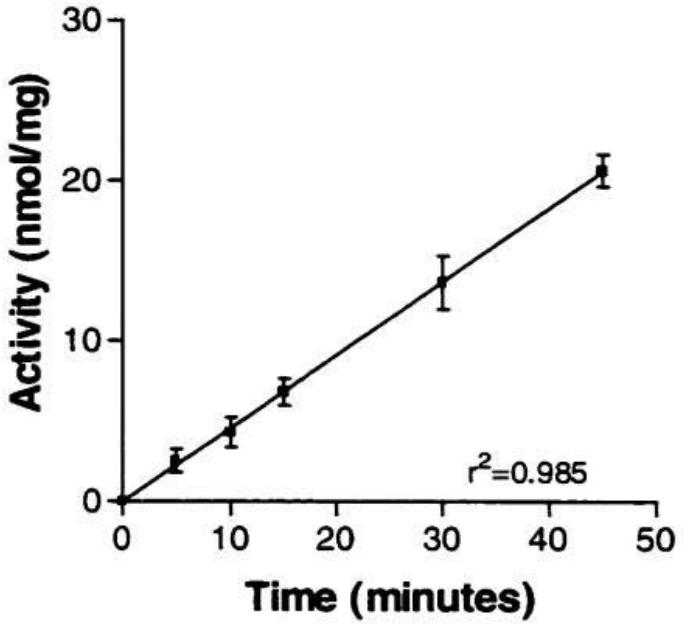
2.4.5 Glutamine Synthetase

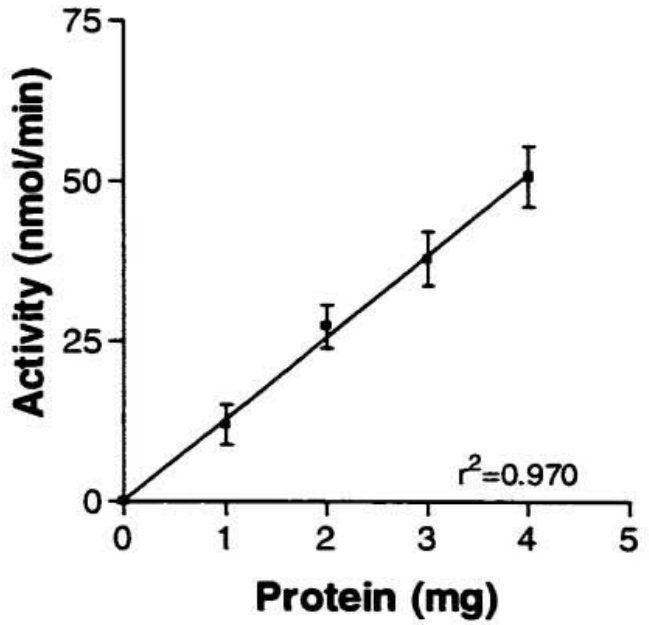
The assay for glutamine synthetase is based upon the formation of γ-glutamylhydroxamate from glutamate and hydroxylamine in the presence of the creatine phosphokinase-based ATP regenerating system as described by Vorhaben et al.[178].

Homogenate or hepatocyte suspensions were diluted 10% ($^w/_v$) in 3.4 mM Tris-acetate buffer, pH 7.4 containing 0.25M sucrose and 1.0 mM EGTA. Samples were freeze-thawed 3X using liquid N_2 , and then centrifuged at 15000 rpm for 10 minutes. The cytosolic supernatant was removed and used for

Figure 2.8 Time and Protein Linearity Curves for Ornithine Aminotransferase

Ornithine aminotransferase activity was determined to be linear with time (using 2 mg protein) and protein content (using 30 minute reaction time). Data are presented as mean ± standard deviation, n = 4.


30
20
10
0
Activity (nmol/mg)
r^2=0.985
0
10
20
30
40
50
Time (minutes)


75
50
25
0
Activity (nmol/min)
r^2=0.970
0
1
2
3
4
5
Protein (mg)

determination of enzyme activity.

An assay cocktail composed of 56 mM Tris-acetate, pH 7.4; 27 mM hydroxylamine; 14 mM $MgSO_4 \bullet 7H_2O$; 14 mM β-mercaptoethanol; 42 mM potassium L-glutamate; 7 mM ATP; and 7 mM creatine phosphate was mixed with 4 mg of creatine phosphokinase. In 25 mL Erlenmeyer flasks, 700 µL of the assay cocktail was allowed to equilibrate to 37°C, before 2 mg of protein was added to start the reaction. After 25 minutes in a shaking water-bath at 37°C, the reaction was terminated with 1.5 mLs of ferric chloride solution (0.37M ferric chloride;0.67N HCl; 0.2M TCA) and then allowed to stand for 45 minutes for complete color development. Samples were centrifuged for 10 minutes in a clinical centrifuge to pellet particulate matter and the absorbance of the supernatant was read at 500nm to determine the quantity of glutamylhydroxamate formed. Glutamylhydroxamate quantity was obtained from a standard curve of L-glutamic acid-mono-hydroxymate (Figure 2.9). The activity of glutamine synthetase was determined to be linear with time and protein (Figure 2.10).

2.4.6 Ornithine Transcarbamylase

Ornithine transcarbamylase (OTC) was assayed by measuring the citrulline synthesized from ornithine and carbamyl phosphate[131]. L-ornithine (7.5

Figure 2.9 Typical Standard Curve for L-Glutamic acid-mono-hydroxymate.

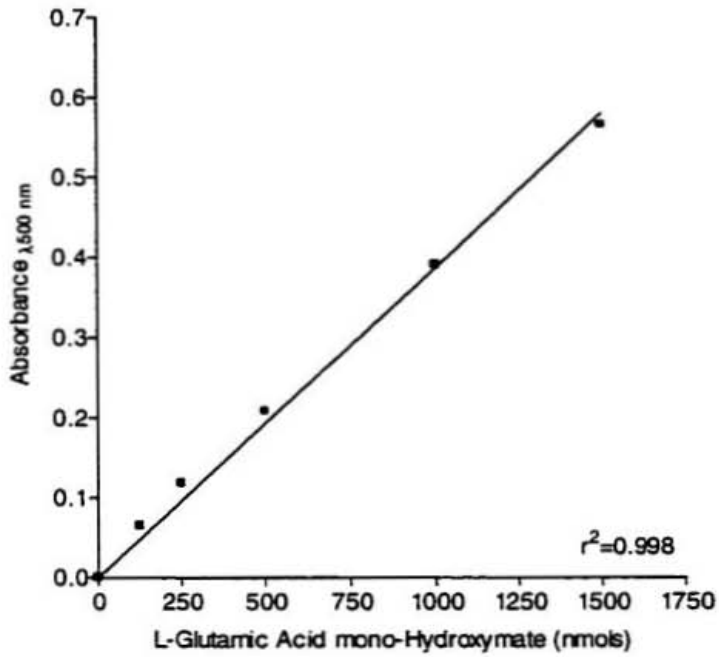
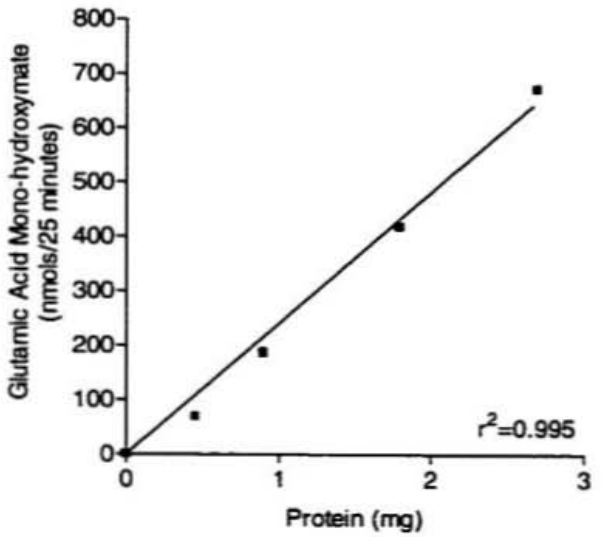
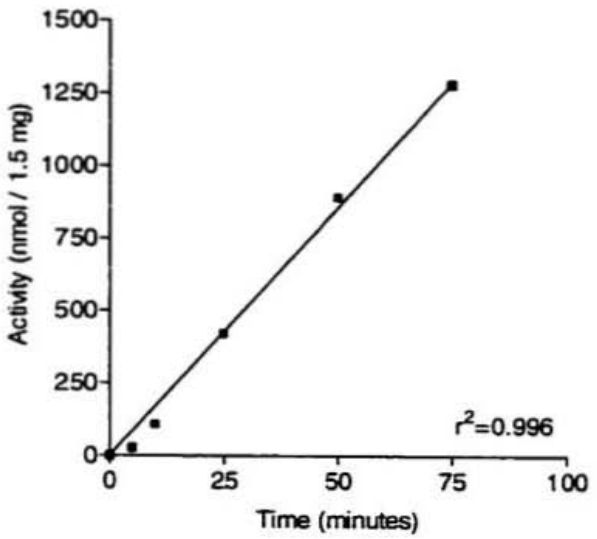

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Absorbance λ500 nm
r²=0.998
0
250
500
750
1000
1250
1500
1750
L-Glutamic Acid mono-Hydroxymate (nmols)

Figure 2.10 Time and Protein Curves for Glutamine Synthetase.

Glutamine synthetase activity was linear with respect to time and protein. The data are presented as the mean ± standard deviation, n = 3.


Glutamic Acid Mono-hydroxymate (nmols/25 minutes)
800
700
600
500
400
300
200
100
0
0
1
2
3
Protein (mg)
r^2=0.995


Activity (nmol / 1.5 mg)
1500
1250
1000
750
500
250
0
0
25
50
75
100
Time (minutes)
r^2=0.996

mM) and carbamyl phosphate (15.0 mM) were made fresh each day in 270 mM triethanolamine (TEA) buffer, pH 7.6.

Reactions were carried out in 1.5 mL eppendorf tubes containing 2.5 mM ornithine and 25 μg of protein and proceeded in the presence of 5 mM carbamyl phosphate. This assay required broken mitochondria, so all samples were freeze-thawed (3X) to disrupt the mitochondrial membrane. The tubes were equilibrated to 37°C and the reaction started with the addition of carbamyl phosphate. After incubating for 7 minutes at 37°C, the reaction was terminated by adding 300 μL of 30% perchloric acid (PCA). The tubes were centrifuged for 10 minutes at 10000 X g to pellet the particulate matter. The supernatant was assayed for citrulline content according to protocol described by Herzfeld and Raper[71]. Colorimetric reagents in the assay included i) diacetyl monoxime (0.5% $^{w}/_{v}$ butanedione monoxime in water) and ii) ferric acid solution (250 mLs concentrated sulphuric acid mixed with 200 mLs concentrated phosphoric acid to a final volume of 1 L; after cooling 250 mg of $FeCl_3$ were added per L). The colorimetric reagents were used to make the chromogenic reagent: 5.0 mg of thiosemicarbazide were added to 50 mLs of diacetyl monoxime immediately prior to use; after the thiosemicarbazide had dissolved, 100 mLs of ferric acid reagent was added while stirring (stable for only one hour after preparation).

Citrulline was determined using 100 μL of sample mixed with 150 μL of water in a 13X 100mm glass tube. To these components were added 3.0 mLs of the chromogenic reagent. After vortexing, the tubes were covered with marbles

and heated for 5 minutes in a boiling-water bath. The tubes were cooled to room temperature, re-vortexed and the absorbance read at 530 nm. The quantity of citrulline in the samples was determined using a citrulline standard curve (Figure 2.11). The assay was examined for linearity with respect to time and protein (Figure 2.12).

2.5 Isolation and Incubation of Hepatocytes

2.5.1 The Two Step Collagenase Procedure

Hepatocytes were isolated according to the two-step collagenase method of Seglen[163]. Animals were anesthetized with sodium pentobarbital (100 µL / 100 g body weight), and approximately 250 µL of heparin was injected into the femoral vein of the left hind limb. The abdomen was then opened along the midline to expose the portal vein. Surgical ties were loosely placed around the inferior vena cava above the renal artery and around the portal vein (Figure 2.13). Following cannulation of the portal vein, the vena cava was severed below the right kidney and antegrade perfusion commenced (40 mL/min) with Krebs-Henseleit Ca^{2+}-free buffer (121mM NaCl, 4.7mM KCl, 1.2mM $MgSO_4$, 1.2mM KH_2PO_4, 25.2mM $NaHCO_3$) containing 2mM EGTA, 20 mM DL-glucose, 2.1 mM lactate and 0.3 mM pyruvate, equilibrated with 95/5% O_2/CO_2. The chest cavity was immediately opened and the vena cava cannulated via the right atrium. After ties around the inferior vena cava were securely fastened, a non-recirculating

Figure 2.11 Typical Standard Curve for Citrulline Determination.

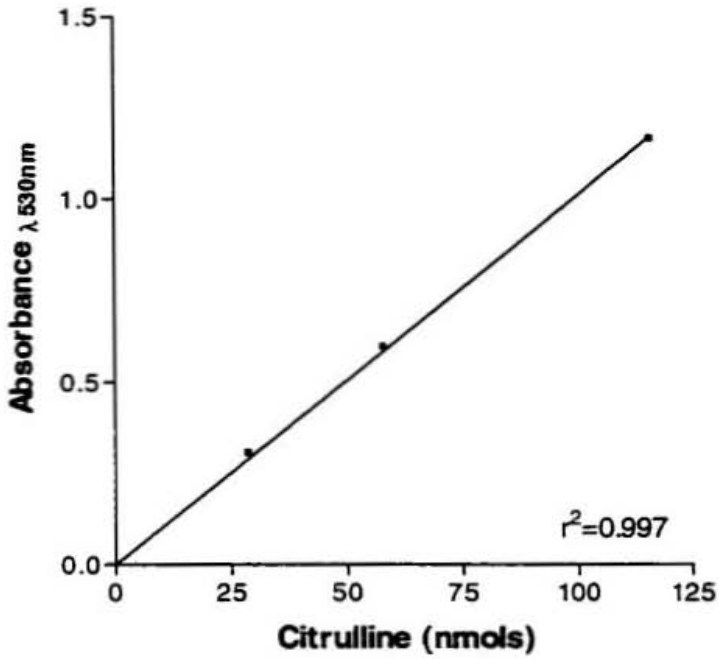
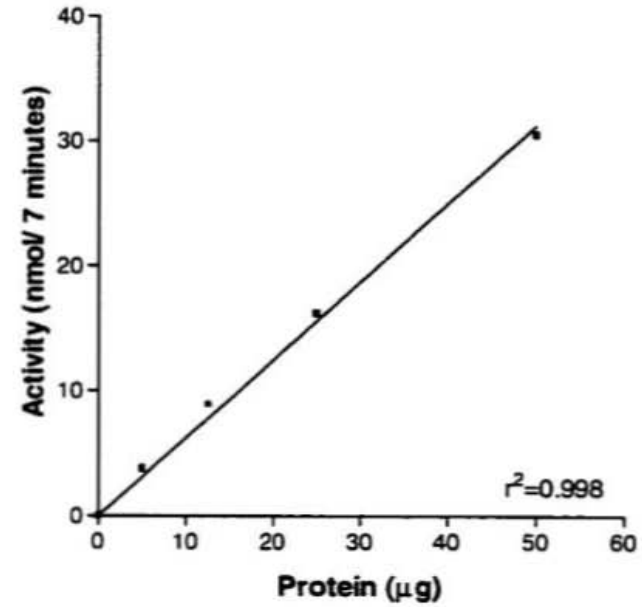

1.5
1.0
0.5
0.0
Absorbance λ 530nm
r²=0.997
0
25
50
75
100
125
Citrulline (nmols)

Figure 2.12 Time and Protein Curves for Ornithine Transcarbamylase.

The activity of ornithine transcarbamylase was linear with time and protein. The data are typical values for the assay and are the mean of duplicates.


Activity (nmol/ 7 minutes)
40
30
20
10
0
0
10
20
30
40
50
60
Protein (µg)
r²=0.998

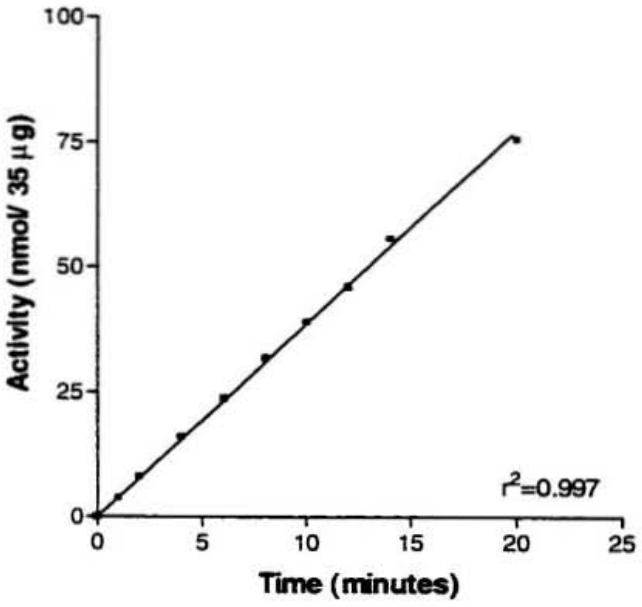
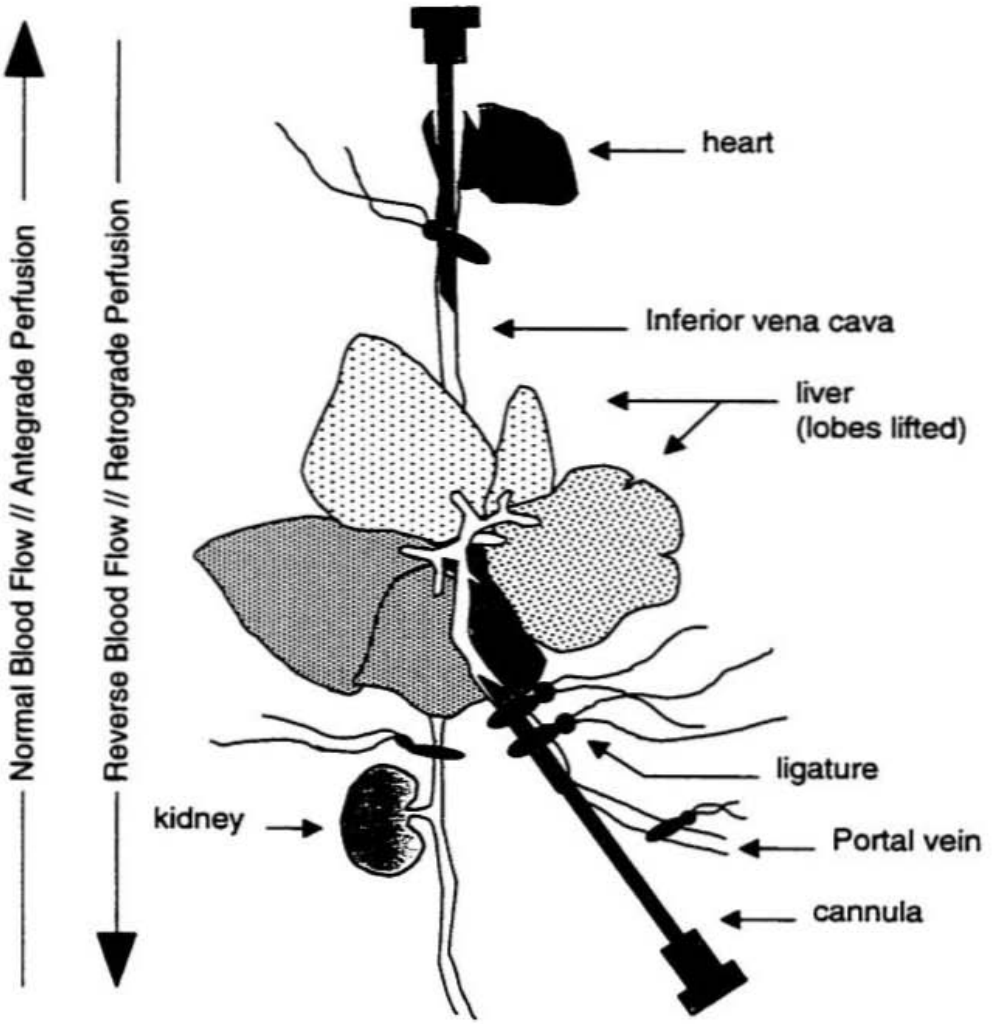

Activity (nmol/ 35 µg)
100
75
50
25
0
0
5
10
15
20
25
Time (minutes)
r²=0.997

Figure 2.13 Outline of Surgical Procedure for the Isolation of Rat Hepatocytes.


Normal Blood Flow // Antegrade Perfusion
Reverse Blood Flow // Retrograde Perfusion
heart
Inferior vena cava
liver
(lobes lifted)
ligature
kidney
Portal vein
cannula

perfusion was established (flow rate of 40 mL/min). At this time, a single lobe may have been tied off and removed and the tissue sample preserved in 10% buffered formalin for histological analysis. Approximately 500 mL of the Ca^{2+} free medium (37°C) was allowed to perfuse the liver before an additional 500 mL of Krebs-Henseleit buffer containing calcium (1.3 mM) was circulated through the liver in a similar non-recirculating fashion. To separate hepatocytes, a recirculating flow of Krebs-Henseleit Ca^{2+} plus buffer containing 0.25% BSA (Fraction V, Boehringer-Mannheim, Germany) and 25mg/100mL collagenase (CLS2) (Worthington Biochemical Corporation, Lakewood, NJ) was maintained until the liver was soft and began to fall apart. At this time, the liver was carefully removed and gently teased apart with gloved fingers in the collagenase medium to release cells. The cells were incubated in a shaking water bath at 37°C for 10 minutes while slowly gassed with 95/5% O_2/CO_2. Cells were then filtered through a nylon mesh and centrifuged at 600 rpm for two minutes. The supernatant was decanted and cells resuspended in fresh Krebs-Henseleit Ca^{2+}-plus buffer, centrifuged and resuspended. Cells were then washed in Krebs-Henseleit Ca^{2+}-plus buffer containing 2.5% BSA. The supernatant was decanted and cells resuspended in the 2.5% BSA medium (diluted 1:18 $^{w}/_{v}$). Resuspended cells were filtered through a double layer of cheesecloth prior to incubation. Samples of cell suspensions (3.0 mls) were taken for the determination of dry weight. These cells were aliquoted into aluminum drying pans and heated at 50°C for at least 24 hours to determine dry weight (samples of the BSA solution (-

hepatocytes) were also dried to correct for the weight of added BSA). Trypan blue exclusion analysis routinely demonstrated greater than 95% viability of isolated hepatocytes.

2.5.2 The Percoll® Wash

Percoll, a colloidal suspension of silica particles coated with polyvinylpyrrolidine, is unable to enter either damaged or intact cells[12]. The damaged swollen cells, due to the loss of cytoplasmic protein and larger water space, have a lower density than intact cells and will float in the 1.06 g/cm^3 density (ρ) isotonic Percoll solution while the intact cells pellet[163].

Isotonic Percoll solution was prepared[12] by combining 90 mL Percoll (Pharmacia), 10 mL stock balanced salt solution (1.37M NaCl, 50mM KCl, 8.1 mM) and 2 mL stock phosphate buffer (100 mM Na_2HPO_4, 15 mM KH_2PO_4) and adjusting the pH to 7.4 with 0.1 M HCl. The final cell suspension (30 mg dry weight/mL) was diluted 1:1 ($^v/_v$) with the wash medium (Ca^{2+}-plus Krebs Henseleit buffer). Equal volumes of cell suspension and Percoll solution were added to centrifuge tubes, gently mixed and centrifuged at 0-4°C for ten minutes at 50 % g. Undamaged cells were found in the pellet and other damaged cells, non-parenchymal cells, cell aggregates and cell debris floated to the upper region of the tube.

The Percoll was removed by aspirating the supernatant and gently resuspending the cells in wash medium. The suspension was centrifuged for three minutes at 40 × g (4°C). The pelleted cells were washed one more time prior to assessment of viability by Trypan blue exclusion.

2.5.3 Definition of Viability

The viability of isolated hepatocytes has been determined by the technique of Trypan blue exclusion as described by Seglen[163] and Berry[12]. Cells that are damaged will take up the dye and stain blue. Undamaged cells do not take up the dye and remain opaque. Briefly, 25 µL of diluted hepatocytes were combined with 100 µL of Trypan blue (0.1% ($^{w}/_{v}$) in Krebs-Ringer phosphate { 0.154M NaCl, KCl, KH_2PO_4,$MgSO_4 \bullet 7H_2O$, 0.11M $CaCl_2$, and 0.1 M NaH_2PO_4/HCl buffer pH 7.4 }). The cells (at least 200) were counted in 4 squares of a counting chamber and the percentage of cells staining with the dye calculated. Typical preparations in our lab demonstrate ≥ 95% viability. Cells prepared after chemical insult demonstrate ≥ 90% viability.

2.5.4 Amino Acid Production in Isolated Hepatocytes

Isolated hepatocytes (diluted 1:18 in Ca^{2+} Krebs-Henseleit buffer containing 2.5% BSA) were used to investigate amino acid production from a variety of substrates. A typical assay was carried out in stoppered 25 ml Erlenmeyer flasks in a shaking water bath at 37°C: 400 µL of Ca^{2+} Krebs-Henseleit buffer was

equilibrated to 37°C prior to addition of 500 µL of freshly swirled hepatocytes. The system was gassed slowly with 95/5% O_2/CO_2 for 15 seconds prior to and following the addition of cells or substrate. The cells were shaken for 10 minutes to allow them to equilibrate to 37°C. Cells were incubated with 2.5 mM DL-P5C or 1.0 mM solution of proline, glutamate or ornithine for 15 or 30 minutes. The reaction was terminated with the addition of 250µL of 50% TCA. The contents were decanted into an eppendorf and allowed to sit on ice for 20 minutes. Following this incubation, the tubes were centrifuged for five minutes at 15000 × g. The production of proline in isolated hepatocytes was determined to be linear with time and protein (Figure 2.14) and demonstrated a sigmoidal-response with increasing concentrations of DL-P5C (Figure 2.15). Amino acid concentrations were determined by HPLC analysis[94] or by amino acid analysis[102].

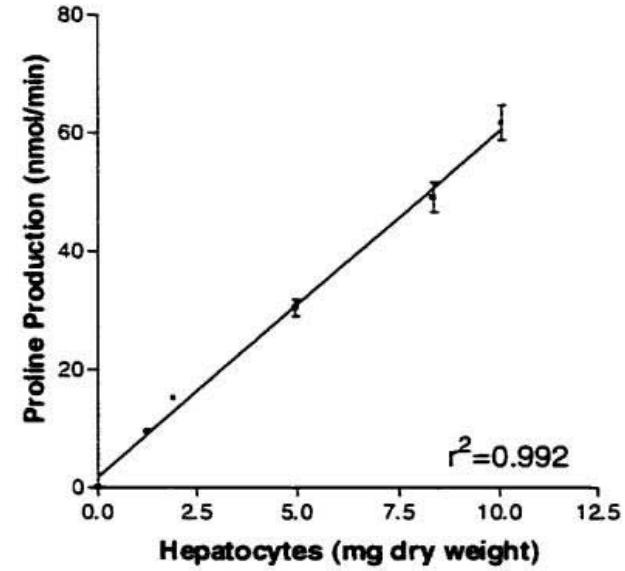
2.6 Antegrade or Retrograde Perfusion with Digitonin

2.6.1 Preparation of Digitonin

Digitonin, (Calbiochem, La Jolla, CA, USA) high purity grade, was prepared according to Berry et al.[12]. A digitonin stock solution (50 mg/mL) was prepared fresh just before use by dissolving 100 mg of digitonin in 2 mL of MOPS buffer (20 mM MOPS {prepared digitonin-free at 22°C and pH adjusted to 6.7; the pH shifts to 7.0 at the working temperature, 37°C}, 3 mM EDTA). The mixture, a white suspension, was boiled for several minutes until it became clear.

Figure 2.14 Time and Protein Linearity for Proline Production in Isolated Hepatocytes.

Proline production from DL-P5C in isolated hepatocytes was due to the combined actions of the P5C transporter and P5C reductase. Production of proline in isolated hepatocytes was linear with time (using ~ 5 mg hepatocytes) and protein (dry weight of hepatocytes; 15 minute incubation period).The data are presented as the mean ± standard deviation, n = 4.


Proline Production (nmol/min)
80
60
40
20
0
0.0
2.5
5.0
7.5
10.0
12.5
Hepatocytes (mg dry weight)
r²=0.992

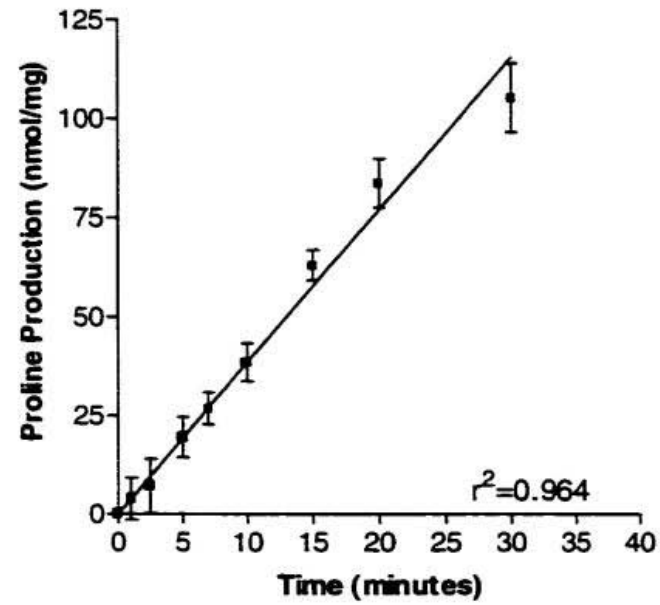
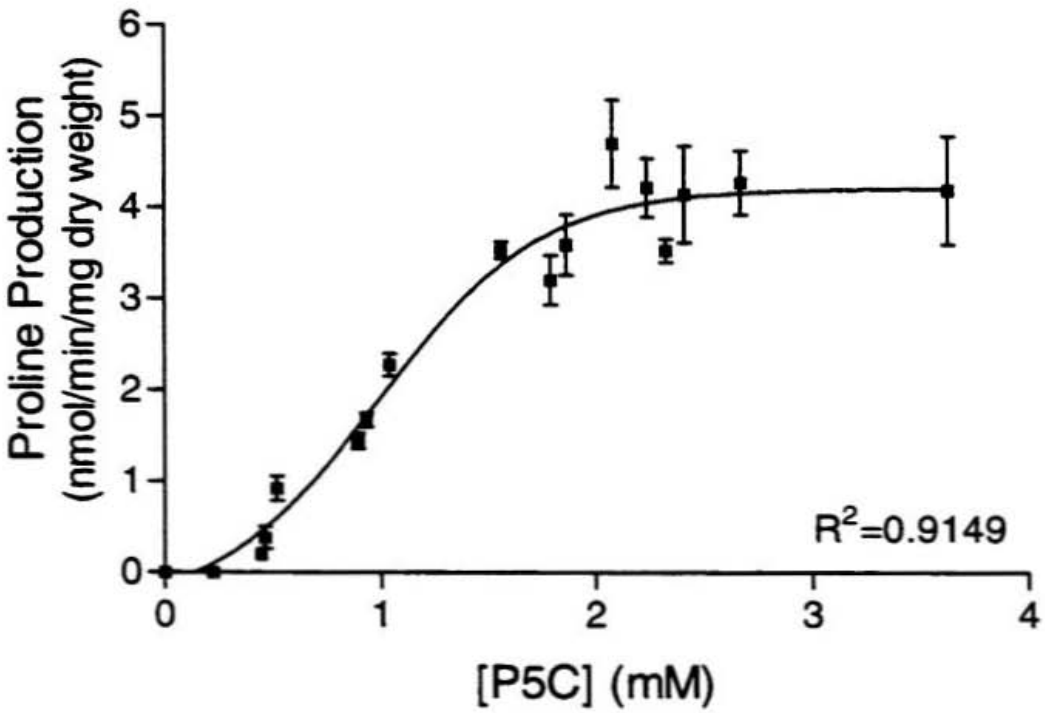

Proline Production (nmol/mg)
125
100
75
50
25
0
0
5
10
15
20
25
30
35
40
Time (minutes)
r²=0.964

Figure 2.15 Proline Synthesis in Isolated Rat Hepatocytes as a Function of DL-P5C Concentration

Isolated hepatocytes (~ 5 mg) were prepared and incubated with DL-P5C for 15 minutes at 37°C in a shaking water bath. Proline was quantified by the acid-ninhydrin method. Data are presented as mean ± standard deviation, n=3.


Proline Production
(nmol/min/mg dry weight)
6
5
4
3
2
1
0
0
1
2
3
4
[P5C] (mM)
R^2=0.9149

The working solution (5 or 10 mg/ml) was prepared by dilution of the stock in Ca^{2+}-free Krebs-Henseleit buffer, followed by filtration through a 0.45 micron syringe tip filter prior to perfusion.

2.6.2 Digitonin Infusion

Digitonin perfusion was performed essentially as described by Quistorff[145]. Following stabilization of the non-recirculating perfusion with Ca^{2+} free Krebs-Henseleit buffer, a single lobe was (randomly) taken for histological analysis. Digitonin was infused at 1.1 mL/min into the perfusion flow using a Harvard Apparatus infusion pump. Retrograde infusions of digitonin for 15, 30, or 45 seconds were employed for enriched preparations of periportal cells. Enriched preparations of perivenous cells were prepared in a similar fashion following antegrade infusion of digitonin (90 second infusion). A second lobe was taken for histological analysis following digitonin infusion, but prior to perfusion with Ca^{2+}-plus Krebs-Henseleit buffer. The addition of the Ca^{2+}-plus medium appears to enhance the removal of cytoplasmic debris as it is released from damaged cells. This was noted as a loss of the characteristic pattern of damage following digitonin infusion and a general (uniform) pale discoloring of the liver when Ca^{2+}-plus medium was infused.

2.7 Histological Analyses

Liver samples taken for histological analysis were immediately placed in 10% buffered formalin and kept in the cold. These samples were prepared for histological analysis by Mr. Ed Evelyn, Mrs. Judy Foote and Mr. Mike Goldworthy of the Histology Unit of the Health Sciences Complex (St. John's, Newfoundland). Liver sections were stained with haematoxylin and eosin for the examination of necrotic damage and with oil red O for fat deposition. Processed slides were first checked by the staff of the histology unit for quality of the sample; the staff knew that some specimens were treated with toxins and the tissue may be more delicate for processing. Samples were assessed by light microscopy to determine extent of necrosis.

3 Results

3.1 Selective Zonal Necrosis with Hepatotoxins

Specific toxins may be used to produce necrosis in the liver. This technique has been applied to investigations of hepatic zonation. Acinar localization of the enzymes involved in hepatic proline metabolism was initially investigated using three particular toxins. Carbon tetrachloride[65] and bromobenzene[89] were used to induce necrosis in the perivenous zone. Allyl alcohol[78] was used to induce necrosis in the cells of the periportal zone.

3.1.1 Selective Zonal Necrosis with Carbon Tetrachloride

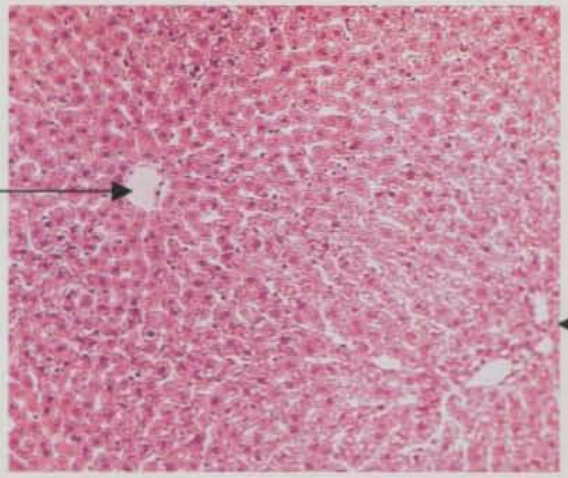
Pathologically, the administration of CCl_4 leads to fatty liver and destruction of the cells in the perivenous region of the liver acinus[150]. Presumably, the perivenous location of enzymes involved in the detoxification or biotransformation of CCl_4 leads to increased susceptibility of the perivenous region to damage from this compound or its related metabolites. In this study, CCl_4 was administered to selectively damage cells in the perivenous region to examine the acinar distribution of enzymes involved in proline metabolism.

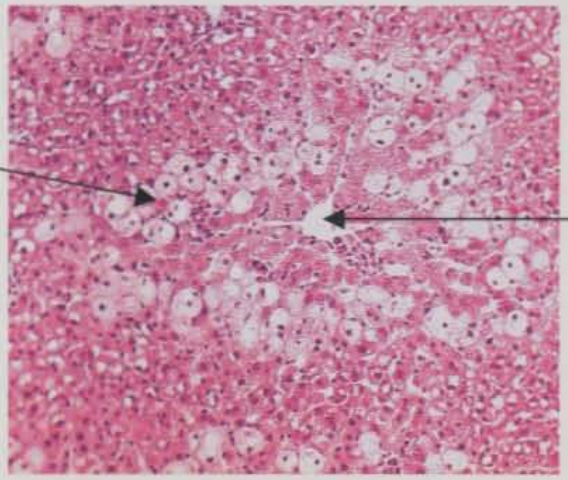
3.1.1.1 Results of the CCl_4 Experiments

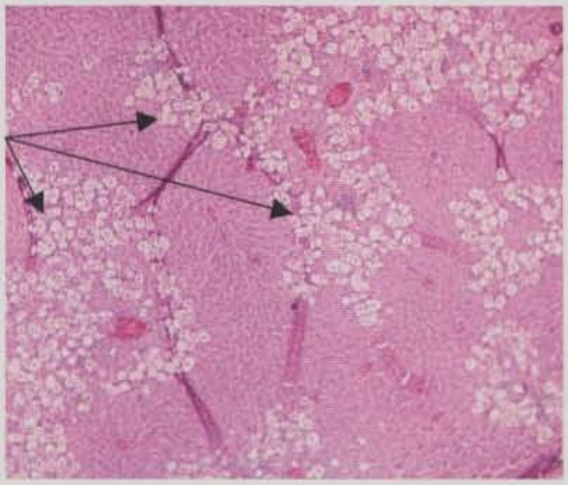
Intoxication with CCl_4 lead to diffuse damage in the perivenous region of the acinus as indicated by histological analysis following staining with haematoxylin and eosin (Figure 3.1) . The livers of treated animals were enlarged and showed

Figure 3.1 Selective Perivenous Damage with Carbon Tetrachloride

Following a 24 hour fast, rats were administered carbon tetrachloride (1.0 mmol/100 g body weight) by gastric gavage to induce perivenous zone damage. Rats were killed 24 hours later and portions of the livers preserved in 10% buffered formalin for histological analysis. Liver sections were stained using haematoxylin and eosin to determine the extent of necrosis. The upper panel is typical of a control animal liver (magnification 40X). The middle panel illustrates a typical liver following administration of CCl_4 (magnification 40X), note the "balloon cells" surrounding the hepatic venule. The bottom panel also shows a liver following CCl_4 intoxication (16X); note the diffuse pattern of damage.


Hepatic
Venule
Portal
Triad


Necrotic
Cells
Hepatic
Venule


Necrotic
cells

the characteristic pale discolourings on the liver surface associated with perivenous damage.

Initial experiments with CCl_4 poisoning utilized liver homogenates from non-fasted animals for the analysis of enzyme activity. The results of these preliminary experiments are presented in Table 3.1. No significant differences were seen with marker enzymes for either the perivenous or periportal zone. However, a two fold increase was seen in the activity of P5C reductase. These initial experiments were repeated and similar results observed (data not shown).

Subsequent experiments included a period of fasting and the preparation of isolated hepatocytes to investigate zonation of P5C metabolism. Following preparation of isolated hepatocytes, broken cells were used to determine the activity of several enzymes, including markers of the perivenous and periportal zones (Table 3.2). Glutamine synthetase and ornithine aminotransferase activities were significantly decreased by 58% and 48% respectively. The activity of ornithine transcarbamylase was increased by 28% following intoxication with CCl_4. The production of proline was increased by 60% when compared to controls.

3.1.2 Selective Zonal Necrosis with Bromobenzene

Bromobenzene, like carbon tetrachloride has been reported to selectively cause damage in the perivenous zone of the hepatic acinus[89]. James et al.[78]

Table 3.1 Zonation Studies in Liver Homogenates after CCl_4 Induced Cirrhosis

Enzyme	Zonal Location	Control	CCl_4 Treated
P5C Reductase[1]	Unknown	44.18 ± 9.77	79.96 ± 10.69*
Glutamine Synthetase[1]	PV	19.19 ± 4.54	19.26 ± 10.55
Ornithine Aminotransferase[2]	PV	17.22 ± 2.31	12.79 ± 5.24
Ornithine Transcarbamylase[2]	PP	784.1 ± 174.1	722.0 ± 63.23

1 P5C reductase and glutamine synthetase were assayed with cytosolic protein.
2 Ornithine aminotransferase and ornithine transcarbamylase were assayed with mitochondrial protein.
* Data are significantly different from controls, Student's t-test ($p<0.05$).
Data are presented as Mean ± Standard deviation (nmol/min/mg protein), n = 4 in each group.
PV perivenous hepatocytes.
PP periportal hepatocytes.

Table 3.2 Zonation Studies in Isolated Hepatocytes after CCl_4 Induced Cirrhosis

Enzyme	Zonal Location	Control	CCl_4 Treated
Proline Production[1] *(P5C Transport + P5C reductase)* nmol/min/mg dry weight	Unknown	1.42 ± 0.34	2.34 ± 0.50*
Glutamine Synthetase[2] nmol/mg/min	PV	21.34 ± 3.71	12.29 ± 7.23*
Ornithine Aminotransferase[2] nmol/min/mg	PV	2.14 ± 0.46	1.02 ± 0.57*
Ornithine Transcarbamylase[2] nmol/min/mg	PP	971.3 ± 211.7	1246 ± 145.6*

1 Intact hepatocytes.
2 Broken hepatocytes.
* Data significantly different from controls, Student's t-test ($p < 0.05$).
Data are presented as Mean ± Standard deviation, n = 4 in each group.
PV perivenous hepatocytes.
PP periportal hepatocytes.

characterized CCl_4 induced necrosis as midzonal to centrilobular; damage caused by bromobenzene intoxication however, provided a more tightly circumscribed area of damage around the hepatic venule. In this study, gastric gavage of bromobenzene was used to provide a more compact model of perivenous localized damage to examine the localization of enzymes involved in proline metabolism.

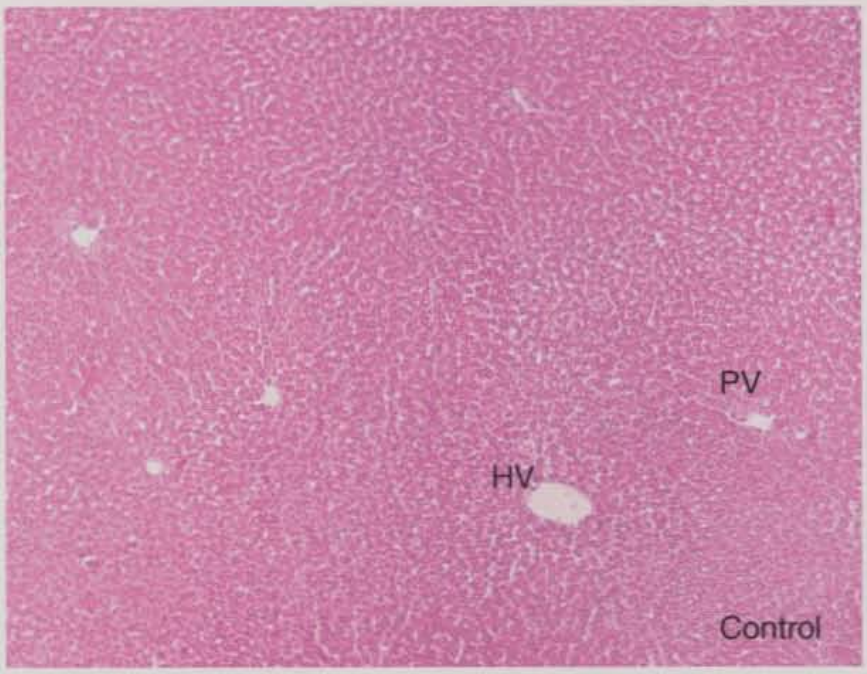
3.1.2.1 Results of the Bromobenzene Experiments

The administration of bromobenzene to rats resulted in extensive damage to the perivenous zone which appeared as large necrotic areas around the hepatic venule (Figure 3.2). The livers of rats treated with bromobenzene did not appear enlarged, contrary to the effects of CCl_4 administration. The livers did show the characteristic pattern of discolored areas over the liver surface associated with damage to the perivenous zone.

The activity of the perivenous zone cytoplasmic marker enzyme, glutamine synthetase was decreased by 64% following bromobenzene treatment, while the perivenous mitochondrial marker, ornithine aminotransferase, was not significantly decreased (Table 3.3). When these enzyme activities were compared using the relative DNA concentration of the protein samples, glutamine synthetase activity decreased by greater than 50% and ornithine aminotransferase activity decreased by greater than 20% (Table 3.4) Ornithine transcarbamylase activity was not significantly affected by bromobenzene

Figure 3.2 Selective Perivenous Damage with Bromobenzene

Following a 24 hour fast, rats were administered bromobenzene (3.8 mmol/kg body weight) by gastric gavage to induce perivenous zone damage. Rats were killed 24 hours later and portions of the livers preserved in 10% buffered formalin for histological analysis. Liver sections were stained using haematoxylin and eosin to determine the extent of necrosis. The top panel shows a liver section from a control animal (40X magnification). The bottom panel shows a typical liver section from a bromobenzene treated animal (40X magnification). Note the massive, yet discrete damage around the hepatic venule.


PV
HV
Control

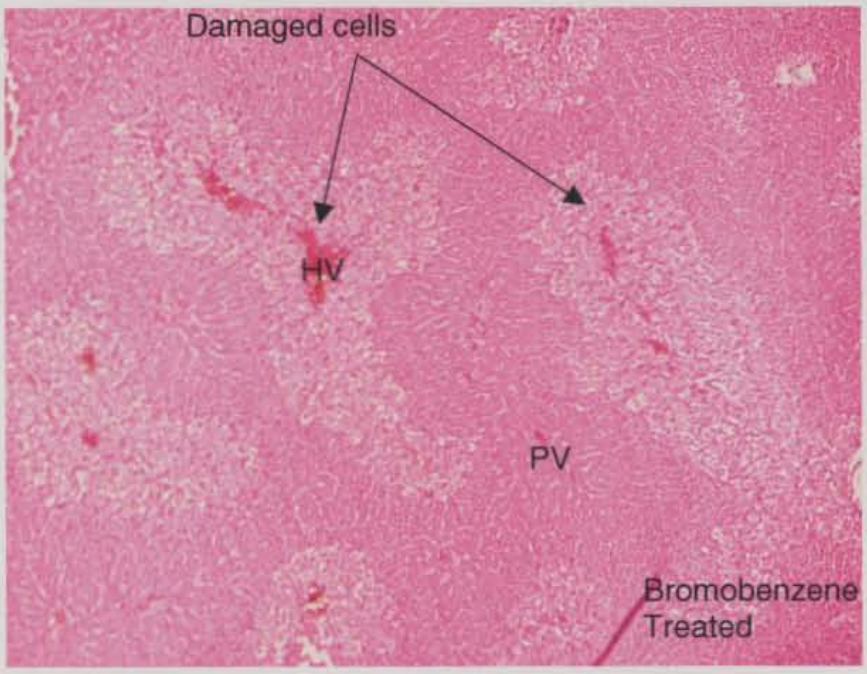

Damaged cells
HV
PV
Bromobenzene
Treated

Table 3.3 Zonation Studies in isolated Hepatocytes after Bromobenzene Induced Cirrhosis (Protein Based Activity)

Enzyme	Zonal Location	Control	Bromobenzene Treated
Proline Production[1]	Unknown	0.92 ± 0.32	3.44 ± 1.21*
Glutamine Synthetase[2]	PV	11.29 ± 2.36	4.05 ± 2.02*
Ornithine Aminotransferase[2]	PV	3.98 ± 0.76	3.56 ± 0.62
Proline Oxidase[2]	Unknown	9.29 ± 0.60	8.26 ± 0.73*
Ornithine Transcarbamylase[2]	PP	860.3 ± 15.53	838.6 ± 39.96

1. Proline production has been measured in intact hepatocytes. Production of proline assumed to be derived from the transport of proline and the activity of P5C reductase. Data are expressed as nmol/min/mg dry weight; mean ± standard deviation.

2. Activity assayed in broken hepatocytes and expressed as nmol/min/mg of broken hepatocytes.

* Test samples are significantly different from controls; $p < 0.05$, student's t-test; n = 8 animals in each group.

Table 3.4 Zonation Studies in isolated Hepatocytes after Bromobenzene Induced Cirrhosis (DNA Activity)

Enzyme	Zonal Location	Control	Bromobenzene Treated
Proline Production[1]	Unknown	0.035 ± 0.012	0.13 ± 0.046*
Glutamine Synthetase[2]	PV	6.53 ± 1.36	2.34 ± 1.17*
Ornithine Aminotransferase[2]	PV	2.30 ± 0.44	2.06 ± 0.36
Proline Oxidase[2]	Unknown	5.37 ± 0.35	4.77 ± 0.42*
Ornithine Transcarbamylase[2]	PP	497.3 ± 8.98	484.7 ± 23.1

1. Proline production has been measured in intact hepatocytes. Production of proline assumed to be derived from the transport of proline and the activity of P5C reductase. Data are expressed as nmol/min/μg DNA; mean ± standard deviation.

2. Activity assayed in broken hepatocytes and expressed as nmol/min/μg DNA.

* Test samples are significantly different from controls; $p < 0.05$, student's t-test; n = 8 animals in each group.

administration. Proline production exhibited more than a three-fold increase following bromobenzene administration (Table 3.3). Proline oxidase activity was decreased by 11% (Table 3.3), this decrease was also evident if the activity of proline oxidase was compared per unit DNA (Table 3.4).

3.1.3 Selective Zonal Necrosis with Allyl Alcohol

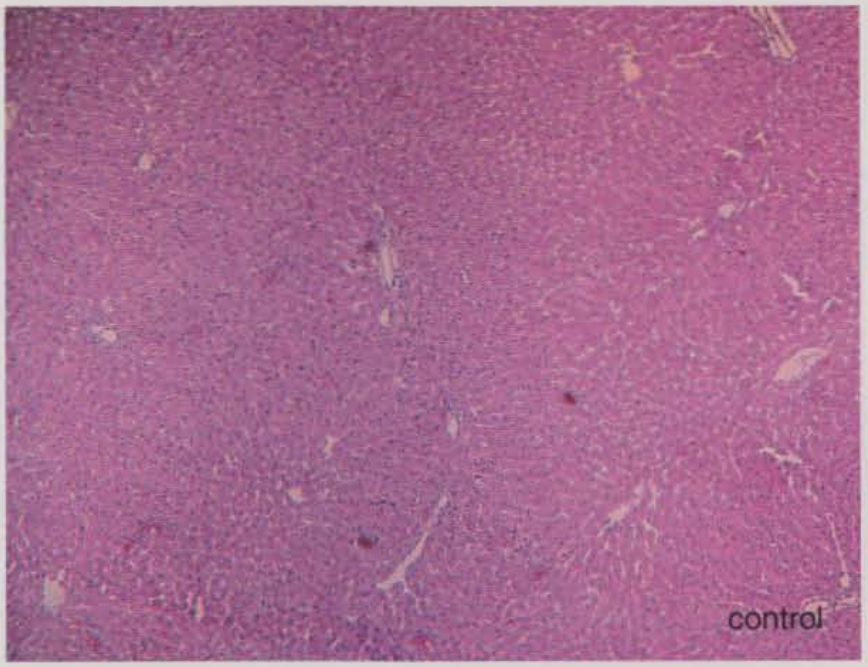
The administration of allyl alcohol has been used to selectively induce necrosis to periportal hepatocytes[78]. Allyl alcohol is converted in the liver to acrolein, the hepatotoxic metabolite, by the action of alcohol dehydrogenase[4]. In this study, allyl alcohol was used to provide an *in vivo* model of periportal necrosis to study the zonal location of proline production and proline oxidase activity.

3.1.3.1 Results of the Allyl Alcohol Experiments

Allyl alcohol administration to rats by gastric gavage resulted in damage to cells in the periportal zone as shown by histological analysis (Figure 3.3). The activity of ornithine transcarbamylase was significantly decreased by 20% in hepatocytes from treated animals compared with controls (Table 3.5 and Table 3.6). The activity of the perivenous markers, OAT and GS were not affected. Proline production and the activity of proline oxidase were also not affected by allyl alcohol administration.

Figure 3.3 Selective Periportal Damage with Allyl Alcohol

Following a 24 hour fast, rats were administered allyl alcohol (1.0 mmol/kg body weight) by gastric gavage to induce perivenous zone damage. Rats were killed 24 hours later and portions of the livers preserved in 10% buffered formalin for histological analysis. Liver sections were stained using haematoxylin and eosin to determine the extent of necrosis. The upper panel photograph shows a typical liver section from a control animal receiving only the corn oil vehicle (40X magnification). The photograph in the bottom panel is from an allyl alcohol-treated animal (40X magnification). Note the diffuse damage in the area periportal (zone 1) region of the liver while the cells around the central vein appear unaffected.


control

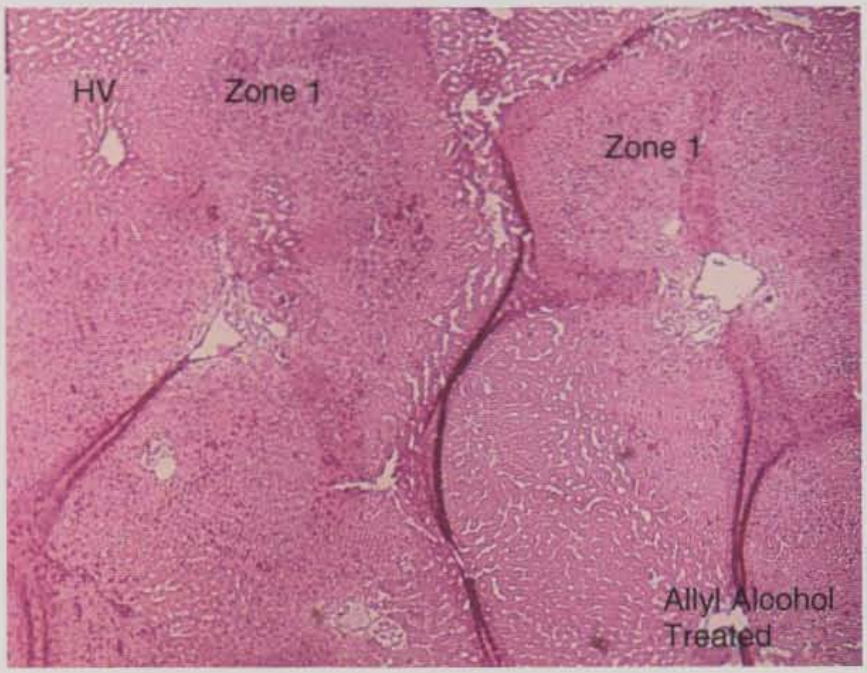

HV
Zone 1
Zone 1
Allyl Alcohol
Treated

Table 3.5 Zonation Studies in Isolated Hepatocytes after Allyl Alcohol Induced Liver Cirrhosis (Protein Activity)

Enzyme	Zonal Location	Control	Allyl Alcohol Treated
Proline Production[1]	Unknown	0.88 ± 0.18	1.01 ± 0.27
Glutamine Synthetase[2]	PV	10.44 ± 4.01	11.71 ± 3.28
Ornithine Aminotransferase[2]	PV	5.35 ± 1.79	4.73 ± 1.11
Proline Oxidase[2]	Unknown	11.71 ± 3.44	10.39 ± 3.08
Ornithine Transcarbamylase[2]	PP	1036 ± 84.55	831.3 ± 112.2*

1. Proline production has been measured in intact hepatocytes. Production of proline assumed to be derived from the transport of proline and the activity of P5C reductase. Data are expressed as nmol/min/mg dry weight; mean ± standard deviation.

2. Activity assayed in broken hepatocytes and expressed as nmol/min/mg of broken hepatocytes.

* Test samples are significantly different from controls; $p < 0.05$, student's t-test; $n = 4$ in the control group, $n = 9$ animals in the allyl alcohol group.

Table 3.6 Zonation Studies in Isolated Hepatocytes after Allyl Alcohol Induced Liver Cirrhosis (DNA Activity)

Enzyme	Zonal Location	Control	Allyl Alcohol Treated
Proline Production[1]	Unknown	0.032 ± 0.0065	0.037 ± 0.0098
Glutamine Synthetase[2]	PV	6.03 ± 2.32	6.77 ± 1.90
Ornithine Aminotransferase[2]	PV	3.09 ± 1.03	2.73 ± 0.64
Proline Oxidase[2]	Unknown	6.77 ± 1.99	6.06 ± 1.78
Ornithine Transcarbamylase[2]	PP	598.8 ± 48.87	480.5 ± 64.86*

1. Proline production has been measured in intact hepatocytes. Production of proline assumed to be derived from the transport of proline and the activity of P5C reductase. Data are expressed as nmol/min/μg DNA; mean ± standard deviation.

2. Activity assayed in broken hepatocytes and expressed as nmol/min/μg of DNA.

* Test samples are significantly different from controls; $p < 0.05$, student's t-test; $n = 4$ in the control group, $n = 9$ animals in the allyl alcohol group.

3.1.4 Discussion of the *In Vivo* Selective Zonal Necrosis Experiments

Chemically induced hepatotoxicity has been employed as a model for the investigation of hepatic metabolic zonation. The molecular and cellular events which define the induction of a particular necrotic event are impacted upon by different factors including enzyme induction and inhibition as well as the nutritional status of the animal [117].

In regard to the effects of the toxins employed in this study, the dietary status of the animal was relevant. Preliminary experiments (Table 3.1) with CCl_4 poisoning utilized animals with *ad libitum* access to food. Treated animals demonstrated a substantial decrease in food intake compared to control animals. To control for decreased food intake, animals were subsequently fasted prior to and following CCl_4 administration. McLean and McLean [112] demonstrated that rats experienced heightened sensitivity to CCl_4 following a starvation period of 18 hours. Similar heightened sensitivity was also demonstrated by bromobenzene treated rats fasted prior to exposure[139]. Strubelt et al.[172] demonstrated a moderate increase in allyl alcohol toxicity with fasting, whereas Hanson and Anders[59]showed a protection against allyl alcohol toxicity by fasting. However, it has been shown that increased allyl alcohol toxicity is correlated with diminished levels of glutathione[59;151]. Pessayre and colleagues[139] were able to show that basal hepatic glutathione was decreased by 40% after 18 hours of fasting and 60% after 42 hours of fasting. Diminished levels of glutathione have been directly linked to increased toxicity of CCl_4 and bromobenzene[139]. Allyl alcohol infusion

through an isolated perfused rat liver was shown to dramatically decrease glutathione levels by 95%, but the decreased levels of glutathione were not correlated with an increased allyl alcohol toxicity[8]. However, depletion of available glutathione is generally thought to result in decreased binding of reactive metabolites to glutathione, thus increasing the binding of these reactive metabolites to proteins or lipids which subsequently enhances the hepatotoxicity of these reactive metabolites[139]. Thus it seems that the combined effects of fasting and toxin administration should have provided an acceptable environment in the livers of treated rats to generate the desired necrotic effects. It was on this premise that the described dietary regime and toxin administration was implemented.

The results of the toxin experiments suggest that the majority of the enzymes involved in the metabolism of P5C are enriched in the perivenous zone. Glutamine synthetase activity, the definitive marker of the perivenous cells most proximal to the terminal hepatic venule, was significantly decreased following CCl_4 (43%) and bromobenzene (64%) treatment. Ornithine aminotransferase activity was also decreased following these toxin treatments, 52% following CCl_4 treatment and 11% following bromobenzene treatment. Ornithine transcarbamylase activity, a marker of the periportal region[122], was unaffected by the bromobenzene treatment. However, a 28% increase in activity was noted following CCl_4 treatment. This increase may simply reflect the increased ratio of periportal to perivenous cells which normally results from damage to the

perivenous region. These results suggest that a significant amount of damage had occurred in those cells immediately surrounding the terminal hepatic venule, with lesser damage occurring in cells distal to the terminal hepatic venule.

A 20% decrease in OTC activity was noted following allyl alcohol treatment. The markers of the perivenous region, glutamine synthetase and ornithine aminotransferase were not affected by allyl alcohol treatment. These results suggested that a significant proportion of periportal cells were damaged, while the perivenous region remained intact. The degree of allyl alcohol-induced periportal damage cannot be ascribed to a specific locale in terms of cells proximal to the portal vein versus cells which may be described as more distal or even mid-zonal (*zone 2*). A specific marker (protein, mRNA or enzyme activity) for cells occupying *zone 2* has not been described. Ornithine transcarbamylase has been ascribed to both zones 1 and 2[122], thus we were unable to define the relative area (proximal or distal to the portal vein) of damage.

Proline oxidase activity was significantly decreased following administration of bromobenzene. Other studies have also demonstrated decreased proline oxidase activity following damage to the perivenous zone. Ehrinpreis and colleagues[34] used CCl_4 for seven weeks to induce cirrhosis in young rats. During this study, they noted that liver proline oxidase activity was dramatically reduced 24 hours after the initial administration of the toxin. Although they were unable to identify the cause of the decrease, CCl_4 is now known to specifically destroy cells in the perivenous region of the liver acinus[78].

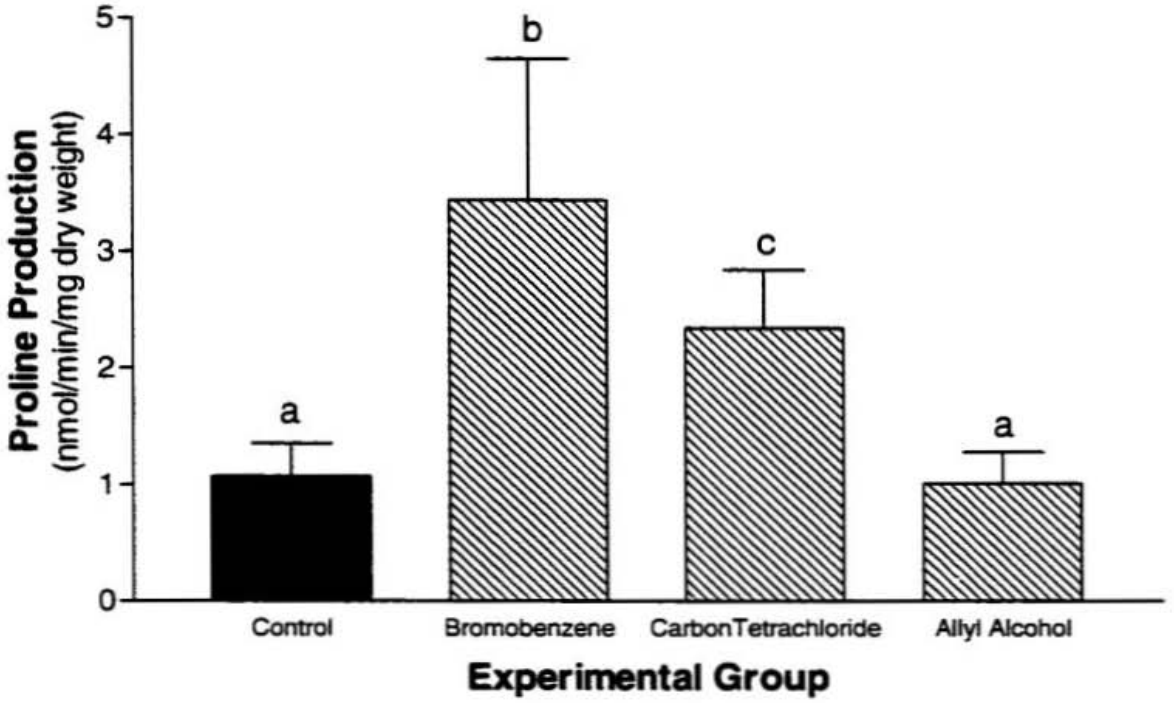
In agreement with these results, the activity of proline oxidase was not affected by damage to the periportal zone by allyl alcohol. The activity of OAT was decreased in the bromobenzene treated rats by 11%. The activity of proline oxidase decreased in a similar fashion (11%). Taken together, the results of the toxin experiments have provided evidence to suggest a perivenous enrichment of proline oxidase. The enriched zone is not as discrete as the glutamine synthetase zone, but rather is probably as broad as the ornithine aminotransferase positive zone.

The production of proline from P5C in hepatocytes isolated from toxin treated rats was a combination of P5C transport across the plasma membrane of the hepatocyte and the action of P5C reductase in the cytosol. Figure 3.4 provides a summary of the data for proline production in isolated hepatocytes from toxin-treated animals.

From these data, it can be seen that significant bromobenzene-induced perivenous zone destruction increased proline production from P5C. Damage to the perivenous and mid-zones by CCl_4-induced necrosis demonstrated a decreased capacity (relative to bromobenzene data) for proline production, although a significant increase compared to control hepatocytes was still seen. Finally, damage to the periportal cells following allyl alcohol-induced damage results in a further decrease in proline production relative to bromobenzene and carbon tetrachloride results, but there was no difference when compared to

Figure 3.4 Proline Production in Isolated Hepatocytes From Toxin-Treated Rats.

Data from the toxin experiments have been summarized and analyzed using one-way ANOVA (Tukey post-test). Bars with different letters are significantly different ($p<0.05$). Control data from individual experiments were pooled ($n = 16$) (no difference was noted when control groups from different experiments were compared), bromobenzene treated ($n = 8$), carbon tetrachloride treated ($n = 4$), allyl alcohol treated ($n = 9$). Data are presented as mean ± standard deviation.


Proline Production
(nmol/min/mg dry weight)
5
4
3
2
1
0
a
b
c
a
Control
Bromobenzene
CarbonTetrachloride
Allyl Alcohol
Experimental Group

controls. These results appear to suggest a periportal enrichment of proline synthesis. However, three confounding variables are possible. First, as the perivenous zone was damaged, activity of enzymes enriched in this zone was subsequently lost or decreased. Second, damage to the perivenous zone could cause an induction of proline synthesis. Third, toxin-induced damage could cause a loss of a transport barrier which may be limiting for proline synthesis.

The enzymes which would normally metabolize P5C or proline in the perivenous zone, specifically P5C dehydrogenase[132] and proline oxidase were significantly decreased following damage to the perivenous zone. Thus, the effects of bromobenzene- and carbon tetrachloride-induced necrosis may confound the data regarding proline production from P5C by decreasing the utilization of P5C for other pathways and by decreasing the catabolism of proline produced from P5C. In this regard, more proline would accumulate. Allyl alcohol treatment would decrease the ability to convert P5C to proline (assuming P5C reductase was localized to the periportal zone). Also, the ability to catabolize proline, via the action of proline oxidase, or to alternatively metabolize P5C, via P5C dehydrogenase or ornithine aminotransferase, was still intact. The actions of these enzymes could decrease the amount of P5C available for conversion to proline.

The transport of proline P5C across the plasma and mitochondrial membranes may be affected by the administration of toxins. Following hepatic perfusion of ethanol, Fellenius et al.[39] observed an 80% inhibition of proline

consumption. Ethanol treatment has been shown to significantly decrease the transport of amino acids via the Na^+-dependent System A transporter system in primary cultures of hepatocytes[32;154] and in basolateral (sinusoidal) rat liver plasma membrane vesicles[130]. Blocking the metabolism of ethanol with pyrazole failed to alleviate the inhibition of α-amino isobutyric acid (AIB) transport, suggesting that the presence of ethanol, rather than its metabolism, was the factor contributing to the inhibition of transport[154]. If the administration of bromobenzene or carbon tetrachloride produced similar effects, then the decrease in proline consumption/transport in the liver would need to be compensated by an increase in P5C transport. P5C transported into hepatocytes could then be utilized by P5C reductase in the cytosol to produce proline. Phang demonstrated that there was no shared carrier for P5C and any other amino acid and that entry into the [Chinese Hamster Ovary] cell was concomitant with its conversion to proline[119].

Short-term administration of ethanol (14 days) does not result in necrosis of any hepatocyte populations[11], suggesting that necrosis of hepatocytes can not entirely account for decreased amino acid transport across the sinusoidal and plasma membrane of hepatocytes. Smith and Ploch[168] discuss the possibility of altered membrane fluidity as a mechanism of modifying amino acid transport across the plasma membrane. The peroxidative damage of structural phospholipids in hepatocytes is a common characteristic of hepatotoxicity by CCl_4[150], bromobenzene[24] and allyl alcohol[4]. It is not inconceivable then, that if

administration of a toxin does not lead to necrosis (generously assuming that focal necrosis is dependent upon the concentration of toxic intermediate encountered), significant lipid peroxidation may occur as to alter membrane fluidity. Such an event could increase vertical (or lateral) displacement of integral membrane (transport) proteins thus impeding binding to the active site and altering transport of the amino acid.

These results provide strong evidence for the perivenous localization of proline oxidase activity in a pattern similar to that of ornithine aminotransferase. The localization of P5C reductase, based upon the production of proline, appears to be enriched, but not exclusively, to the periportal zone. Because of the inconclusive results derived from the *in vivo* results, we sought an *in vitro* approach which would provide better control of enzyme or transport activities that may be affected by the influence or presence of particular hepatotoxins. We sought a method which was very rapid, therefore allowing no time for the induction of enzymes which may have occurred in the hepatotoxin studies.

3.2 Selective Zonal Necrosis using Digitonin Perfusion

The administration of the zone selective toxins carbon tetrachloride, bromobenzene, and allyl alcohol generated results which must be interpreted with care. The administration of these toxins may provide an *in vivo* effect not directly related to the extent of site-directed necrosis, but which may independently affect the activity of the marker enzymes or the systems being

examined. To counter this problem, we undertook a series of experiments to utilize an *in vitro* model of selective zonal damage. Quistorff et al.[146] optimized a model based upon perfusion of the liver with digitonin. This perfusion resulted in the release of the cytosolic constituents. More interestingly, the damage could be directed to the perivenous or periportal zones depending on the direction and duration of digitonin perfusion. This permitted the isolation of proteins and other cellular constituents from specific zones of the liver. We have developed a model using retrograde perfusion of the rat liver (*in situ*) for the purposes of 1. isolating enriched populations of periportal hepatocytes with minimal perivenous cell contamination; 2. Using the enriched populations of periportal cells to investigate the zonal characteristics of proline metabolism. We have also utilized antegrade digitonin perfusion to perform similar investigations.

3.2.1 Digitonin Perfusion to Destroy Perivenous Cells

Rat livers were perfused in the retrograde direction with digitonin (5.0 mg/ml) to damage cells in the perivenous zone. The damage caused by digitonin perfusion demonstrated typical cellular ultra-structural changes. As digitonin was perfused into the liver, the effluent became cloudy and the liver surface became dotted with pale discolored spots (Figure 3.5). Sections from digitonin (retrograde) perfused livers stained with haematoxylin and eosin confirmed the perivenous localized damage (Figure 3.6).

The duration of the digitonin perfusion was varied to determine the optimal time of cell exposure to digitonin. The extent of damage was gauged by the

Figure 3.5 Retrograde Digitonin Perfusion - Surface Pattern

Rat livers perfused retrograde with digitonin show a characteristic pattern on the liver surface. Livers were perfused and photographed *in situ*; panel A: a typical rat liver perfused with Ca^{2+}-free Krebs-Henseleit buffer. Note the clear liver surface. Panel B; magnified view of the same normal liver. Panel C; the same liver following retrograde perfusion with digitonin (5.0 mg/ml) for 45 seconds. Note the spotted pattern seen over the entire liver surface. The upper lobes have been raised to reveal the same pattern is uniform throughout the liver. Panel D; magnification of the same liver to reveal the distinct pattern associated with retrograde digitonin perfusion, and damage to the perivenous zone.

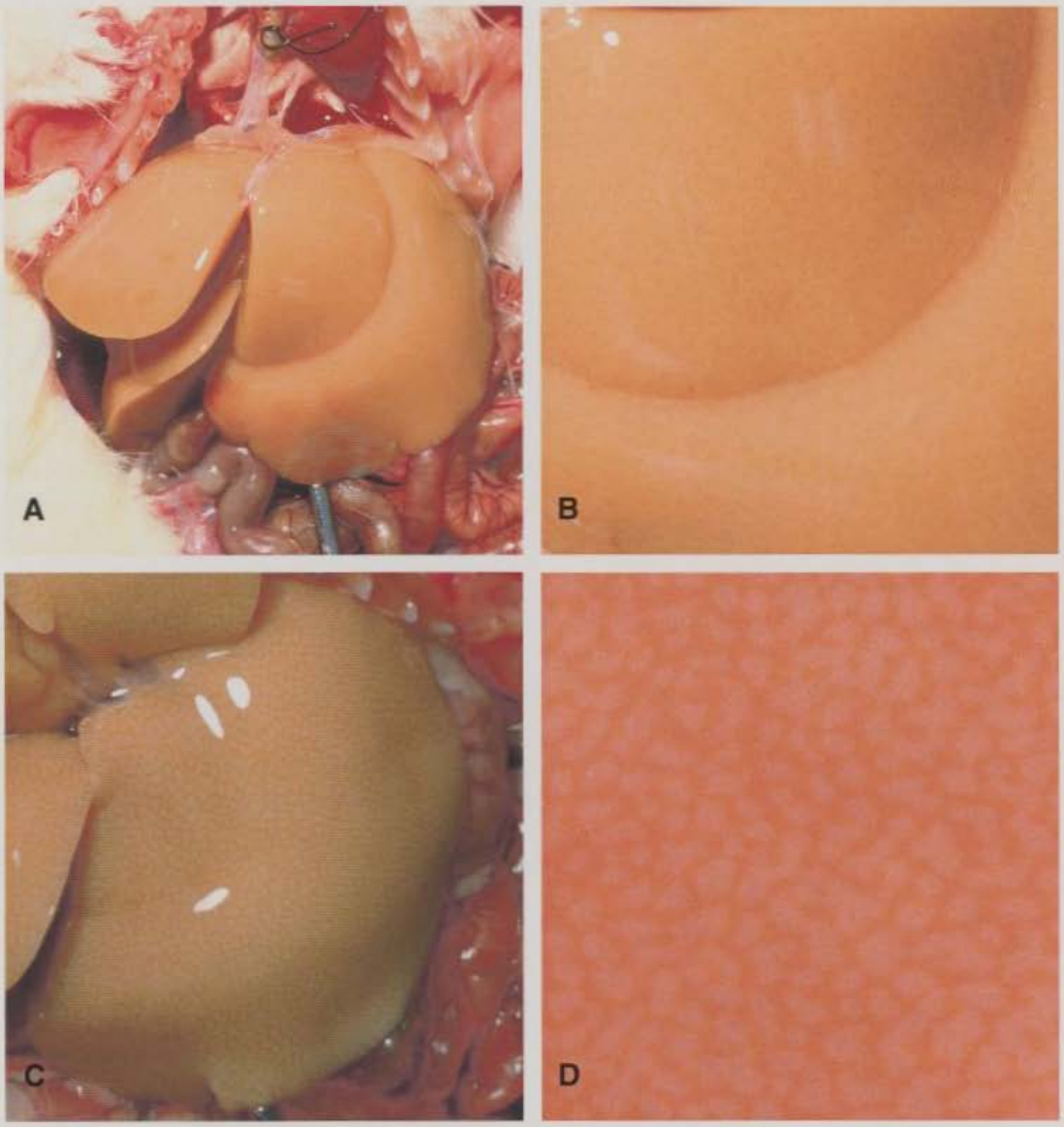
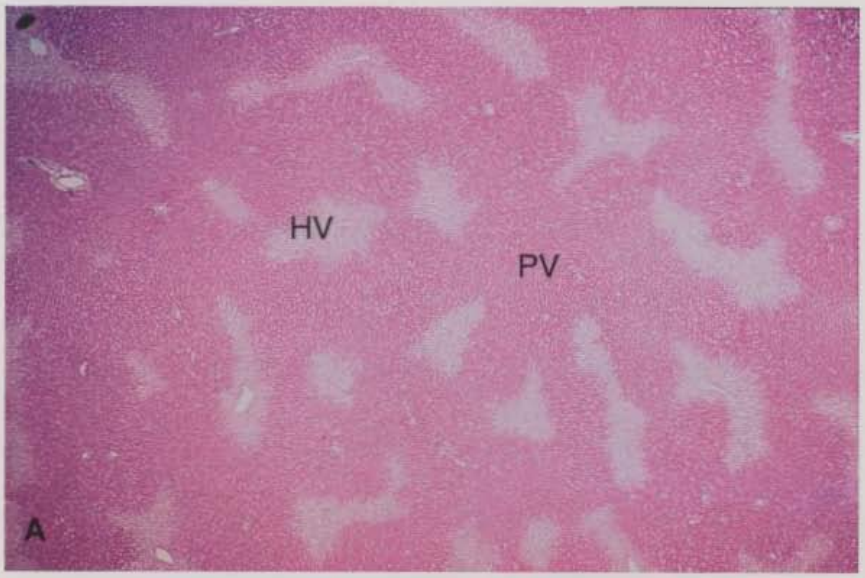
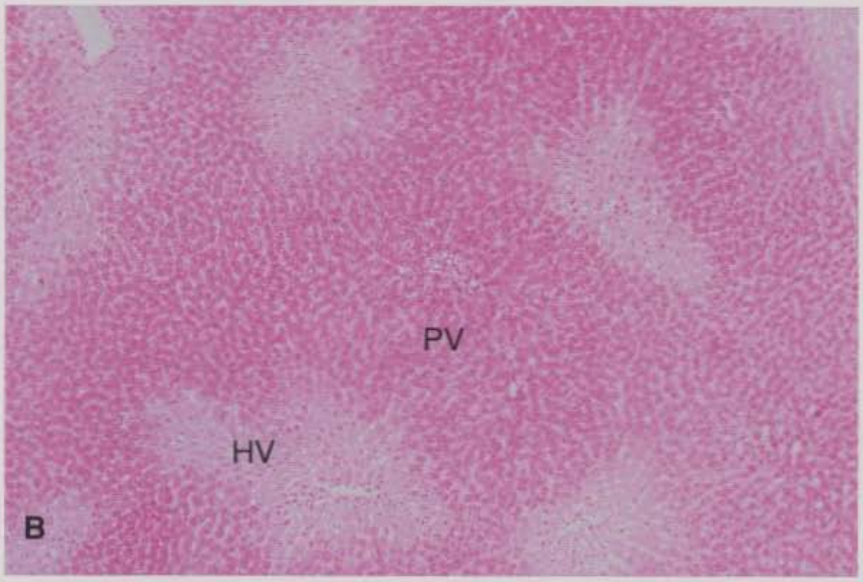

A
B
C
D

Figure 3.6 Selective Perivenous Damage with Retrograde Digitonin Perfusion

Livers were perfused with digitonin (5.0 mg/ml) for 45 seconds to elicit damage in the perivenous zone. Following digitonin perfusion, portions of the livers were preserved in 10% buffered formalin for histological analysis. Liver sections were stained using haematoxylin and eosin to determine the extent of damage. The upper panel (A) photograph shows a typical liver section from an animal perfused with digitonin (magnification 8X). The lower panel (B) is a magnification (40X) of the same section to further demonstrate damage in the perivenous zone.


HV
PV
A


PV
HV
B

decrease in glutamine synthetase activity (Figure 3.7). The activity of glutamine synthetase decreased to an apparent minimum of ~5-10% control activity (30 - 90 seconds perfusion time). The extent of damage was not increased if higher concentrations of digitonin (7.5 or 10 mg/mL) were perfused for similarly timed perfusions (data not shown). Initial experiments (15 second digitonin perfusion) demonstrated an approximate 50% decrease in glutamine synthetase activity (Figure 3.8). However, ornithine aminotransferase activity, which is resident in a slightly broader zone[95], was not significantly decreased, nor was the activity of proline oxidase. The activities of OAT and PO were then determined following 30 and 45 second retrograde perfusion with digitonin (Figure 3.8). It was decided that 45 seconds perfusion was optimal for the maximal destruction of perivenous cells with minimal destruction of periportal cells. The data expressed in Figure 3.8 illustrate the loss of activity of the perivenous-localized enzymes glutamine synthetase and ornithine aminotransferase. In addition the activity of proline oxidase was also decreased with prolonged exposure to digitonin.

Following optimization of the retrograde digitonin-collagenase perfusion technique for the isolation of enriched populations of periportal cells, the zonal characteristics of proline oxidase were investigated. Rats were perfused with digitonin (5.0 mg/mL) for 45 seconds in the retrograde fashion to selectively destroy perivenous cells. Hepatocytes were then isolated and the activities of several enzymes determined. Tables 3.7 and 3.8 outline the results of this experiment. Glutamine synthetase activity was decreased approximately 95%.

Figure 3.7 Glutamine Synthetase Activity Decreases with Increased Duration of Retrograde Digitonin Perfusion

Rat livers were perfused in the retrograde direction with digitonin (5.0 mg/ml) to selectively destroy perivenous hepatocytes. Hepatocytes were isolated and glutamine synthetase activity assayed to determine the extent of perivenous damage. Values are normalized to control activity and expressed as mean ± standard deviation; n=3 for controls and n=4 for test values. Main graph illustrates the decrease in glutamine synthetase activity as a function of digitonin perfusion duration; the smaller graph illustrates the same data expressed as the extent of decrease in glutamine synthetase activity. Asterisks indicate values are significantly different from control activity.

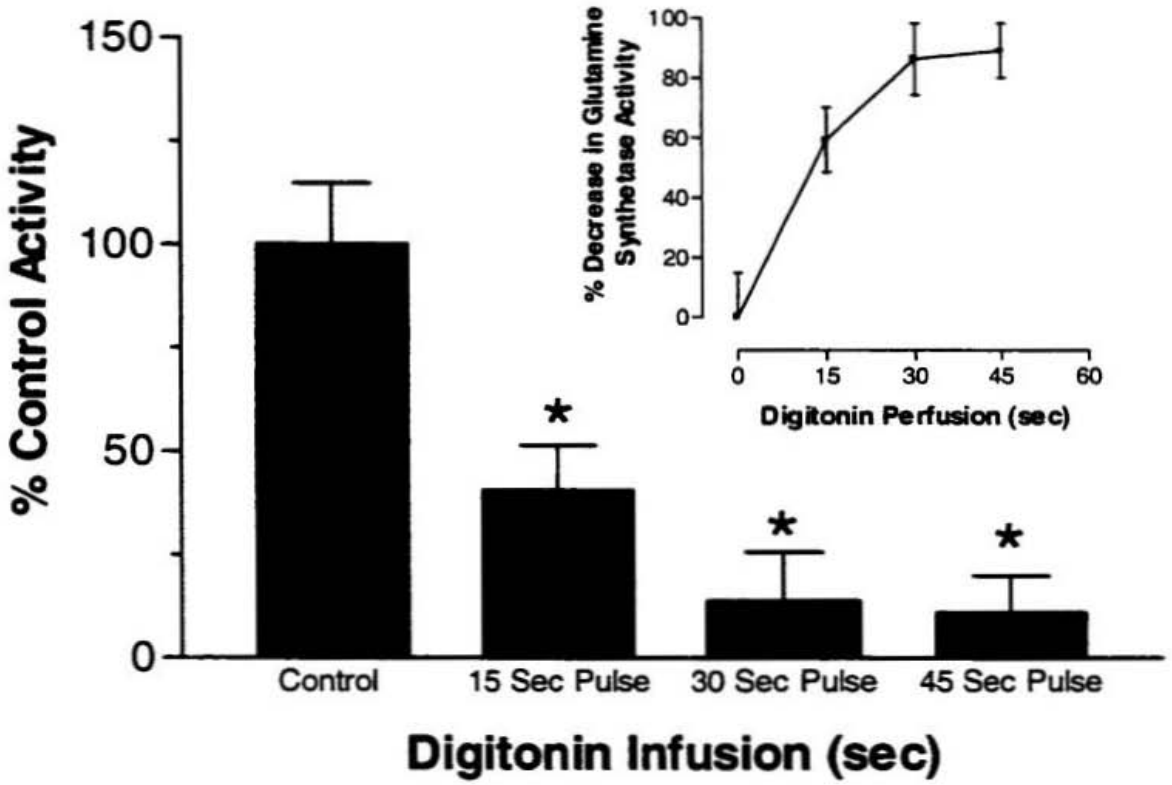

150
100
50
0
% Control Activity
Control
15 Sec Pulse
30 Sec Pulse
45 Sec Pulse
Digitonin Infusion (sec)
*
*
*
% Decrease in Glutamine Synthetase Activity
100
80
60
40
20
0
0
15
30
45
60
Digitonin Perfusion (sec)

Figure 3.8 Decreased Activities of Ornithine Aminotransferase, Proline Oxidase and Glutamine Synthetase Following Prolonged Digitonin Perfusion.

Rat livers were perfused in the retrograde direction with digitonin (5.0 mg/ml) for prolonged periods (0 seconds(control), 30 and 45 seconds) to selectively destroy perivenous hepatocytes. Hepatocytes were isolated and the activities of proline oxidase (A), ornithine aminotransferase (B) and glutamine synthetase (C) determined. Values are expressed as mean ± standard deviation; n=6 for controls, n=4 for 30 seconds and n = 8 for 45 seconds. Asterisks indicate values are significantly different from control activity ($p<0.05$).

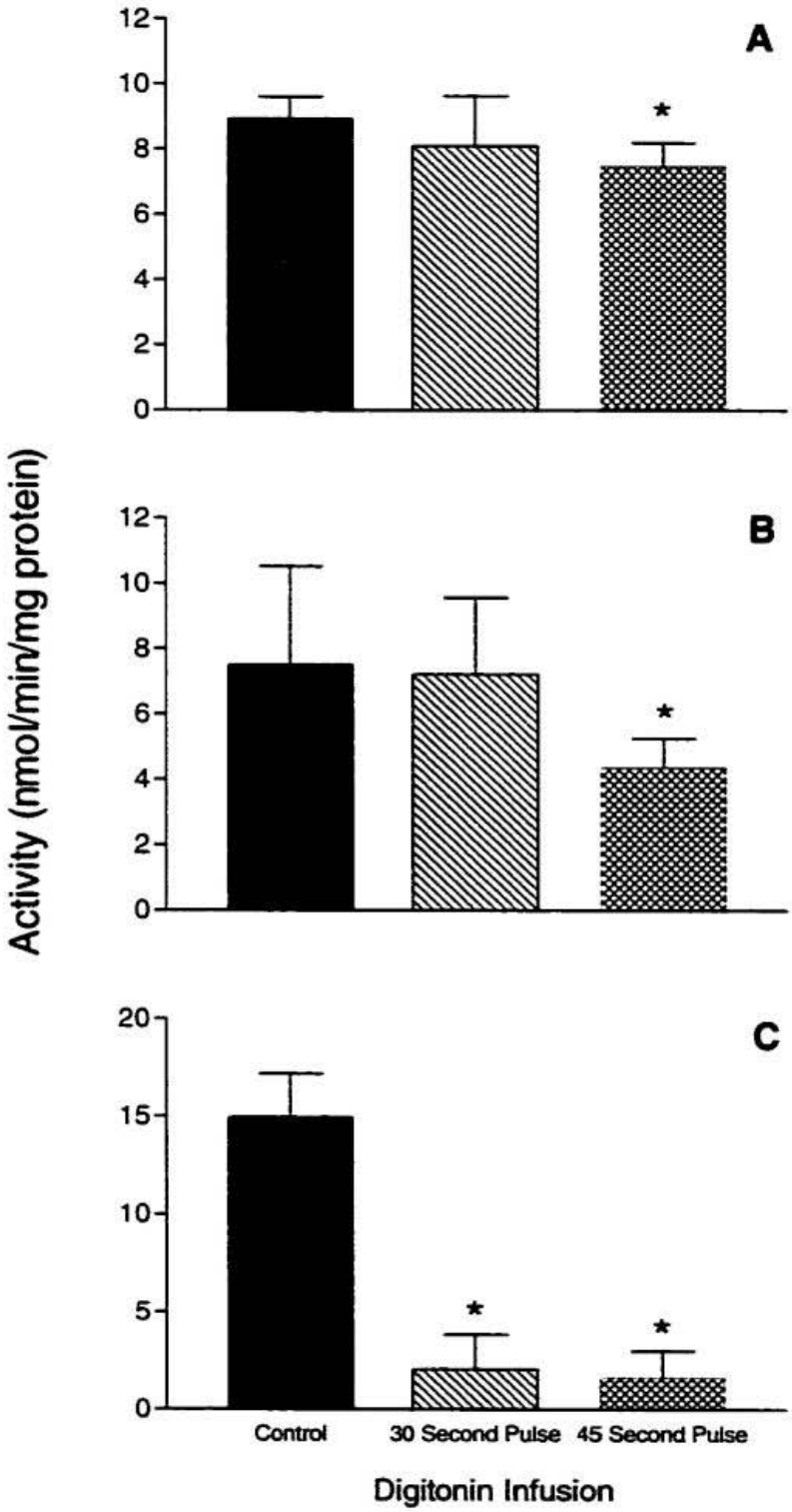

A
B
C
12
10
8
6
4
2
0
20
15
5
*
Activity (nmol/min/mg protein)
Control
30 Second Pulse
45 Second Pulse
Digitonin Infusion

Table 3.7 Zonation Studies in Isolated Hepatocytes following Retrograde Digitonin Perfusion (Protein Based Activity)

Enzyme	Zonal Location	Control	Digitonin Treated
Proline Production[1]	Unknown	3.39 ± 0.80	2.16 ± 0.77*
Glutamine Synthetase[2]	PV	14.23 ± 3.31	0.83 ± 0.76*
Ornithine Aminotransferase[2]	PV	5.24 ± 0.83	3.47 ± 0.82*
Proline Oxidase[2]	Unknown	8.46 ± 1.13	6.10 ± 1.06*
Ornithine Transcarbamylase[2]	PP	1139 ± 212.4	1255 ± 223.2

1. Proline production has been measured in intact hepatocytes. Production of proline assumed to be derived from the transport of proline and the activity of P5C reductase. Data are expressed as nmol/min/mg protein dry weight; mean ± standard deviation.

2. Activity assayed in broken hepatocytes and expressed as nmol/min/mg protein.

* Test samples are significantly different from controls; $p < 0.05$, student's t-test; $n = 6$ animals in the control group and $n = 8$ in the treated group.

Table 3.8 Zonation Studies in Isolated Hepatocytes following Retrograde Digitonin Perfusion (DNA Based Activity)

Enzyme	Zonal Location	Control	Digitonin Treated
Proline Production[1]	Unknown	0.138 ± 0.032	0.088 ± 0.031*
Glutamine Synthetase[2]	PV	12.48 ± 2.90	0.73 ± 0.67*
Ornithine Aminotransferase[2]	PV	4.60 ± 0.73	3.04 ± 0.72*
Proline Oxidase[2]	Unknown	7.42 ± 0.99	5.35 ± 0.93*
Ornithine Transcarbamylase[2]	PP	999.1 ± 186.3	1100 ± 195.8

1. Proline production has been measured in intact hepatocytes. Production of proline assumed to be derived from the transport of proline and the activity of P5C reductase. Data are expressed as nmol/min/µg DNA; mean ± standard deviation.

2. Activity assayed in broken hepatocytes and expressed as nmol/min/µg of DNA.

* Test samples are significantly different from controls; $p < 0.05$, student's t-test; $n = 6$ animals in the control group and $n = 8$ in the treated group.

The activities of ornithine aminotransferase and proline oxidase were decreased 34% and 28% respectively. Ornithine transcarbamylase activity was not affected. The production of proline from P5C in these enriched populations of periportal hepatocytes was significantly decreased by 36%.

3.2.2 Digitonin Perfusion to Destroy Periportal Cells

The results of the retrograde digitonin-perfusions encouraged us to attempt to selectively destroy the periportal cells using antegrade digitonin-perfusion. The photographs in Figure 3.9 illustrate the type of damage observed following antegrade digitonin-perfusion. Contrasting the spotted pattern observed following retrograde digitonin-perfusion, livers perfused with digitonin in the antegrade fashion show a matrix or hatched pattern on the surface of the liver (Figure 3.9, panel A,B). The damaged areas appear shortly after the digitonin perfusion commenced. The liver photographed in panel A (Figure 3.9) appears mottled with patches of liver seemingly not perfused with digitonin, marked by interruptions in the matrix pattern. The interrupted hatched pattern was also observed on a microscopic level (Figure 3.9, panel C).

Analysis of enzyme activity for glutamine synthetase, ornithine aminotransferase, proline oxidase and ornithine transcarbamylase revealed no significant change between control and digitonin-perfused animals (Table 3.9 and Table 3.10). Analysis of proline production from P5C in these animals also showed no significant effect.

Figure 3.9 Antegrade Digitonin Perfusion - Surface and Histological Pattern

Rat livers perfused antegrade with digitonin show a characteristic pattern on the liver surface. Livers were perfused and photographed *in situ*; panel A: a typical rat liver following antegrade perfusion with digitonin (5.0 mg/ml) for 90 seconds. Note the matrix-like pattern seen over the liver surface. Panel B; magnification of the same liver to reveal the distinct pattern associated with antegrade digitonin perfusion, and damage to the periportal zone. Panel C: Following digitonin perfusion, portions of the livers were preserved in 10% buffered formalin for histological analysis. Liver sections were stained using haematoxylin and eosin to determine the extent of damage. The photograph shows a typical liver section from an animal perfused with digitonin in the antegrade direction (magnification 16X). The lighter areas represent the damaged cells; a portion of the lighter area is outlined in the lower right corner. Note the same matrix-like pattern as in panels A and B.

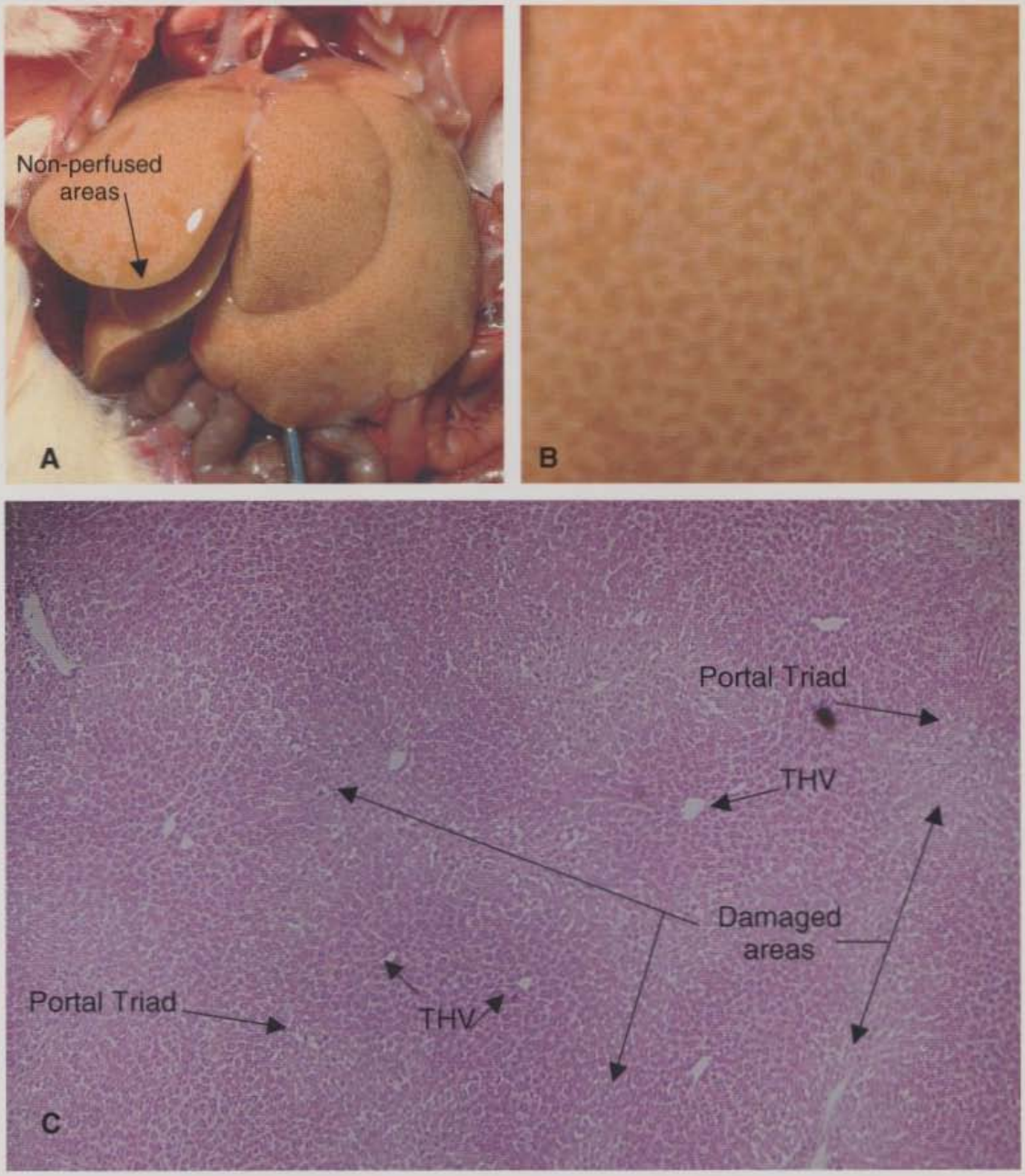

Non-perfused
areas
A
B
Portal Triad
THV
Damaged
areas
Portal Triad
THV
C

Table 3.9 Zonation Studies in Isolated Hepatocytes following Antegrade Digitonin Perfusion (Protein Based Activity)

Enzyme	Zonal Location	Control	Digitonin Treated
Proline Production[1]	Unknown	2.95 ± 1.08	2.55 ± 0.59
Glutamine Synthetase[2]	PV	13.54 ± 5.83	18.51 ± 8.63
Ornithine Aminotransferase[2]	PV	5.39 ± 0.50	7.07 ± 1.29
Proline Oxidase[2]	Unknown	8.29 ± 0.93	9.62 ± 1.13
Ornithine Transcarbamylase[2]	PP	1049 ± 176.5	1129 ± 175.9

1. Proline production has been measured in intact hepatocytes. Production of proline assumed to be derived from the transport of proline and the activity of P5C reductase. Data are expressed as mean ± standard deviation, nmol/min/mg dry weight.

2. Activity assayed in broken hepatocytes and expressed as mean ± standard deviation, nmol/min/mg protein.

   N = 3 animals in each group.

Table 3.10 Zonation Studies in Isolated Hepatocytes following Antegrade Digitonin Perfusion (DNA Based Activity)

Enzyme	Zonal Location	Control	Digitonin Treated
Proline Production[1]	Unknown	0.120 ± 0.044	0.104 ± 0.024
Glutamine Synthetase[2]	PV	11.88 ± 5.11	16.24 ± 7.57
Ornithine Aminotransferase[2]	PV	4.73 ± 0.44	6.20 ± 1.13
Proline Oxidase[2]	Unknown	7.27 ± 0.82	8.44 ± 0.99
Ornithine Transcarbamylase[2]	PP	920.2 ± 154.8	990.4 ± 154.3

1. Proline production has been measured in intact hepatocytes. Production of proline assumed to be derived from the transport of proline and the activity of P5C reductase. Data are expressed as mean ± standard deviation, nmol/min/µg DNA.

2. Activity assayed in broken hepatocytes and expressed as mean ± standard deviation, nmol/min/µg DNA.

   N = 3 animals in each group.

3.2.3 Discussion of the *In Vivo* Selective Zonal Necrosis Experiments

Perfusion of rat liver with digitonin has been utilized in the past to describe the zonal characteristics of amino acid transport[21], lipogenic enzymes[37] and gluconeogenesis[143] in freshly isolated hepatocytes. Typically, the enrichment of particular populations of hepatocytes have been confirmed using cytosolic marker enzymes[145;145;146]. The experiments described here, use both cytosolic and mitochondrial marker enzymes of the perivenous zone (glutamine synthetase[164] and ornithine aminotransferase[137] respectively). Enriched periportal cell preparations are normally contaminated with perivenous cells or perivenous cell "ghosts"* containing mitochondrial enzyme activity. To decrease the contamination by damaged cells, hepatocyte preparations were further processed on a Percoll gradient[12;93].

Initial experiments employing retrograde digitonin-perfusion provided evidence that the perivenous zone could be selectively damaged. The characteristic discrete pale discolourings or spots on the liver surface, typical of perivenous damage associated with CCl_4 and bromobenzene treatment, were observed within 20 seconds of the initiation of digitonin perfusion (Figure 3.5). The same trend was also noted on a microscopic level (Figure 3.6) (compare with CCl_4 (Figure 3.1) and bromobenzene (Figure 3.2) treated livers).

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* Perivenous cell "ghosts": Plasma membranes of cells affected by digitonin become leaky, mitochondrial membranes do not become leaky. Thus, while the cytosolic constituents are lost, the mitochondria may be retained in the hepatocyte ghost. These ghosts may then demonstrate mitochondrial enzyme activity[146].

The results of experiments to optimize the retrograde digitonin-perfusion are illustrated in Figures 3.7 and 3.8. These efforts demonstrated that retrograde perfusion of rat liver with digitonin at a concentration of 5.0 mg/mL for 45 seconds provided enrichment of periportal cells with minimal contamination of perivenous hepatocytes. More specifically, the graphs in Figure 3.8 demonstrate that cells proximal to the terminal hepatic venule (those cells having positive glutamine synthetase activity) were almost completely abolished following retrograde digitonin perfusion. The zone of positive OAT activity however, was slightly larger than the glutamine synthetase-positive zone demonstrated by smaller decreases in the activity of ornithine aminotransferase (no significant difference and 42% decrease) than glutamine synthetase (91% and 94% decrease) following 30 and 45 second digitonin perfusions. These results are in agreement with the results of Kuo et al.[95] who demonstrated the co-localization of mRNA and enzyme activities of glutamine synthetase and ornithine aminotransferase. The results also provide evidence that both mitochondrial enzymes were not removed as completely as the cytoplasmic glutamine synthetase. Proline oxidase activity was decreased by 16% following retrograde digitonin-perfusion for 45 seconds, no significant difference was seen after a 30 second pulse (Figure 3.8). The activity of proline oxidase thus appeared to follow a similar perivenous enrichment to ornithine aminotransferase; i.e. the zone of cells containing positive activity was larger than the zone of cells containing positive glutamine synthetase activity. As more of the perivenous zone was

destroyed, the activity of GS, OAT and PO decreased in proportion to the (original) size of positive zonal activity. The data reported in Tables 3.7 and 3.8 include the activity of the periportal marker ornithine transcarbamylase, which was not significantly affected by retrograde digitonin-perfusion. Maintenance of the ornithine transcarbamylase activity following retrograde perfusion with digitonin demonstrated that the periportal zone (zones 1 and 2) was not significantly damaged during digitonin perfusion.

Hepatocytes isolated from livers perfused with digitonin in the retrograde manner demonstrated a decreased capacity to produce proline from P5C (Table 3.7 and 3.8). These data are in conflict with the toxin data which suggest that proline production from P5C was increased when the perivenous zone was damaged. However one must consider the differences in the experimental design. The toxin experiments had two factors which could potentially confound the data - 1) potential induction of proline synthesis (P5C transport and / or P5C reductase) by carbon tetrachloride and bromobenzene; 2) destruction of a limiting transport barrier for entry of P5C into hepatocytes by carbon tetrachloride and bromobenzene, but not allyl alcohol. The digitonin studies were very short (45 s versus 48 hours), so there was little time to induce either step in proline synthesis. Digitonin may disrupt a transport barrier, but if so, it did not increase proline synthesis from exogenous P5C.

The periportal zone is typically regarded to be significantly larger than the perivenous zone. Haussinger[66] states that the glutamine synthetase-positive cells

represent ~7% of all hepatocytes of an acinus. The periportal zone is usually considered to account for the remaining 90%. To accommodate the larger volume of cells to be perfused by digitonin, the digitonin concentration was increased to 10 mg/mL (~ 8 mM) and the duration of perfusion to 90 seconds. Other researchers have utilized increased digitonin concentrations and perfusion times for antegrade perfusion to generate periportal damage; Burger et al[21] used a 7 mM (8.6 mg/mL) solution of digitonin infused for 75 - 105 seconds to destroy zonal populations and Chen and Katz[25] utilized 2 mLs of a 10 mg/mL digitonin preparation (20 mg of digitonin in total compared with 16.5 mg in the design described in our design). We therefore expected that these parameters would elicit an adequate amount of damage to the periportal zone while leaving the perivenous zone relatively undamaged. The ultra-structural and microscopic analyses indicated this to be true (Figure 3.9). Unfortunately, the enzymatic results did not reflect a significant enrichment in perivenous hepatocytes (Tables 3.9 and 3.10). The fact that the perivenous-localized enzyme activities (glutamine synthetase, ornithine aminotransferase and proline oxidase) remain unchanged indicated that the perivenous zone was intact; perivenous cell enrichment would have been reflected by a significant decrease in the activity of ornithine transcarbamylase. Also, slight increases in the activities of the perivenous zone might have been expected due to an increased perivenous: periportal cell ratio.

Antegrade digitonin-perfusion resulted in a surface pattern consistent with descriptions by Quistorff and colleagues[143;145 145](Figure 3.9). Periportal zone

damage was much more evident and defined on a microscopic level for the antegrade digitonin-perfused livers compared with the allyl alcohol-induced periportal zone damage (compare Figures 3.3 and 3.9). The concentration of digitonin was increased to 10 mg/mL and the duration of digitonin perfusion was extended by an additional 45 seconds, relative to the digitonin preparation used during retrograde perfusion to damage the perivenous zone. These adjusted parameters were thought to be adequate due to the quality of the digitonin preparation (the digitonin was of an ultra-pure quality and was filtered prior to perfusion; unfiltered preparations typically resulted in poor perfusions, presumably due to a blocked hepatic vascular system) and to the rapid solubilization of cell membranes in the perivenous zone following retrograde perfusion of digitonin at 5 mg/mL for only 45 seconds. However, the activity of OTC was not significantly decreased under these conditions (Tables 3.9 and 3.10). Cheng et al[26] investigated the zonation of cholesterol synthesis and found that the de novo synthesis of this sterol was significantly higher in periportal hepatocytes (PP/PV = 1.67). Thus, when the digitonin bound the cholesterol in the plasma membrane of periportal hepatocytes, cholesterol inside these cells became available targets for the infused digitonin. A greater amount of digitonin would then have to be perfused to facilitate extensive damage to the periportal zone. Apparently, 20 mg of digitonin was not enough to damage a significant proportion of the periportal cells to elicit a significant decrease in the activity of ornithine transcarbamylase.

3.3 Amino Acid Production in Isolated Hepatocytes

Amino acid production was assessed in hepatocytes isolated from rats under a variety of experimental conditions.

3.3.1 Amino Acid Production in Enriched Acinar Populations of Hepatocytes

Following administration of the toxins bromobenzene and allyl alcohol, hepatocytes were incubated with DL-P5C to assess the production of proline. Subsequent analyses were performed using HPLC to assess the metabolic fate of P5C and whether this fate was affected by damage to a particular zone of the hepatic acinus.

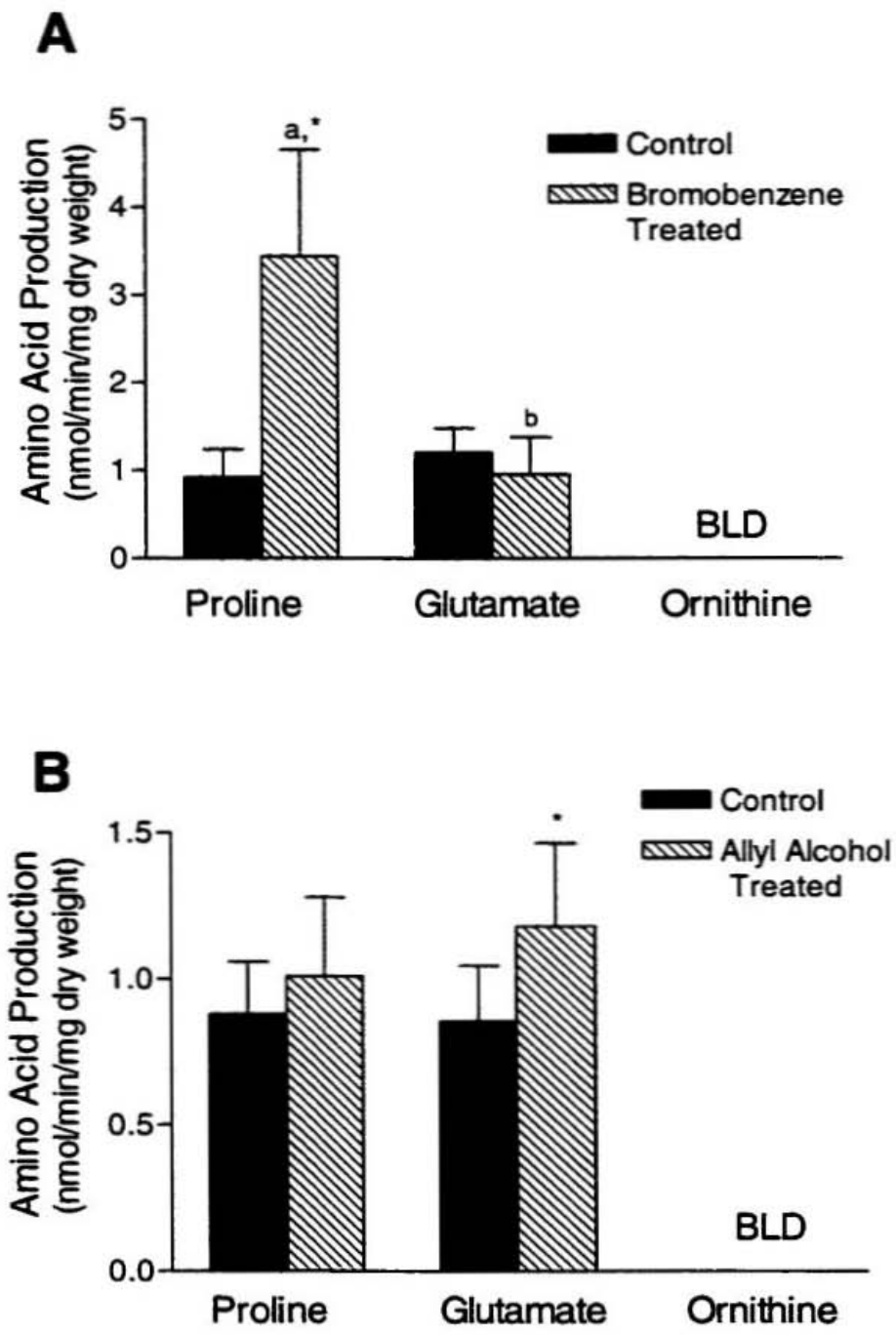
Hepatocytes isolated from control, 48-hour fasted rats, were incubated with DL-P5C and produced proline and glutamate at similar rates but there was no detectable production of ornithine (Figure 3.10A,B). Following bromobenzene intoxication, hepatocytes produced proline at a significantly higher rate (>3X) than glutamate (Figure 3.10A). After allyl alcohol intoxication, proline production was not significantly altered while glutamate production was increased by almost 40% (Figure 3.10B) compared to control. Ornithine production was again below the level of detection.

3.3.2 Amino Acid Production in Isolated Hepatocytes from Fed Rats

A second set of experiments were performed to compare amino acid

Figure 3.10 Amino Acid Production in Isolated Hepatocytes Incubated with DL-P5C Following Intoxication with Allyl Alcohol and Bromobenzene

Experimental hepatic cirrhosis was induced with intragastric administration of either bromobenzene (3.8 mmol/kg body weight) or allyl alcohol (1.0 mmol/kg body weight). Toxins were diluted in Mazola corn oil and control animals received corn oil only. Animals were fasted for 24 hours prior to and after intoxication to reduce variability of response among animals. Hepatocytes were isolated by the collagenase method and incubated with 2.5 mM DL-P5C for 15 minutes at 37°C. Amino acids were quantified by HPLC analysis. Proline was quantified by the acid-ninhydrin method. Bars with different letters are significantly different; * - significantly different from control. Values are presented as mean ± standard deviation; controls (n=4), treated animals (n=8);. Significance was set at $p < 0.05$, Student's t-test.


A
Amino Acid Production
(nmol/min/mg dry weight)
5
4
3
2
1
0
a,*
b
BLD
Control
Bromobenzene
Treated
Proline
Glutamate
Ornithine
B
Amino Acid Production
(nmol/min/mg dry weight)
1.5
1.0
0.5
0.0
*
BLD
Control
Allyl Alcohol
Treated
Proline
Glutamate
Ornithine

production in hepatocytes isolated from animals neither fasted, nor treated with toxins. Hepatocytes were incubated with a variety of substrates to assess the metabolic fate of each amino acid substrate.

Hepatocytes incubated with DL-P5C under these conditions produced significantly more proline than glutamate; ornithine production was not detected (Figure 3.11). When proline was utilized as the substrate, only marginal glutamate production occurred, ornithine production was not detected (Figure 3.12). Incubation with glutamate or ornithine did not result in significant production of proline, ornithine or glutamate.

3.3.3 Discussion of Amino Acid Production in Isolated Hepatocytes

P5C conversion to either proline, glutamate or ornithine in the isolated hepatocyte involves both transporters and enzymes. Conversion to proline is the simplest route: transport across the plasma membrane and catalysis by cytosolic P5C reductase to generate proline. Conversely, to generate glutamate or ornithine, P5C must be transported across the plasma and mitochondrial membranes. Then catalysis by the mitochondrial enzymes P5C dehydrogenase or ornithine aminotransferase converts P5C to glutamate and ornithine respectively. P5C transport across the plasma membrane of Chinese hamster

Figure 3.11 Amino Acid Production in Hepatocytes Isolated From Fed Rats and Incubated with DL-P5C

Hepatocytes were prepared by the two-step collagenase procedure and incubated for 30 minutes at 37°C (with gentle agitation) in the presence of 3.0 mM DL-P5C. Values are mean ± standard deviation; n=4.

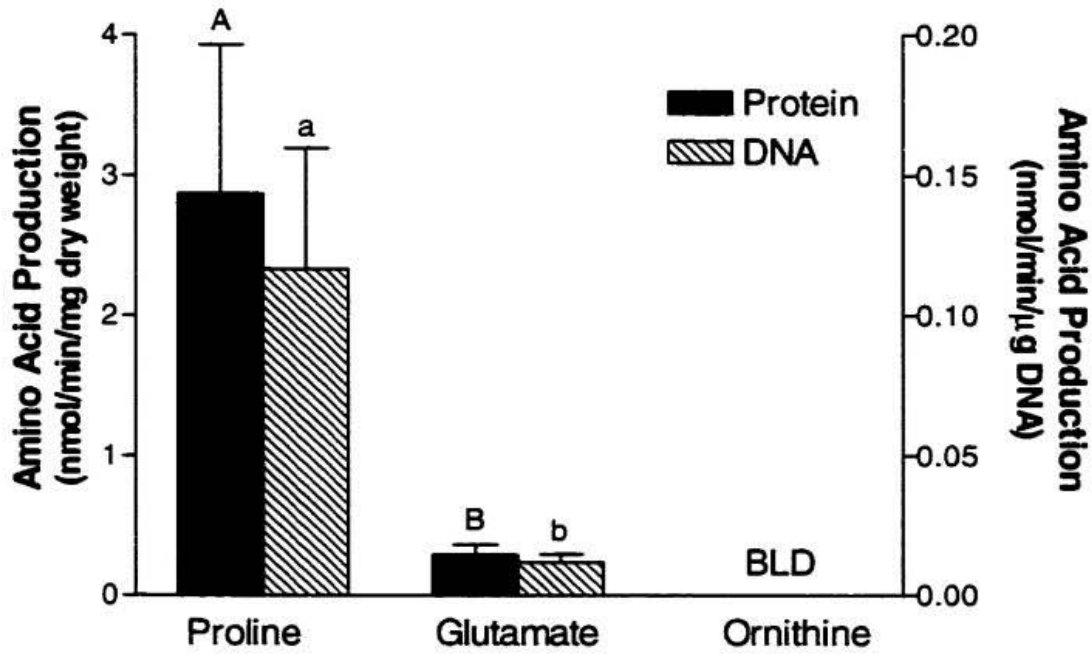
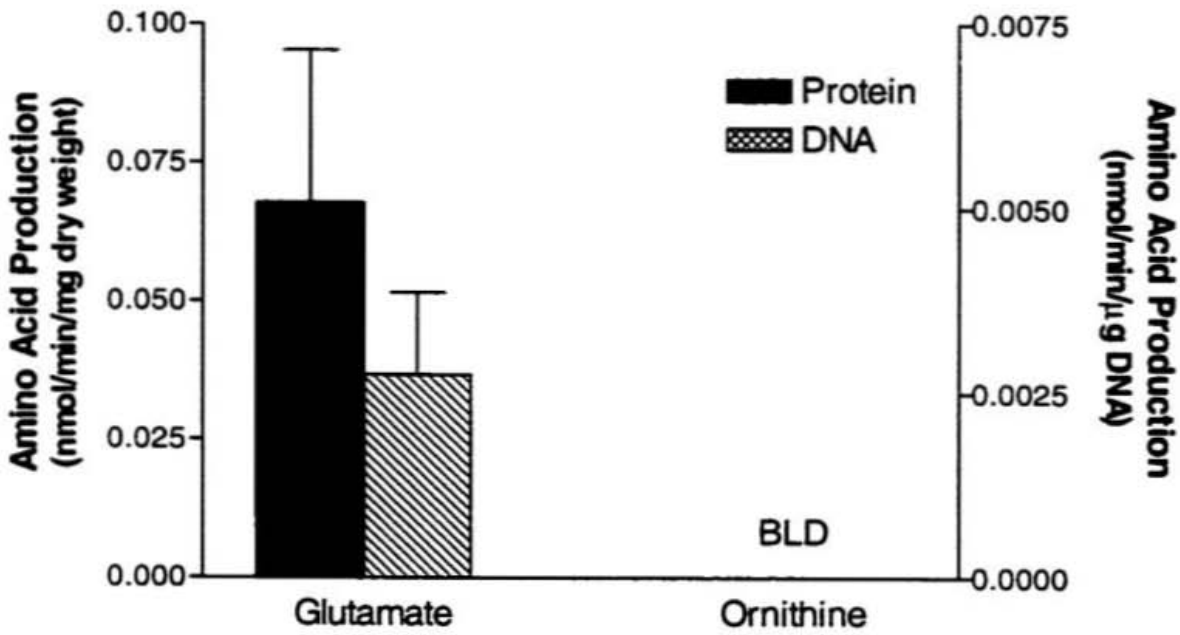

Amino Acid Production
(nmol/min/mg dry weight)
4
3
2
1
0
A
a
B
b
BLD
Protein
DNA
0.20
0.15
0.10
0.05
0.00
Amino Acid Production
(nmol/min/µg DNA)
Proline
Glutamate
Ornithine

Figure 3.12 Amino Acid Production in Isolated Hepatocytes From Fed Rats and Incubated with L-Proline

Hepatocytes were prepared by the two-step collagenase procedure and incubated for 30 minutes at 37°C (with gentle agitation) in the presence of 1.0 mM L-Proline. Values are mean ± standard deviation; n=4.


Amino Acid Production
(nmol/min/mg dry weight)
0.100
0.075
0.050
0.025
0.000
Protein
DNA
BLD
0.0075
0.0050
0.0025
0.0000
Amino Acid Production
(nmol/min/µg DNA)
Glutamate
Ornithine

ovary cells has been demonstrated by Mixson and Phang[119;120]. Here, we have demonstrated that P5C transport across the plasma membrane of rat hepatocytes was possible by its conversion to proline (Figures 3.10 and 3.11). Transport of exogenous P5C across the mitochondrial membrane was suggested by the production of glutamate when P5C was the sole substrate (Figures 3.10 and 3.11). Alternatively, P5C transported across the plasma membrane was converted to proline which was then transported across the mitochondrial membrane. Proline oxidase could then act upon the proline, convert it to P5C and the newly formed P5C be utilized by P5C dehydrogenase to synthesize glutamate. Hagedorn and colleagues[57;58] have provided evidence which strongly supports a cycle in which proline is transported into mitochondria, enzymatic action by proline oxidase and then transport of P5C out of mitochondria into the cytosol. Under their conditions, 60% of the P5C was recycled to proline. They note that the mitochondrial transport of P5C remains uncharacterized but that P5C dehydrogenase activity is most likely an important regulatory site[57].

The acinar localization of P5C reductase is unknown; thus proline production in isolated hepatocytes was used to investigate the zonal characteristics of this enzyme. As mentioned earlier, the production of proline in isolated hepatocytes was due to both P5C reductase activity and transport of P5C across the plasma membrane.

Hepatocytes isolated from the livers of fed rats demonstrated a preferential (approximately ten-fold greater) production of proline from exogenously added

DL-P5C compared to the production of glutamate and ornithine (Figure 3.11). Following a 48 hour fast, proline production from DL-P5C was decreased to one third that of the fed state; glutamate production was increased more than two-fold (Figure 3.10). As a result, following a 48 hour fast, P5C was converted to proline and glutamate at similar rates. Ornithine production, compared to proline and glutamate production, was not detected, similar to the fed state. Arginine was not produced in either set of experiments.

These results suggest that P5C metabolism was different in the fasted state compared to the fed state. Both proline and glutamine are gluconeogenic substrates via α-ketoglutarate. Thus one may assume that during periods of fasting, the capacity to utilize these substrates for glucose or glycogen production would be increased. It follows then that P5C conversion to proline and glutamine would be stimulated during periods when increased demands for gluconeogenic substrates are prevalent. In the fed state, P5C was equally converted to proline and glutamate (in these experiments, glutamate refers to glutamine and glutamate), whereas in the fasted state P5C was converted to proline at a markedly increased rate. The effects of fasting on the activities of P5C reductase and P5C dehydrogenase have not been investigated previously.

Figure 3.10 presents the data for the bromobenzene experiment; as noted above, hepatocytes isolated from control animals produced proline and glutamate equally. Treatment with bromobenzene successfully damaged the perivenous zone, decreasing the activity of the markers glutamine synthetase

and ornithine aminotransferase (see Tables 3.3 and 3.4). Following destruction of the perivenous zone, there was a large increase in proline production from P5C compared to controls. Carbon tetrachloride poisoning also resulted in increased proline production (Table 3.2). These results indicate a possible periportal enrichment of the transporter(s) involved in P5C transport or removal of a transport limitation. The enzyme P5C reductase, could also be enriched in the periportal zone. This conclusion assumes that capacity for proline synthesis from P5C is unchanged by the toxins. It should be noted that, following selective damage to the perivenous zone, cells in the perivenous zone would have a decreased capacity to oxidize proline produced from P5C in the periportal zone by P5C reductase.

Allyl alcohol treatment resulted in no significant change in the rate of proline production. However, the rate of proline production was significantly decreased following treatment with allyl alcohol compared to bromobenzene or carbon tetrachloride treatment. Considering that ornithine transcarbamylase activity was decreased by 20% (Tables 3.5 and 3.6), and the zonal distribution of OTC is >1 (PP:PV)[122], a significant portion of cells were potentially still able to convert P5C to proline. Thus, although proline production was not reduced following allyl alcohol treatment, the trend of decreasing proline production with decreased perivenous damage (i.e. the degree of perivenous damage was bromobenzene > carbon tetrachloride > allyl alcohol) was maintained (Figure 3.4).

Glutamate production from P5C was significantly increased (27%) following periportal damage by allyl alcohol (Figure 3.10B). This result provides further evidence for a perivenous enriched P5C dehydrogenase. Damage to the periportal zone will increase the perivenous:periportal cell ratio. Thus the relative concentration of P5C dehydrogenase protein should be increased and hence glutamate production should have increased accordingly.

Figure 3.12 illustrates amino acid production from L-proline in hepatocytes from fed rats. L-glutamate and L-ornithine, when used as substrates, did not contribute to any significant production of amino acids in hepatocytes isolated from fed animals. The conversion of glutamate to P5C requires the activity of P5C synthase, which does not occur in liver[180]. Thus, we did not expect any production of proline or P5C from glutamate. Similarly, we did not detect any production of ornithine from any supplied substrates. This result agrees with the findings of Herzfeld and Raper[70] who could not detect any reversal of OAT in liver (only in the small intestine of rats). The quantity of amino acids produced from L-proline was negligible compared to that seen with hepatocytes incubated with DL-P5C (Figure 3.11). These results suggest that under these experimental conditions, the inter-conversion of proline, glutamate and ornithine was negligible. Yet, under the same conditions, P5C was converted to proline (~90% of total amino acids produced) and glutamate (~10% of total amino acids produced) with no detectable amount of ornithine being produced (Figure 3.12). Mixson and Phang[119] noted in their study involving Chinese hamster ovary cells

incubated with radiolabelled P5C, that radiolabelled proline accounted for the total intracellular accumulation of radiolabelled P5C. Hagedorn and Phang[57] calculated that in isolated mitochondria (from rats) 60% of P5C produced via the action of proline oxidase was recycled to back to proline (under conditions which decreased flux of P5C to glutamate). Hensgens et al.[68] incubated isolated hepatocytes from 18-24 hour-starved rats with 5 mM proline and determined that glutamate was produced at a rate of ~ 1 nmol/min/mg dry weight. Our results indicate that hepatocytes isolated from fed rats produce glutamate at a rate of ~0.07 nmol/min/mg dry weight when incubated with 1 mM proline. Under these same conditions, hepatocytes produced glutamate at a rate of ~ 0.3 nmol/min/mg dry weight when incubated with 3.0 mM DL-P5C. Isolated hepatocytes from rats fasted for 48 hours produced glutamate from DL-P5C at a rate of ~ 1 nmol/min/mg dry weight. These results, both ours and others, indicate that P5C conversion to glutamate may be dependent upon the dietary status of the animal.

4 Summary and Conclusions

4.1 General Discussion

Pyrroline-5-carboxylate is a common metabolic intermediate in the inter-conversion of proline, glutamate and ornithine (arginine). Metabolism of these amino acids is heterogeneous across the liver acinus, the functional unit of the liver. Considerable knowledge has been accrued regarding the zonation of the metabolism of both glutamate and ornithine in the liver. However, less is known about the acinar heterogeneity of P5C and proline metabolism.

The synthesis of glutamate and glutamine, catalyzed by glutamate dehydrogenase[147], glutamine synthetase[46] respectively, occurs predominantly in the perivenous zone. Similarly, catabolism of these amino acids is also zoned. Glutaminase[67], for example, has been shown to be localized only in the proximal periportal zone. The transport of glutamate and glutamine in the liver has been linked with three different carrier systems. System G- is involved in the perivenous transport of glutamate[21;135]. Glutamine transport has been shown to be heterogeneous across the liver acinus[88] and recently, the expression of mNAT, (for N-system amino acid transporter) was shown to be zonated[54].

The ornithine cycle has been extensively studied[31;121;132]. Dingemanse et al[31] used *in situ* hybridization to demonstrate that expression of the mRNAs for all the enzymes involved in urea synthesis were zonated. The messages were concentrated in the periportal hepatocytes and declined toward the terminal hepatic venule, and were absent from the glutamine synthetase-positive hepatocytes.

The periportal zone is characterized by a high capacity for uptake and catabolism of many amino acids[66,83] with the exception of glutamate[64,21] arginine[132]. Gluconeogenesis from amino acids occurs predominantly in the periportal zone via the action of several aminotransferases. The activities of the gluconeogenic enzymes such as glucose-6-phosphatase[17], fructose 1,6-bisphosphatase[99], and phosphoenolpyruvate carboxykinase[116] have been localized to the periportal zone. Proline is a gluconeogenic amino acid, therefore the periportal catabolism of this amino acid would be logical. However, proline is also a member of the glutamate family of amino acids. The catabolism of these amino acids occurs in different zones across the acinus. Arginine[132], ornithine[95] and glutamate[110] are catabolized in the perivenous zone. Histidine[158] and glutamine[62,182] are catabolized in the periportal zone.

These precedents led us to investigate another facet of ornithine and glutamine metabolism, the P5C - proline role. In particular, was metabolism of P5C and proline zonated across the liver acinus.

4.2 Zonation Experiments

A variety of techniques are available for investigation of liver cell heterogeneity and metabolic zonation. In these experiments, specific populations of hepatocytes were isolated following selective zonal damage either by toxin administration or by controlled digitonin perfusion of the liver. Zone-specific marker enzymes were assayed in conjunction with histological examinations to

assess the extent of zonal damage following treatment. The generalized results of these experiments are presented in Table 4.1.

Damage to the perivenous zone resulted in significant reduction of the perivenous markers glutamine synthetase and ornithine aminotransferase. The periportal marker, ornithine transcarbamylase activity was either unaffected or slightly increased. Selective damage to the periportal zone was not, unfortunately, as effective. Allyl alcohol caused a significant decrease in ornithine transcarbamylase activity; however, antegrade digitonin-perfusion of the liver did not significantly decrease the activity. The failure to significantly damage the periportal zone may be attributed to the much larger proportion of this hepatic zone. Neither of the perivenous marker enzyme activities were significantly altered by damage to the periportal zone.

Proline oxidase activity was significantly decreased following damage to the perivenous zone by both bromobenzene and retrograde-digitonin perfusion. Damage to the periportal zone had no effect on the activity of proline oxidase. These results provide convincing evidence that proline oxidase activity is

Table 4.1 Summary of Zonation Experimental Results

Enzyme	Zone	Treatment				
		Allyl Alcohol	**Carbon Tetrachloride**	**Bromo-benzene**	**Retrograde Digitonin Perfusion**	**Antegrade Digitonin Perfusion**
		PP Zonal Damage	*PV Zonal Damage*	*PV Zonal Damage*	*PV Zonal Damage*	*PP Zonal Damage*
Glutamine Synthetase	PV	---	↓	↓	↓	---
Ornithine Aminotransferase	PV	---	↓	↓	↓	---
Ornithine Transcarbamylase	PP	↓	↑	---	---	---
Proline Oxidase	***PV***	---	↓ (from ref 34)	↓	↓	---
Proline Production (P5C Transport and P5C Reductase activity)	***PP or Zone 2***	---	↑	↑	↓	---

PV - enriched in the perivenous zone; PP - enriched in the periportal zone.
↑ indicates an increase in activity or production; ↓ indicates a decrease in activity or production; --- no change.
Bolded letters - zone enrichment based on present data.

heterogeneousiy distributed along the hepatic acinus. The greatest activity is localized to the perivenous zone and follows a gradient pattern simiiar to that of ornithine aminotransferase. The amino acid production data also suggested a perivenous enrichment of P5C dehydrogenase in agreement with the results of O'Sullivan et al[132]. From our data, the relative sizes of positive enzyme activity about the terminal hepatic venule may be described as P5CDh > PO > OAT > GS.

P5C reductase activity was indirectly investigated via the transport of P5C and subsequent production of proline when DL-P5C was the sole substrate. P5C was able to be converted to proline following selective perivenous and periportal damage, thus one may assume that P5C transport is possible across the entire acinus. However the nature of the zonal gradient for P5C transport, if any, remains unclear. Assuming P5C transport was possible across the entire acinus and unaffected by toxin administration, P5C reductase activity increased following toxin-induced damage to the perivenous zone, but remained unchanged following allyl alcohol-induced damage to the periportal zone. These results may suggest a slight periportal enrichment, assuming that damage by allyl alcohol did not cause enough damage to the periportal zone to cause a decrease in P5C reductase activity.

Examination of the amino acids produced from P5C following bromobenzene-induced perivenous damage illustrated that the majority of P5C was being converted to proline. Conversely, allyl alcohol-induced periportal

damage resulted in an increase in glutamate production; proline production was still increased but the total production was decreased considerably from the bromobenzene experiment. These results may suggest that P5C reductase zonation may follow a uniform or U-shaped gradient (greatest enrichment in the mid-zone and lower enrichments about the portal triad and terminal hepatic venule) zonation patterns across the acinus.

Damage to the perivenous zone following retrograde-digitonin perfusion caused a decrease in proline production, while antegrade-digitonin perfusion had no effect on the rate of proline production from P5C. These results contrast the results of the toxin experiments. The dietary status of the animal and *in vivo* interactions of the toxins must be considered here. The digitonin-perfused animals were in the fed state while the toxin-treated animals underwent a 48 hour fast to facilitate selective zonal damage[139]. P5C conversion to proline was increased three-fold in the fed state (in control animals) compared to 48 hour fasted controls. The dietary status of the animal could alter the zonal characteristics of P5C reductase. Moorman et al[123] demonstrated that normal carbamoylphosphate synthetase mRNA expression (decreasing in the periportal to perivenous direction) across the acinus was reversed following starvation. In addition, administration of toxins *in vivo* results in the biotransformation of one compound into possibly several metabolites. Any one of these toxins may provide a stimulatory or inhibitory effect on proline production via P5C reductase activity or on P5C transport.

These experiments do not provide solid evidence for a particular zonal enrichment of P5C reductase. Periportal enrichment of the enzyme activity appears logical however. A periportal-localized P5C reductase activity permits primary utilization of P5C for proline synthesis compared with glutamate or ornithine synthesis (perivenous events). In this scenario, P5C that enters the liver is preferentially converted to proline which may be utilized for reparatory collagen synthesis during liver cirrhosis. A periportal localization of P5C reductase would also segregate proline synthesis and catabolism across the acinus. A similar arrangement is seen with glutamine, enzymes for synthesis and degradation are oppositely zoned[181].

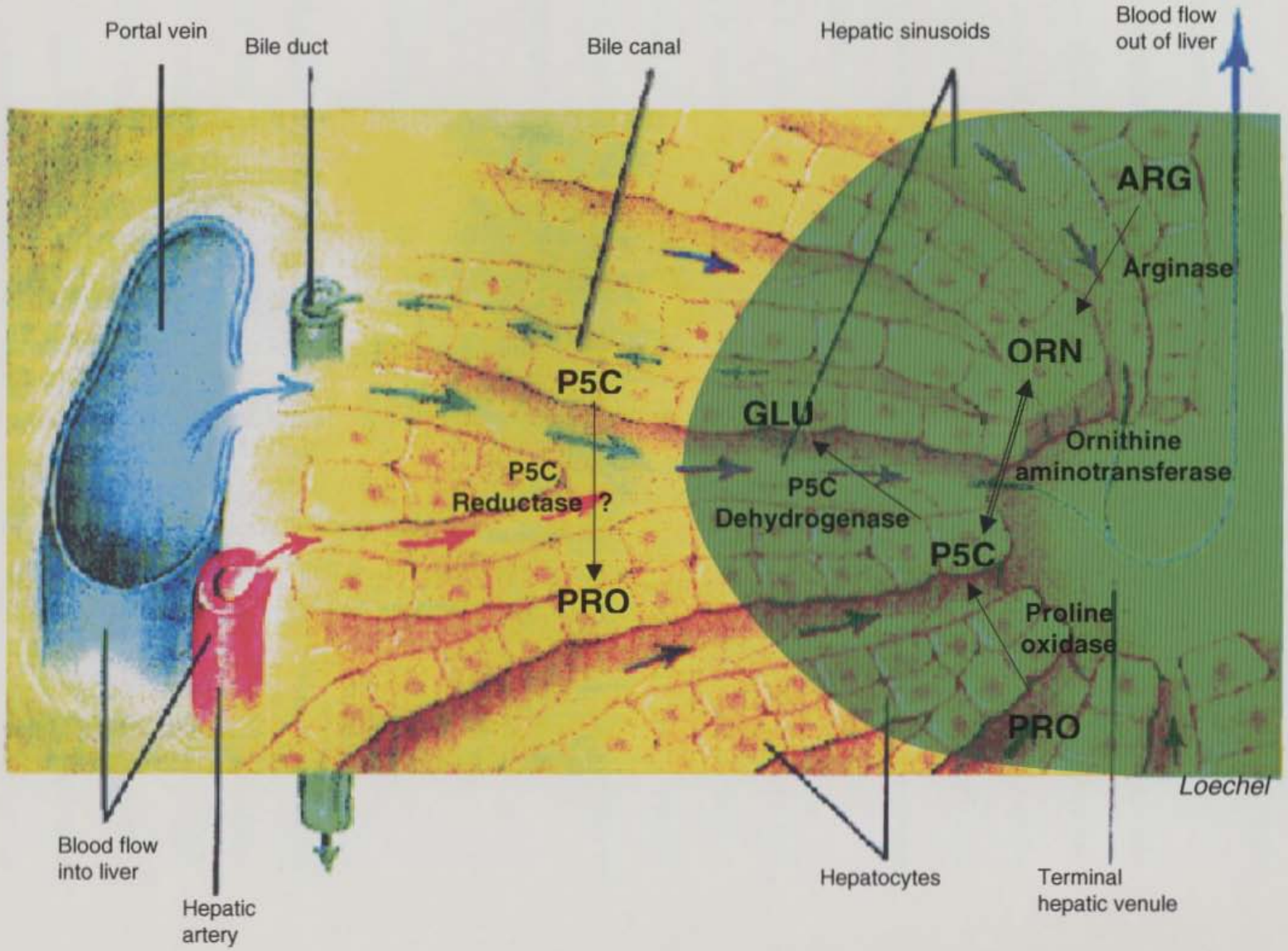
4.3 Summary

From these conclusions and hypotheses, the scheme presented in Figure 4.1 was developed. In this acinar model, proline synthesis occurs in the periportal zone via cytosolic P5C reductase activity. Thus circulating and intestinal-derived P5C[40] can be used for proline synthesis.

Proline catabolism occurs in the perivenous zone via the mitochondrial enzyme, proline oxidase. P5C generated from proline oxidation is then available to P5C dehydrogenase, also mitochondrial, for glutamate synthesis. P5C dehydrogenase may occupy a shallow gradient similar to ornithine aminotransferase and proline oxidase. In this way, glutamate generated in the perivenous zone by P5C dehydrogenase can be combined with NH_4^+ and utilized

Figure 4.1 Perivenous Localization of P5C Metabolism

Cross section of a liver acinus as proposed by Rappaport[149]. The pathway for the metabolism of P5C is shown in the perivenous zone (overlying green area). The localization of P5C reductase is shown to be periportal, but this has not been confirmed. Adapted from Hole[74].


Portal vein
Bile duct
Bile canal
Hepatic sinusoids
Blood flow out of liver
ARG
Arginase
ORN
P5C
GLU
Ornithine aminotransferase
P5C Reductase ?
P5C Dehydrogenase
P5C
PRO
Proline oxidase
PRO
Loechel
Blood flow into liver
Hepatic artery
Hepatocytes
Terminal hepatic venule

directly for glutamine synthesis by glutamine synthetase. In this way, P5C dehydrogenase mediates the scavenging function of glutamine synthetase, especially under pathological conditions when additional sources of glutamate are required to neutralize ammonium ions. Alternatively, P5C may be converted to ornithine via the activity of ornithine aminotransferase. However, OAT activity in the liver is generally considered to catalyze ornithine conversion to P5C[132] (the reaction equilibrium strongly favours P5C formation[113]), thus mediating arginine degradation to glutamate.

It is interesting to note that in this scheme, P5C synthesis from proline occurs in the mitochondrion of perivenous cells. Proline synthesis may occur in the cytosol of cells of the periportal and/or mid-zone. Thus the P5C - proline inter-conversion is similar to the glutamate - glutamine inter-conversion which is also segregated by acinar zone and organelle.

4.4 Future Considerations

In lieu of the results of these experiments, there are still questions to be resolved. The zonation of P5C reductase and P5C dehydrogenase activity remains unclear, as does the zonation of P5C transport. The transport of P5C can be examined using radioactively labeled P5C generated via purified OAT[169]. Purified radiolabelled P5C can then be incubated with enriched populations of either perivenous or periportal hepatocytes, and transport measured as

described by Kilberg[88]. Localization of P5C transport may aid interpretation of P5C reductase activity. P5C dehydrogenase activity in the liver is currently under investigation in our lab and results are forthcoming.

Additionally, localization of mRNA expression of proline oxidase, P5C reductase and P5C dehydrogenase remains to be shown. Fortunately, molecular probes for these messages have been generated and the results are forthcoming. It will be possible to compare the distribution of mRNA for these enzymes with the activities assessed in the present study.

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6 Appendix

6.1 Bromobenzene Biotransformation

Mitchell and Jollow[118] have described the accumulated literature regarding the enzymatic metabolism of bromobenzene and other halobenzenes to the actual toxic metabolite(s). Figure 6.1 illustrates their proposed mechanism of the biotransformation of bromobenzene to compounds which cause the selective perivenous necrosis.

6.2 Allyl Alcohol Biotransformation

As stated in the title of the review, Badr[6] described a myriad of proposed mechanisms to account for the selective periportal toxicity associated with allyl alcohol. The roles of glutathione, calcium homeostasis, and oxygen and lipid peroxidation are reviewed and applied to liver toxicity.

6.3 Acid Ninhydrin Determination of Proline

Goodwin[51], described a procedure for the determination of proline in plasma and urine which greatly reduced the interference from other primary amines. The basic chemistry of this technique is depicted in Figure 6.3.

Figure 6.1 Biotransformation of Bromobenzene in Rats.

Outlined is the proposed mechanism of bromobenzene biotransformation in rats. Adapted from Fig 9., Mitchell and Jollows[118]

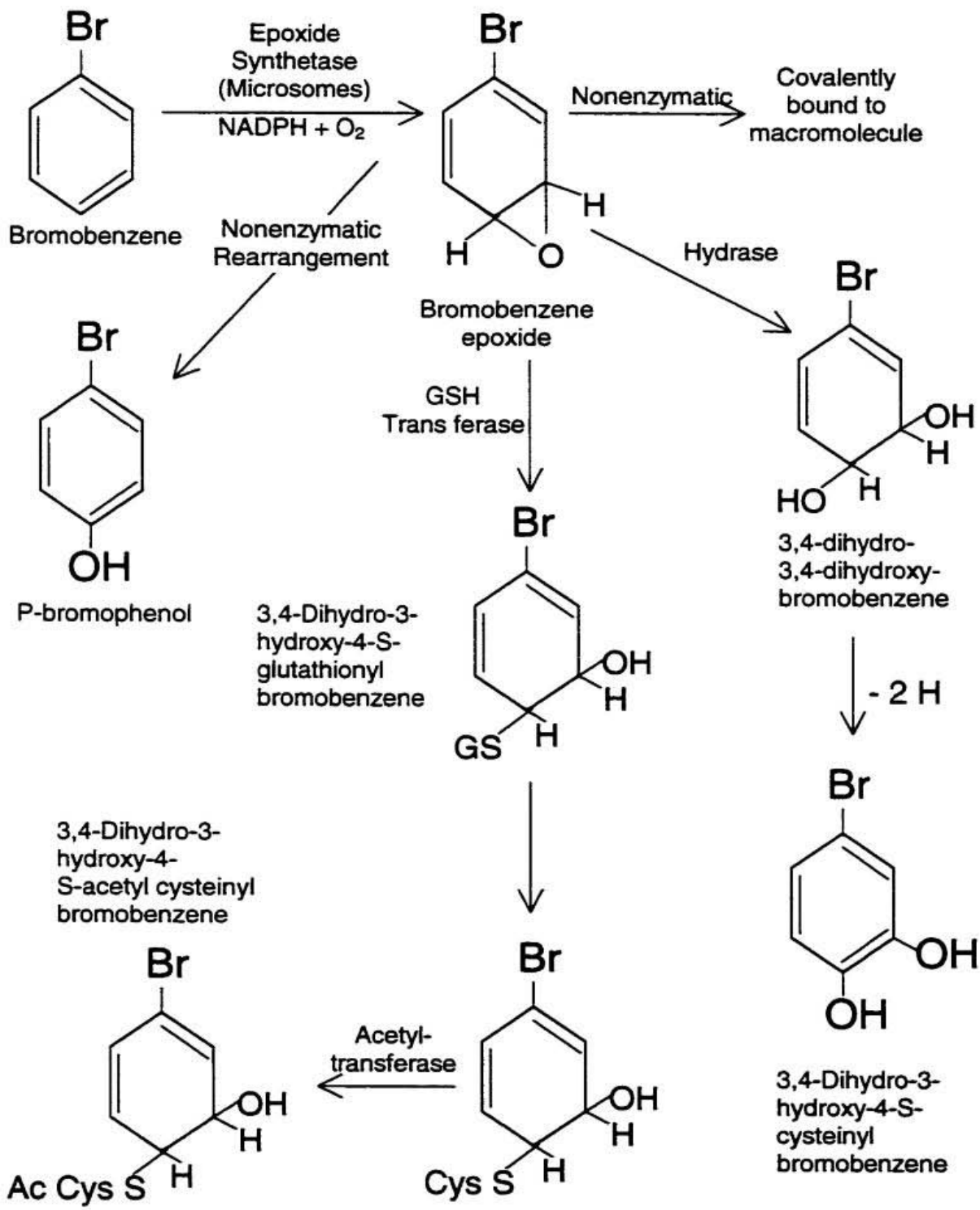

Br
Bromobenzene
Epoxide
Synthetase
(Microsomes)
NADPH + O2
Br
H
H
O
Bromobenzene
epoxide
Nonenzymatic
Covalently
bound to
macromolecule
Nonenzymatic
Rearrangement
Hydrase
Br
OH
P-bromophenol
GSH
Trans ferase
Br
OH
H
HO
H
3,4-dihydro-
3,4-dihydroxy-
bromobenzene
3,4-Dihydro-3-
hydroxy-4-S-
glutathionyl
bromobenzene
Br
OH
H
GS
H
- 2 H
Br
OH
OH
3,4-Dihydro-3-
hydroxy-4-
S-acetyl cysteinyl
bromobenzene
Br
OH
H
Ac Cys S
H
Acetyl-
transferase
Br
OH
H
Cys S
H
3,4-Dihydro-3-
hydroxy-4-S-
cysteinyl
bromobenzene

Figure 6.2 Biotransformation of Allyl Alcohol.

Outlined is the proposed mechanism of allyl alcohol biotransformation. Adapted from Figure 1, Badr[6].

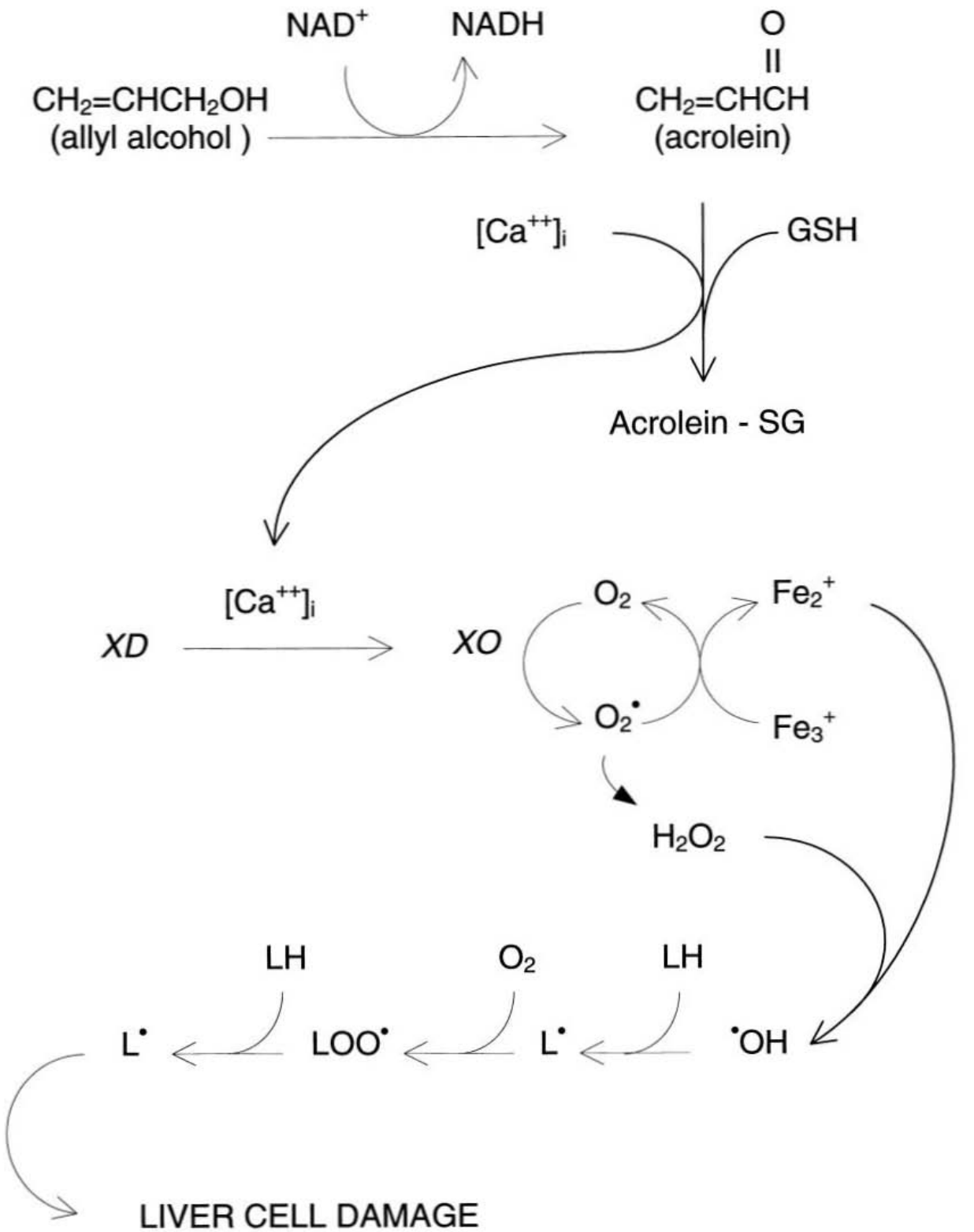
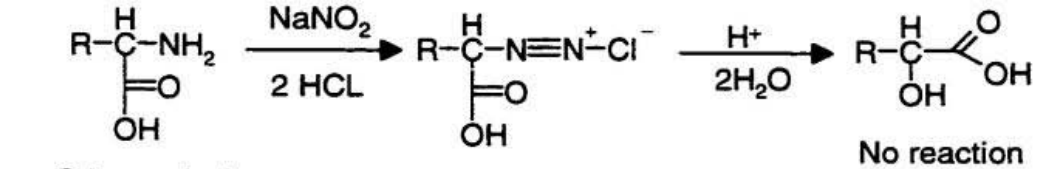
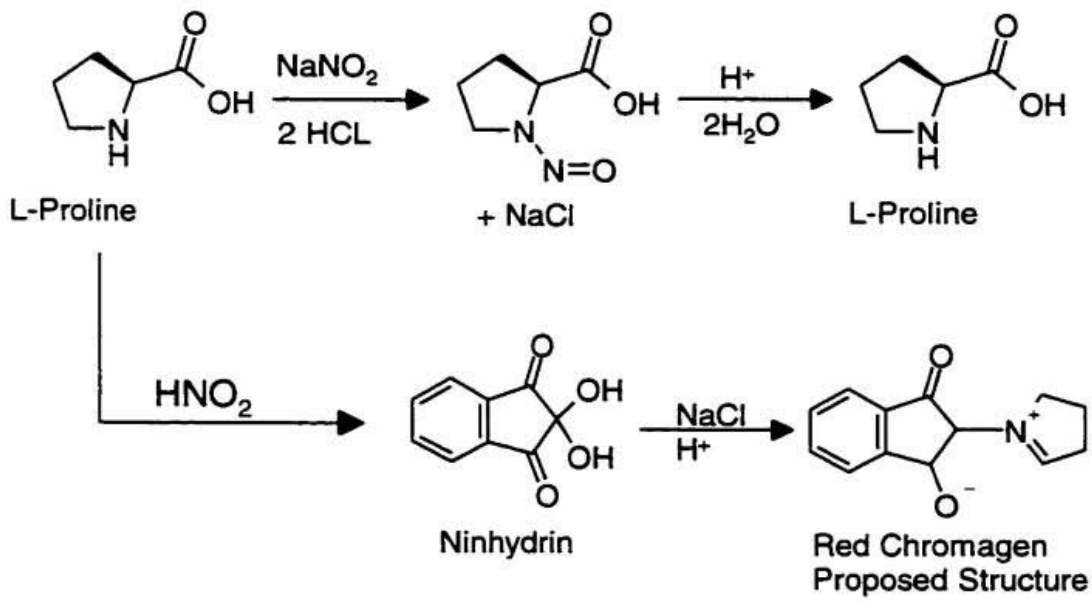

NAD+
NADH
O
II
CH2=CHCH2OH
(allyl alcohol)
CH2=CHCH
(acrolein)
[Ca++]i
GSH
Acrolein - SG
[Ca++]i
XD
XO
O2
Fe2+
O2•
Fe3+
H2O2
LH
O2
LH
L•
LOO•
L•
•OH
LIVER CELL DAMAGE

Figure 6.3 Acid-Ninhydrin Method for Proline Determination

Chemical reactions for the proposed acid-ninhydrin determination of L-Proline. The top portion illustrates the basic chemical reaction of primary amines, namely the amino acids. Below tis is the specific reaction of proline and the proposed structure of the red chromagen. Adapted from Goodwin[51].


NaNO2
2 HCL
H+
2H2O
Primary Amine
No reaction
with ninhydrin


NaNO2
2 HCL
H+
2H2O
L-Proline
+ NaCl
L-Proline
HNO2
NaCl
H+
Ninhydrin
Red Chromagen
Proposed Structure

6.4 Explanation of Expression of Hepatocyte Activity

Enzyme activity has been expressed per mg protein of broken hepatocytes as well as per μg DNA. Proline production has been expressed per mg dry weight of hepatocytes as well per μg DNA. The values in Table 6.1 describe the data determined in the current series of experiments. It is important to note the basis of expression relative to the nutritional status of the animal; the protein content of the rat hepatocyte can decrease by 3 - 10% during a 24 - 48 hour fast[60], however the DNA content of the liver is not altered.

Table 6.1 Basis for Expression of Activity

Parameter	Nutritional Status: Fasted	Fed
Dry Weight[1] (mg dry weight/mL suspension)	9.00 ± 0.71	10.04 ± 0.49
DNA[2] (µg / mL suspension)	247.8 ± 78.64	246.6 ± 32.48
DNA (µg)/Dry Weight(mg)[3]	27.53 ± 8.33	24.62 ± 3.55
Protein Concentration[4] (mg/mL)	14.76 ± 2.84	24.89 ± 7.13
DNA (µg) / Protein (mg)[5]	1.73 ± 0.60	1.14 ± 0.66

1. Hepatocytes were prepared and diluted in Krebs-Henseleit medium containing 2.5% BSA. 3 mLs of this suspension were dried overnight in an aluminum weighing pan at 50°C; 3 mLs of the Krebs-Henseleit with BSA were also dried overnight. The weight of the BSA was subtracted from the hepatocyte weight.
2. DNA content was assessed in aliquots of freshly isolated hepatocytes according to the methods of Schneider[161] and Burton[23].
3. DNA content : Dry weight ratios were determined and compared on an individual sample basis.
4. Protein concentration was determined using the Biuret method[52] after solubilization with deoxycholate[77] either in freshly isolated hepatocytes (corrected for BSA) or in diluted aliquots (corrected for BSA) used for enzyme activity analysis.
5. DNA content : Protein concentration ratios were determined and compared on an individual sample basis.

Values are presented as Mean ± Standard deviation, Fasted n = 15; Fed n = 8.