EFFECTS OF ETHANOL ON GLYCOGEN METABOLISM

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ABSTRACT

The effects of alcohol on glycogen structure and metabolism in fed, starved and starved-refed animals were studied in rats, taking into account factors such as post mortem degradation, careful isolation (native glycogen), and the separate structures and metabolism of low (cytosolic) and high (lysosomal) molecular weight glycogen. These studies were performed using the technique of density gradient ultracentrifugation.

In fed animals, rats were administered doses of ethanol (intragastically) of either 2, 4, or 6 g/kg. The glycogen decreasing effect of ethanol was dose dependent. The lowest ethanol dose (2 g/kg) depleted liver glycogen content by 7-27%, while the highest dose (6 g/kg) showed 60-78% depletion. Ethanol doses of 4 g/kg and 6 g/kg decreased both low and high molecular weight glycogen almost evenly. There was slightly more low molecular weight glycogen loss than high with a 2 g/kg ethanol dose. In time course experiments, maximal glycogen depletion was observed at 90 minutes after an ethanol dose of 6 g/kg. After 24 hours, over-production of glycogen content was seen in ethanol treated rats. However, after 48 hours, liver glycogen content had returned to fed values in ethanol treated rats, although the content of low molecular weight glycogen was elevated relative to high molecular weight. .

Starvation of rats for 48 hours decreased both body weight and liver weight. The hepatic and skeletal muscle glycogen concentrations were decreased by 95% and 55% respectively. The livers of rats starved for 72 hours contained more liver glycogen than those starved for 24 hours and 48 hours. Ethanol accelerated glycogen degradation in the fed-to-starved transition. After 3 hours starvation, liver glycogen content had decreased to about half of the fed levels in ethanol treated rats. However, at 24 hours, glycogen content increased in the ethanol treated rats, to as much as twice that in the control animals. The rate and extent of depletion was greater in LMW glycogen than HMW glycogen at 6 hours and 12 hours.

Studies on the effects of ethanol on the starved-to-refed transition were undertaken using two different protocols, chow refeeding and glucose administration by intragastric intubation. On chow refeeding after 48 hours starvation, liver glycogen repletion at 5 hours was decreased by about 30% in animals treated with ethanol dose of 4 g/kg. At

longer time intervals there was no significant inhibition of glycogen resynthesis. The Inhibition of glycogen resynthesis at 5 hours was probably due both to a decrease in food intake in the treated animals and to inhibition of glycogen synthesis by ethanol. The rate and extent of resynthesis of high molecular weight glycogen was slower in treated rats than in control rats indicating that ethanol might preferentially inhibit the synthesis of high molecular weight glycogen, possibly through disruption or prevention of formation of disulphide bonds in the protein component of high molecular weight glycogen. Unlike liver, intragastric administration of 4 g/kg ethanol before chow refeeding following 48 hours starvation decreased muscle glycogen repletion until 24 hours refeeding, compared to the respective control rats.

A single dose of intragastric administration of ethanol (3.45 g/kg) 1 hour before glucose refeeding by intragastric intubation decreased liver glycogen resynthesis by between 20-40% during the 2 hours after glucose administration. Ethanol probably delayed the peak reached in liver glycogen content by either decreasing glucose absorption, by inhibiting gluconeogenesis or glycogen synthesis, or a combination of all these factors. The overall effect of ethanol in inhibiting glycogen synthesis was not, however, nearly as great as that reported previously in similar experiments.

In experiments where rats were given repeated doses of ethanol for 7 days, liver glycogen content was as much as 25 % higher in treated animals than in control animals at 24 and 48 hours after the last ethanol dose. Both low and high molecular weight glycogen had increased almost uniformly at 48 hours in the ethanol treated rats. Ethanol treatment had, however, decreased kidney glycogen content by 6-26% in the treated rats compared with the control rats, but the content of heart and muscle glycogen was not changed.

The results of this research show that ethanol-induced overproduction of glycogen was seen in fed, fed-starved and starved-refed animals and also in repeated dose experiments. This finding is potentially of great importance in exercise physiology and sports science, in helping to develop recommendations for alcohol intake during training regimes.

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ADH	Alcohol dehydrogenase
ADP	Adenosine 5' - diphosphate
Ag_2SO_4	Silver Sulphate
AlDH	Aldehyde dehydrogenase
AMP	Adenosine 5' - monophosphate
ATP	Adenosine 5' - triphosphate
° C	degrees celsius
CAC	Citric acid cycle
cAMP	Cyclic adenosine 3', 5' - monophosphate
cm	Centimetre
CoA	Coenzyme A
DNA	Deoxyribonucleic acid
Fru-2,6-P_2	Fructose 2,6-bisphophate
g	gram
GLC	Glucose
Glc-6-P	Glucose 6-phosphate
GLY	Glycogen
h, hrs	Hour
H_2O	Water
H_2O_2	Hydrogen Peroxide
KOH	Potassium Hydroxide
LAC	Lactate
MDH	Malate dehydrogenase
MEOS	Microsomal ethanol oxidising system
mg	Milligram
min	Minutes
ml	Millilitre
NaCl	Sodium chloride
NAD^+	Nicotinamide adenine dinucleotide (oxidised form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NIDDM	Non-insulin-dependent diabetes mellitus
nm	Nanometre
NMR	Nuclear magnetic resonance
PP_i	Inorganic pyrophosphate

PYR	Pyruvate
RER	Rough endoplasmic reticulum
RNA	Ribonucleic acid
TCA	Trichloroacetic acid
UDP Glc	Uridine diphosphate glucose
UTP	Uridine triphosphate
W/V	Percentage concentration weight for volume
α	Alpha
β	Beta

GENERAL INTRODUCTION

1.1 GLYCOGEN STRUCTURE AND METABOLISM

1.1.1 Introduction

Glycogen is the major storage form of carbohydrate in animals and corresponds to starch in plants. It occurs mainly in liver (6-8% of the fresh weight of a well-fed adult liver) and muscle, where it rarely exceeds 1% (see Table 1.1). However, because of its greater mass, muscle represents 3-4 times as much glycogen as liver. Like starch, glycogen is a branched polymer of glucose. Glycogen in muscle provides a reservoir of glucose for muscular activity, whereas liver glycogen is involved in the regulation of blood glucose levels by storage of excess glucose after meals and the subsequent release of glucose between meals.

Table 1.1 Storage of Carbohydrate in Post Absorptive Normal Adult Humans (70 kg). (Data from Murray *et al.* 1990)

Liver glycogen	~ 6-8% = 100 g (liver weight 1800 g)
Muscle glycogen	~ 1.0% = 400 g (Muscle mass 35 kg)
Extracellular glucose	~ 0.1% = 10 g (Total volume 10 L)

Glycogen is also found in lower concentrations in heart (Bartley and Dean, 1968), brain (Coxan *et al.*, 1965; Strang and Bachelard, 1971), adrenal gland (Bergman, 1983), adipose tissue (Eichner, 1984), placenta (Heikensjold and Gemzell, 1957) and erythrocytes (Sugiyama and Eguchi, 1984).

Glycogen levels are extremely susceptible to the nutritional state of the animal (Geddes and Stratton, 1977; Geddes, 1971; Baud, 1967; Olavarria *et al.*, 1968), temperature (Kepinov, 1960; Chayoth and Cassuto, 1971), time of day (Mizell *et al.*, 1971; Bockman *et al.*, 1971; Cohn and Joseph, 1971; Suda *et al.*, 1979), season (Mizzel *et al.*. 1971), and even persistent noise (Moszcynski, 1980).

Glycogen was first isolated from liver by Claude Bernard in 1857 (Bernard, 1857). The main structural features of glycogen were established many years ago. For a historical perspective see reviews such as Manners (1957, 1962, 1991), Marshall (1974), Whelan (1971, 1976, 1986), Geddes (1985) and Calder (1991). The synthesis and degradation of glycogen in muscle and liver and the hormonal regulation of these processes have been investigated in depth (see Cohen 1982, 1983, for reviews). There have been major advances in our understanding of the structure of glycogen and the relationship between its structure and biosynthesis. The detailed fine structure continues to be the subject of investigation. The physical form of the molecules, which may occur as particles with molecular weights of up to 10^7 (β-particles), or much larger particles (α-particles) is still being studied (see Geddes, 1985; Manners, 1991; Calder, 1991). Despite these advances many biochemists, including those working on ethanol and glycogen metabolism, are unaware of the current understanding (concept) of glycogen structure, and its close relationship with its metabolism. Most of the alcohol researchers who were dealing with glycogen (Walker and Gorden, 1970; Lefèvre *et al.*, 1970; Winston and Reitz, 1980, 1981; Palmer *et al.*, 1991, Cook *et al.*, 1992; Xu *et al.*, 1993), did not consider the relationship between the β and α particles, and the fact that glycogen is structurally and metabolically heterogeneous and exists in both the cytosol and the lysosomes. The two forms differ in molecular weight and protein content. Despite a considerable amount of research on glycogen content and its metabolism, many researchers believe that glycogen is a simple storage molecule or may be regarded as $(glucose)_n$ (Geddes, 1985).

1.1.2 **The Basic Structure of Glycogen**

Liver glycogen is well established as being not only of very high molecular weight but also of great polydispersity (Orrell and Bueding, 1964; Parodi *et al.*, 1967; Geddes, 1971; Geddes and Stratton, 1977a,b; Geddes *et al.*, 1977a,b; Bullivant *et al.*, 1983; Lutkic *et al.*, 1980; Geddes, 1985; Geddes *et al.*, 1992). Its molecular weight distribution ranges from below 1×10^6 daltons to greater than 1000×10^6, with a average molecular weight, in normal animals, of 270×10^6 (Geddes, 1985). Subsequent studies have indicated that a similar size range exists in normal mammalian muscle (Calder and Geddes, 1985a,b; 1986).

Figure 1.1 illustrates the structure of two outer branches of a glycogen molecule. Chemical studies showed that the macromolecules were composed of linear chains normally containing an average of 10-14 (1-4)-linked α-D-glucose residues. These were interlinked by (1-6) α-D-glucosidic linkages, to form a branched structure. The early fine structural analyses were carried out by chemical methods such as partial acid analysis, methylation analysis and periodate oxidation. More recently, enzymes have

been commonly used in the structural analysis of glycogen, including α-amylase, glucoamylase, debranching enzymes, and glycogen phosphorylase (see reviews by Whelan, 1971; Marshall, 1974; Manners, 1991).

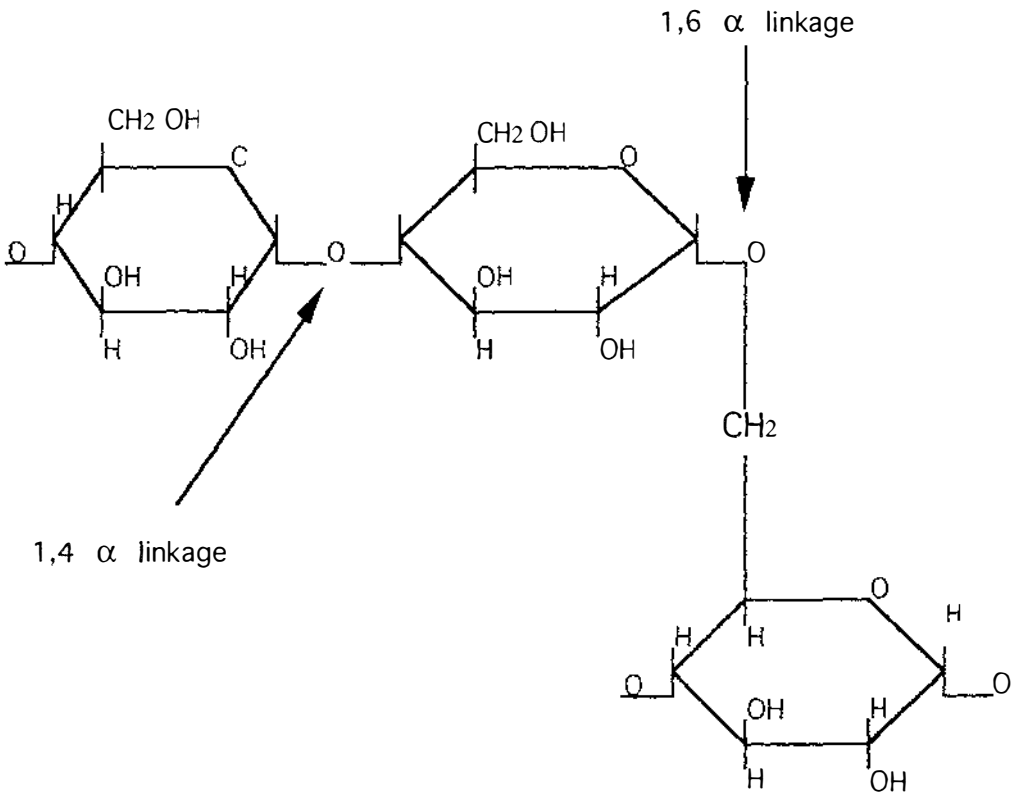

Figure 1.1 Structure of Two Outer Branches of a Glycogen Molecule

Most of the glucose units in glycogen are linked by α-1,4 glycosidic bonds. At about every tenth residue, a branch is created by an α-1,6 glycosidic bond. The polysaccharide components of glycogen are thought to consist nearly entirely of glucose units. Glycogen is similar to amylopectin (starch) except that the branch points are much more frequent (about one every 10 glucose residues).

From electron microscopy, it has been observed that glycogen is built of spherical particles of approximately 30 nm diameter (β-particles) and of associations of these spheres into clusters (α-particles) which can approach 200 nm in diameter (Fawcett, 1955; Drochmans, 1962, 1963, 1965; Drochmans and Dantan, 1968; Wanson and Drochmans, 1972). Individual β--particles were later shown to have a molecular weight of approximately 10 x 10^6 (Geddes and Stratton, 1977a).

1.1.3 Effects of Purification Procedures on Molecular Weight of Purified Glycogen

The traditional glycogen extraction technique used hot, concentrated alkali. Table 1.2 shows the molecular sizes of glycogens obtained by this technique. Glycogen obtained using this method has an average molecular weight of 1-10 x 10^6 Da. However, it has been established that this technique causes extensive degradation of glycogen (Stetten and Katzen, 1961). A second technique for glycogen extraction is based upon maceration of the tissue with cold trichloroacetic acid (TCA). The product obtained by TCA extraction is larger than that obtained by KOH extraction (Table 1.2), although yields are lower than with KOH. Aqueous extraction techniques yield products which have molecular weights in excess of those obtained with either KOH or TCA. Lazarow (1942) established that large liver glycogen particles could be extracted with cold water and they believed that glycogen aggregation was due to protein present in the preparation. However, it was later shown that treatment of such glycogen with hydrogen bond-breaking reagents does not lower the molecular weight (Orrell and Bueding, 1964), indicating that glycogen aggregation may be due to more than one factor. A subsequent development was the phenol-cold water method of Laskov and Margoliash (1963). This method relies upon phenol denaturing and extracting enzymes, thereby eliminating sources of glycogen degradation and allowing easy separation of proteins from glycogen. The glycogen isolated using the phenol-cold water technique is the largest yet obtained (Table 1.2) and glycogen ranging in size up to 3000 x 10^6 Da has been described (Geddes *et al.*, 1977a,b).

The phenol-cold water extraction method has been directly compared to the TCA and KOH techniques (Geddes, 1985). A single lobe of a bullock liver was extracted by the TCA, KOH and phenol-cold water methods and the sedimentation coefficient profiles of the products compared (Geddes, 1985). It appears that mild, aqueous extraction techniques are the most acceptable. They produce glycogen which is of a much larger size than the material extracted by the harsh alkali or acid techniques and this material is closest to the native structure of glycogen (Geddes, 1985).

Table 1.2 Comparison of the Products Obtained by Different Extraction Techniques.

Methods	Size (Daltons)	Source	Reference
KOH	1 - 8 x 10^6	Rat liver	Stetten *et al.*, 1958
	3.1 - 3.8 x 10^6	Human liver	Bryce *et al.*, 1958a
TCA	46 - 70 x 10^6	Rat liver	Stetten *et al.*, 1958
	60 - 160 x 10^6	Cat liver	Bryce *et al.*, 1958b
Coldwater extraction technique	10 - 500 x 10^6	Rabbit liver	Orrell and Bueding, 1958, 1964
Phenol - cold water technique	23 - 400 x 10^6	Rat liver	Laskov and Margoliash, 1963
Mercuric chloride extraction	365 x 10^6	Rat liver	Vaillant, 1970b
Mild extraction procedure	3000 x 10^6	Rat liver	Mordoh *et al.*, 1966
Phenol - cold water method	3000 x 10^6	Rat liver	Geddes *et al.*, 1977a; Geddes,1985

1.1.4 The Proteoglucan Nature of Glycogen

That liver glycogen is built upon a protein backbone was first shown by Krisman and Barenco (1975) and subsequently confirmed by others (Chee and Geddes, 1977; Butler *et al.*, 1977; Matcham *et al.*, 1978). It is well established that liver glycogen is composed of an extremely heterogeneous mixture of single polysaccharide spheres (ß-particles) and vast covalently bound aggregations of these spheres (α-particles) (Geddes *et al.*, 1977a;

1977b; Geddes, 1985). Undegraded ß-particles within the α-particles have a molecular weight of approximately 1 x 10^7 (Geddes *et al.*, 1977a) and they are known to be built upon a single protein backbone (Krisman and Barenco, 1975) in groups of two to three (Chee and Geddes, 1977; Geddes *et al.*, 1977a) which may then aggregate by covalent bonding through disulphide bridges to form the large α-particles (Fig. 1.2 and 1.3; Chee and Geddes, 1977; Geddes and Stratton, 1977a). When liver glycogen which had been isolated by the phenol-cold water method was treated with β-mercaptoethanol and iodoacetamide the proportion of high molecular weight glycogen (> 500 x 10^6) decreased from 20 to 2% (Chee and Geddes, 1977).

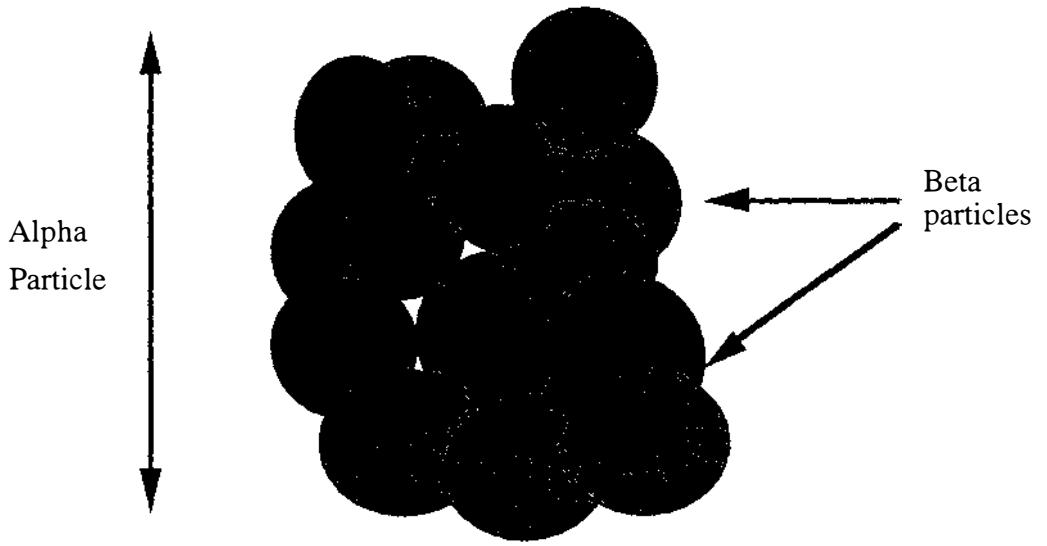

Figure 1.2 Gross Structure of Glycogen Molecule

Large glycogen molecules (alpha particles) consist of aggregates of individual spherical beta particles. The above is similar to electron microscopic observations of alpha particles (Calder, 1991).

Subsequently a similar mode of construction was observed in muscle glycogens (Calder and Geddes, 1985 a,b; 1986). The high molecular weight glycogen (arbitrarily defined as > 500 x 10^6 daltons) is found to be associated with the lysosomal compartment. Minimum estimates suggest that up to 20% of liver glycogen is found in the lysosome (Geddes and Stratton, 1977a,b; Geddes *et al.*, 1983; Geddes and Taylor, 1985a,b; Geddes and Chow, 1994b). Apart from its lysosomal association, high molecular weight glycogen is also distinguished from its lower molecular weight counterpart by having at least double the amount of protein bound to it per gram of polysaccharide (Chee and Geddes, 1977; Calder and Geddes, 1983; Geddes, 1985). A similar phenomenon is observed in muscle tissue (Calder and Geddes, 1985b, 1986).

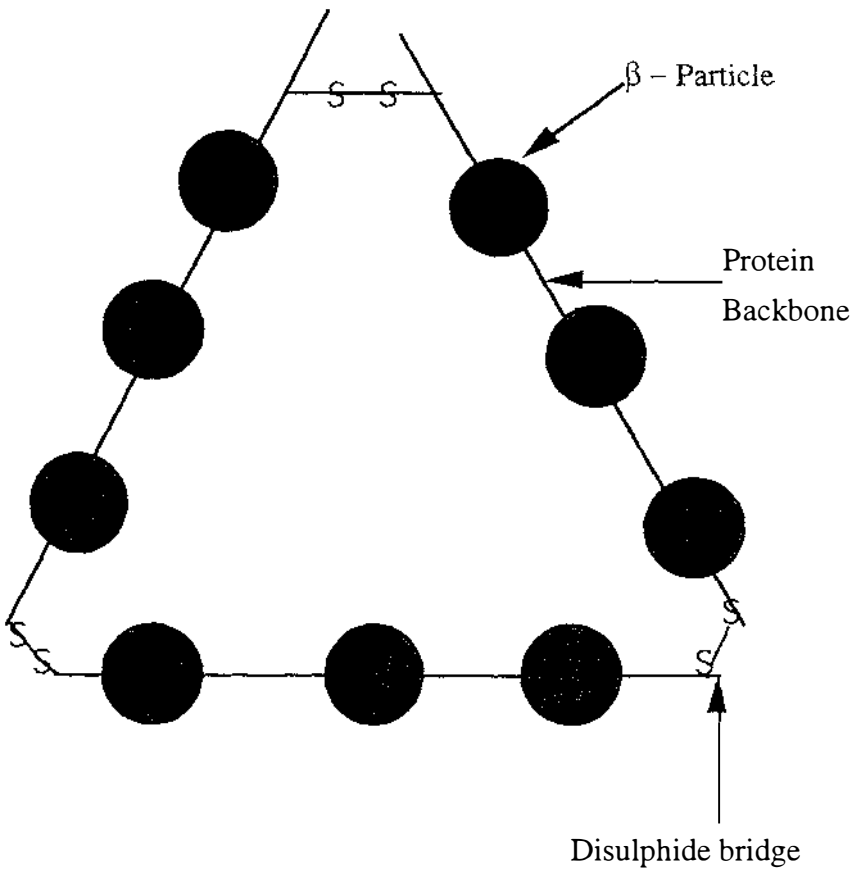

Figure 1.3 A Schematic Representation of the Mode by Which High Molecular Weight Glycogen is Constructed.

The spheres represent glycogen beta particles and these are built around protein (solid black line). 'The fundamental units' of three beta particles on protein backbones form large conglomerates by disulphide (S-S) bridging (from Geddes *et al.*, 1977a). The very large alpha particles are composed of large numbers of these 'fundamental units', up to 40-50. (Note: The drawing is not to scale).

Geddes *et al.* (1977a) and Geddes (1985) reported that low and high molecular weight glycogens are distinct chemical entities and their syntheses must occur separately, perhaps at different cellular sites: i.e. there is pronounced compartmentation of glycogen synthesis (Ching *et al.*, 1985). Indeed, Barber and co-workers (Barber *et al.*, 1967) observed preferential association of phosphorylase with low molecular weight glycogen, and synthase with high molecular weight glycogen.

1.1.5 Glycogen Synthesis and Breakdown

1.1.5.1 Glycogen Synthesis

Glycogen synthesis occurs mainly in muscle and liver. Glucose enters the cell from the bloodstream and is phosphorylated to glucose 6-phosphate by the action of hexokinase in muscle and glucokinase in liver. Glucose 6-phosphate is converted to glucose 1-phosphate in a reaction catalysed by the enzyme phosphoglucomutase. Glucose 1-phosphate is then activated by the reaction with uridine triphosphate (UTP), forming UDP-glucose and inorganic pyrophosphate (PP_i), catalysed by UDP-glucose pyrophosphorylase. In the subsequent step of glycogen synthesis, glycogen synthase catalyzes the addition of the glucose residue from UDP-glucose to the nonreducing end of glycogen (Fig 1.4).

The glycogen synthase reaction is the major regulatory step of the pathway of glycogen synthesis. The enzyme is inactivated by phosphorylation in response to increased cAMP, an effect that is counteracted by insulin (Fig. 1.4). However, other factors such as tissue glycogenin content and the level of lysosomal glycogen storage probably also regulate glycogen synthesis. Glycogen synthase requires a preexisting glycogen primer of at least four glucose residues and the primer consists of up to eight α-(1-4) linked glucose residues. This is attached by the 1-hydroxyl group of the reducing end in a glycosidic linkage to a specific tyrosine residue of the protein glycogenin (37 000 Da). Each completed molecule of glycogen contains a single molecule of glycogenin (see reviews by Smythe and Cohen, 1991; Calder, 1991; Alonso *et al.*, 1995). When the chain has been lengthened to a minimum of 11 glucose residues, a second enzyme, the branching enzyme (amylo (1-4)-(1-6)-transglucosidase) transfers a part of the (1-4) chain (minimum length 6 glucose residues) to a neighbouring chain to form a (1-6) linkage, establishing a branch point in the molecule.

Glycogen synthesis has been observed in both human (Hultman *et al.*, 1971; Bonen *et al.*, 1989) and in rodent muscle (Constable *et al.*, 1984; Kuipers *et al.*, 1986) during exercise when carbohydrates are provided. Even when exogenous carbohydrates are not supplied, glycogenesis is activated throughout exercise in skeletal muscle, and the rate of glycogenesis is increased over that observed at rest (Bonen *et al.*, 1989). It appears that glycogenesis is a dynamic process that occurs at all times (Bonen *et al.*, 1989).

Muscle glycogen restoration from lactate (Glyconeogenesis)

Numerous studies have focussed on the production of lactic acid in skeletal muscle (Fitts and Holloszy, 1976; Juel, 1988; Katz and Sahlin, 1988; Metzger and Fitts, 1987; McDermott and Bonen, 1992). The two primary fates of lactate in skeletal muscle are (1) oxidation and (2) glycogen synthesis (glyconeogenesis). Lactate conversion into muscle glycogen can occur via two distinct pathways, Cori cycle and muscle lactate glyconeogenesis. It has long been known that lactate can be taken up by both inactive and active muscle and oxidized to CO_2 and H_2O (Depocas *et al.*, 1969). Although glucose utilization as a substrate for glycogen synthesis (glycogenesis) has been known for a long time, it is apparent that glycogen can also be synthesised from lactate (glyconeogenesis) (Connet 1979; Bonen *et al.*, 1989; McDermott and Bonen, 1992; Palmer and Fournier, 1997). Glyconeogenesis has also been observed in mammalian skeletal muscle using radiotracer methodology (McLane and Holloszy 1979; Shiota *et al.*, 1984; Pagliossotti and Donovan, 1990; McDermott and Bonen, 1992) which showed that noncontracting, perfused rat hindlimb muscles synthesized glycogen from lactate. Contribution of lactate to glycogen synthesis is matter of controversy. Estimates of lactate conversion to glycogen have ranged from 11% (Bangsbo *et al.*, 1990), to 50% (Astrand *et al.*, 1986), and 95% (Hermansen and Vaage 1977) in human muscle, from 10-66% in rat muscles (McLane and Hollozy, 1979) and 30% in mouse muscle (McDermott and Bonen, 1992). Despite the fact that glyconeogenesis has been observed, the complete biochemical pathway responsible for this process remains unknown. Further investigation is required to determine the regulation of glyconeogenesis in muscle, the pathway involved and an estimation of the significance of lactate in forming glycogen (see section, 1.1.7).

Cell Swelling and Glycogen Synthesis

In hepatocytes from fasted rats, several amino acids such as glutamine, proline, alanine and asparagine are known to stimulate glycogen synthesis (Katz *et al.*, 1976; 1979; Baquet *et al.*, 1990; Meijer *et al.*, 1992; Mouterde *et al.*, 1992; Minassian *et al.*, 1994). Lavoinne *et al.*. (1987) proposed that the regulatory mechanism involved results in an activation of glycogen synthase without a change in glycogen phosphorylase. The mechanism of activation of liver glycogen synthase is by swelling caused by the Na^+-dependent entry of amino acids and by the intracellular accumulation of amino acids which are produced during amino acid catabolism (Baquet *et al.*, 1990). Subsequent

studies (Meijer *et al.*, 1992) revealed that a fall in intracellular Cl^- concentration as well as an increase in intracellular glutamate and aspartate concentrations are observed in swollen hepatocytes. Furthermore, they concluded that the presence of amino acids is responsible, at least in part, for the stimulation of synthase phosphatase.

1.1.5.2 **Glycogenolysis**

Glycogen degradation in liver and muscle occurs mainly via phosphorolysis, catalyzed by glycogen phosphorylase (Fig. 1.4). Glycogen phosphorylase catalyzes the progressive degradation of glycogen chains from the nonreducing ends. The enzyme stops four glucose residues from a branch point (α-(1-6) glucosidic bond), leaving a limit dextrin. Another enzyme (α-(1-4) - α-(1-4) glucan transferase) transfers a trisaccharide unit from one branch to the other, exposing the 1-6 branch points. The hydrolytic splitting of the 1-6 linkages requires the action of a specific debranching enzyme, amylo-(1-6)-glucosidase. Further phosphorylase action can proceed after the removal of the branch. In liver and kidney (but not in muscle), a specific enzyme, glucose 6-phosphatase removes phosphate from glucose 6-phosphate. This enables glucose to diffuse from the cell into the blood. Glycogen phosphorylase is a dimer composed of two identical subunits, with a molecular weight of 97, 444 (842 amino acids) (Sprang *et al.*, 1987; 1991; Johnson, 1992). The properties of the enzyme have been reviewed previously (Madsen, 1986; Johnson *et al.*, 1989; Johnson, 1992; Newgard *et al.*, 1989; Palm *et al.*, 1990).

The role of glycogen in exercise physiology has been intensively investigated for the past three decades. Since about 1980 there have been significant developments in our understanding of muscle glycogen metabolism. The use and deposition of glycogen by muscle have especially received a great deal of attention. Over last two decades, research has shown that a particular combination of diet and exercise results in a significant increase in muscle glycogen levels (oversynthesis of muscle glycogen) (Tarnopolsky *et al.*, 1990; Phillips *et al.*, 1993; Tarnopolsky *et al.*, 1995). This procedure is known as carbohydrate loading or glycogen supercompensation. Carbohydrate loading is one of the more popular methods of nutritional modification used by endurance athletes to improve performance.

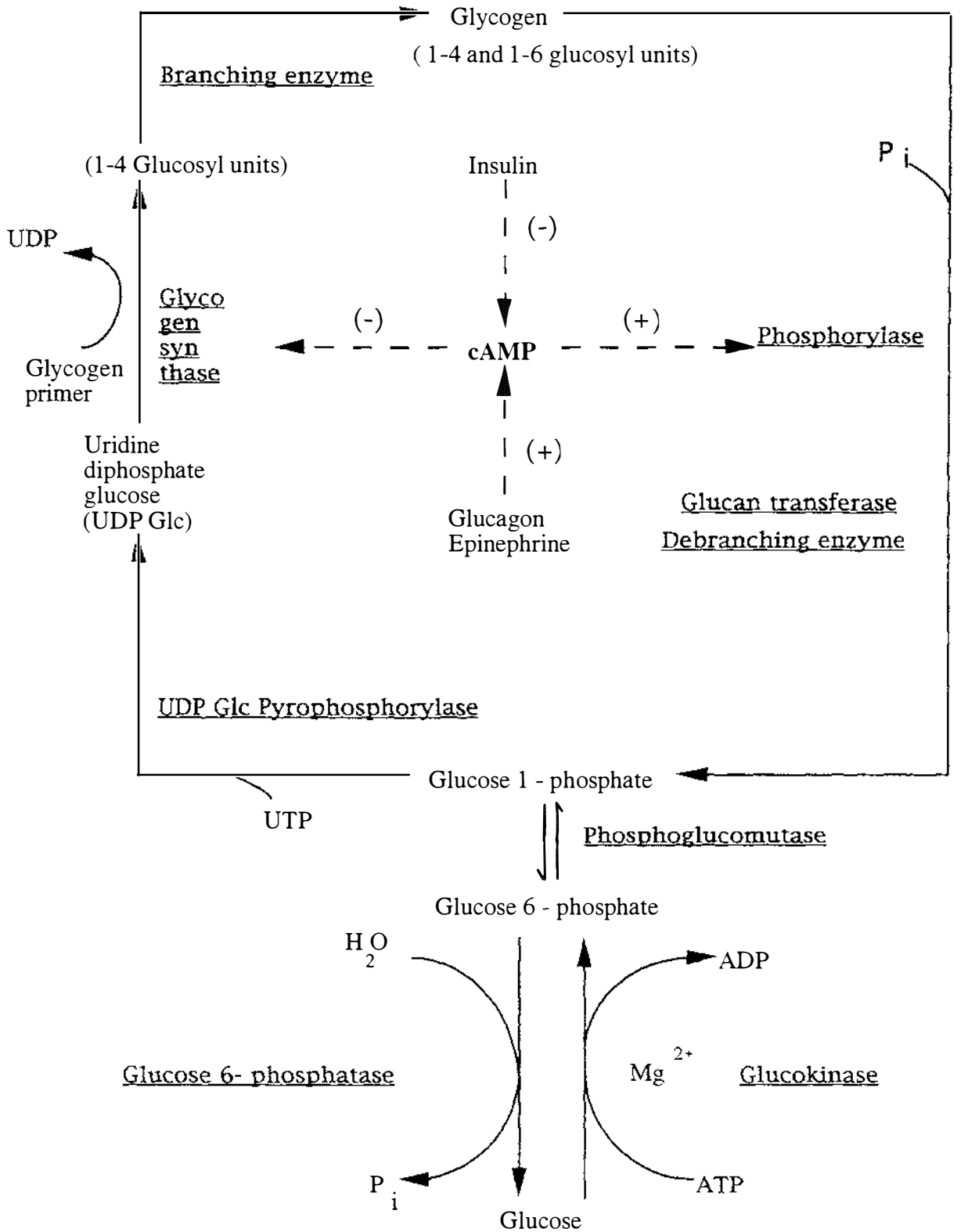

Figure 1.4 Pathway of Glycogenesis and of Glycogenolysis in the Liver

Two phosphates are used in the incorporation of 1 mol of glucose into glycogen. (+), stimulation; (-), inhibition. Insulin decreases the level of cAMP only after it has been raised by glucagon or epinephrine: ie it antagonizes their action . Glucagon is active in heart muscle but not in skeletal muscle. (Redrawn from Murray *et al.*, 1990).

According to the conventional view (1) glycogen concentrations decline during exercise, (2) the more intense the exercise the more rapid the decline in glycogen concentration, (3) when carbohydrates are supplied exogenously, rates of glycogen repletion may be augmented after exercise, and (4) glycogen loss means the muscle has been exercising. These facts have been challenged by many researchers. Glycogen concentrations do not always decline throughout exercise (Bonen *et al.,* 1989).

1.1.5.3 **Glycogenin and Proglycogen**

Glycogenin is a 37-kDa protein upon which new glycogen molecules are considered to be constructed (reviewed by Smythe and Cohen, 1991; Calder, 1991; Alonso *et al.*, 1995). Glycogenin has been most extensively studied in skeletal muscle. Tyrosine is involved in a novel glycosidic linkage between the carbohydrate and protein portions of the molecule (Rodriguez and Whelan, 1985).

The structure of glycogen as proposed by Krisman and Barengo (1975) and Whelan (1976; 1986) show several β-particles being synthesised upon one glycogenin molecule. This assumes more than one glucosylation site per glycogenin molecule, although only a single glucosylation site has been reported for muscle (Smythe *et al.*, 1988; Campbell and Cohen, 1989) and liver (Smythe *et al.,* 1989) glycogenins. However, subsequent findings (Lomako *et al.*, 1991) revealed that muscle glycogenin may in fact be much larger than previously reported and may contain a number of glucosylation sites. Moreover, a self-glucosylating form of glycogenin does not exist in the free state in muscle but only exists in glycogen and in the 400, 000 Da molecular weight proform of glycogen termed "proglycogen" (Lomako *et al.*, 1991).

Amino acid analysis reveals major differences between the protein backbones of rat liver glycogen and rabbit skeletal muscle glycogenin. Rat liver glycogen (Calder and Geddes, 1988) was isolated by the phenol-sulphuric acid method to produce native glycogen with a very high content of high molecular weight glycogen. The rat liver protein was relatively rich in glutamine, serine, glycine and alanine while rabbit skeletal muscle glycogenin contains a relatively higher content of asparagine, alanine and threonine.

The discovery of proglycogen and glycogenin has led to new insights into the regulation of glycogen metabolism (originally known to be regulated at glycogen phosphorylase and glycogen synthase). Proglycogen was proposed to be the stable intermediate for the synthesis and breakdown of depot macroglycogen. According to glucose supply and

energy demand, glycogen oscillates between the macroglycogen (10^7 Da) and proglycogen, but not usually the glycogenin forms (Alonso *et al.*, 1995). It remains unclear what the factors are that regulate the balance between glycogenin, proglycogen, and macroglycogen and the relationship between the β-particles and α–particles. Alonso *et al.* (1995) suggested that the amount of glycogenin will influence how much glycogen the cell can store.

1.1.6 **The Role of Lysosomes in Glycogen Metabolism**

Lysosomes were first recognised as a separate entity by de Duve *et al.*, (1955). All mammalian cells, with the exception of erythrocytes, contain lysosomes (Dean, 1977). They are dense bodies, 0.25 to 0.5 nm in diameter, bounded by a single membrane (de Duve, 1969). In normal animals a portion of the cellular content of glycogen is associated with the lysosomal compartment (Phillips and Unakar, 1967; Fouquet, 1968; Vrensen and Kuyper, 1969; Geddes and Stratton, 1977a; Brown *et al.*, 1978; Knecht and Hernandez, 1978; Garfield and Cardell, 1979; Rybicka, 1981; Geddes *et el.*, 1983; Geddes, 1985; Geddes and Taylor, 1985 a,b; Ching *et al.*, 1985; Calder and Geddes, 1989 a; Geddes and Chow, 1994b).

Glycogen isolated from lysosomal preparations contains a higher proportion of associated protein (reviewed by Geddes, 1985, 1986; Calder, 1991) and is easily distinguishable from its cytosolic counterpart by large molecular size. The modified version of the Cori cycle showing the putative role of the lysosomal pathway in muscle and liver is illustrated in Fig 1.5. The process by which glycogen is compartmentalised in the lysosome has not yet been described. However, some glycogen associated with the rough endoplasmic reticulum of the liver has been observed, and a recent report has found glycogen particles associated with cardiac sarcoplasmic reticulum (Calder and Geddes, 1992).

It is now well established that metabolism of glycogen in the liver (Geddes and Stratton, 1977a,b; Geddes and Taylor, 1985a,b) and muscle (Calder and Geddes, 1985a,b; 1986, Geddes and Chow, 1994b) can proceed hydrolytically through the lysosomal compartment. The enzyme that catalyses this is α-1-4 glucosidase (Fig. 1.5). The regulation of breakdown of lysosomal glycogen is important since the hydrolytic reaction allows the liberation of free glucose. The fact that skeletal muscle lysosomes are involved in glycogen metabolism is of importance because of the metabolic implication of free glucose release within muscle cells (Fournier *et al.*, 1992; Geddes and Chow, 1994b).

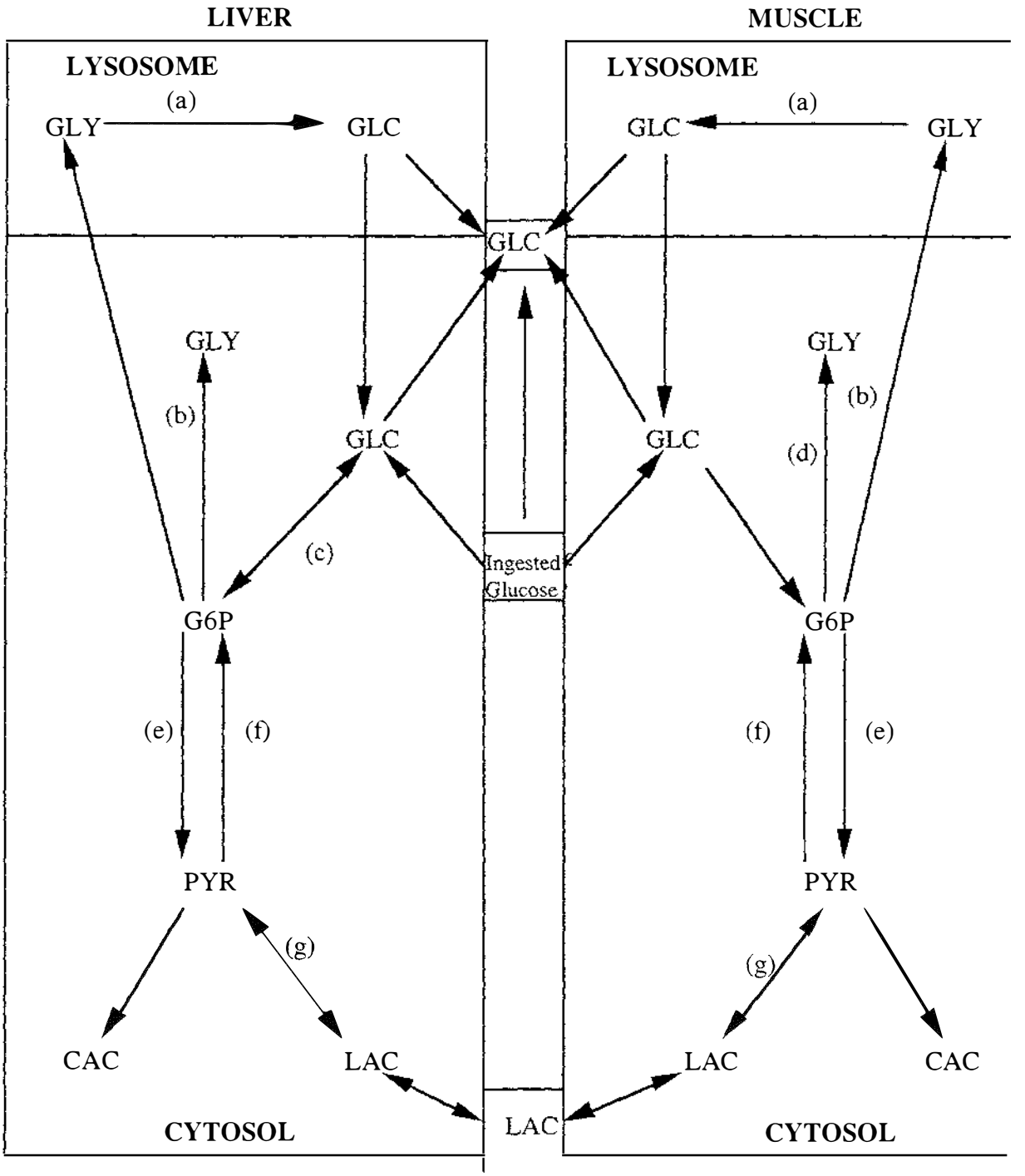

Figure 1.5 A Modified Version of the Cori Cycle Showing the Putative Role of the Lysosomal Compartment in Muscle and Liver.

Note that the release of free glucose into the cytosolic compartment (in both tissues) by the action of debranching enzyme (Geddes, 1986) has been omitted in the interest of clarity. (Redrawn from Geddes and Chow, 1994b)
G6P = glucose-6-phosphate, PYR = pyruvate, LAC = lactate, CAC = citric acid cycle

(a) (1-4)-glucosidase. (b) Phosphoglucomutase. (c) Hexokinase, glucose-6-phosphatase. (d) Hexokinase. (e) Glycolysis. (f) Gluconeogenesis. (g) Lactate dehydrogenase

The glycogen within lysosomes appears to be largely of the α-particulate form (Phillips *et al.*,1967; Taylor 1985). However, although glycogen has been isolated from lysosomal preparations, based on electron microscopy and other techniques, Konishi *et al.*, (1990) observed that there was little or no measurable lysosomal glycogen in normal liver. It appears that intralysosomal storage of glycogen cannot be seen under the electron microscope in the normal liver (Baudhuin *et al.*, 1964; Pfeifer, 1971; Lullmann-Rauch, 1981; Saul *et al.*, 1985). Konishi *et al.* (1990) estimated that about 3% of the glycogen would be hydrolysed by the lysosomal pathway. These results contrasted with numerous other studies which have reported that up to 20% of glycogen may be located in the lysosomal compartment, mainly in the form of high molecular weight glycogen (Geddes, 1986; Calder, 1991; Geddes and Chow, 1994b).

1.1.6.1 **Pompe's Disease** (glycogen storage disease type II)

The congenital absence of lysosomal glucosidase is known to be the enzyme deficiency responsible for glycogen storage disease type II. The disease was first described by Pompe (1932), was termed type II by Cori (1957), and the enzyme deficiency responsible for it was recognised by Hers (1963). The lysosomal nature of this deficiency and the excess glycogen storage resulting from it were reported by Baudhuin *et al.*, (1964).

Electron microscopic observation indicates the presence of significant amounts of glycogen inside the lysosome of many Pompe's disease tissues, including liver (McAdams and Wilson, 1966; Hernandez *et al.*, 1966; Hug *et al.,* 1966; Hug and Schubert, 1967a,b; Garanicis, 1968; Bruni and Paluello, 1970; Walvoort *et al.*, 1985), skin (Hers, 1973), leucocytes (Hers and VanHoof, 1968), placenta (Bendon and Hug, 1985), and skeletal and heart muscle (Zellweger *et al.*, 1965; Cardiff, 1966; Hug *et al.*, 1966; Smith *et al.*, 1966; Hug and Schubert, 1967b; Hers and Van Hoof, 1968; Bruni and Paluello, 1970; Griffin, 1984; Howell *et al.*, 1984; Walvoort *et al.*, 1985).

1.1.7 **The Cori Cycle and its Modifications**

Muscle is generally a glucose-utilizing organ, rather than glucose-producing, because it lacks glucose 6-phosphatase. However, it has now been shown that the source of free glucose in muscle is not only from the liver via the blood stream; there is also an endogenous source. Several publications (Calder and Geddes, 1983; 1985a,b; 1986;

1992; Geddes and Chow, 1994b) have shown that muscle, like liver, stores a significant portion of its glycogen reserves in the lysosomal compartment from whence it can be released as free glucose, via the action of α-D-glucosidase. Consequently, modification of the Cori cycle has been suggested (Geddes, 1986; Geddes and Chow, 1994b) in order to recognise the potential glucogenic capacity of muscle. In addition, a recent publication (Fournier *et al.*, 1992) has shown that frog muscle releases physiologically significant amounts of free glucose when recovering from exercise. These findings indicate the importance of hydrolytic breakdown of glycogen (in addition to phosphorolytic).

Fig 1.5 shows a modified version of the Cori cycle. In addition to degradation of liver glycogen by phosphorylase, hydrolytic degradation steps (debranching by amylo-1,6- α-glucosidase and hydrolysis of lysosomal glycogen by acid 1,4-α-glucosidase) are included. In the liver each of these accounts for approximately 10% of glycogen degradation; 10% of glycogen is at the branch point and will be released by debranching and at least10% is in the lysosome. Thus 20% of liver glycogen may be hydrolytically degraded to glucose and may be transported directly to the blood stream. (Geddes, 1985).

The irreversible steps of glycolysis have been assumed to result in the inability of muscle tissue to convert lactate to glycogen. However, conversion of lactate to glycogen in muscle tissue has been observed (Moorthy and Gould, 1969; Bendall and Taylor, 1970; Hermansen and Vaage, 1977; Mclane and Holloszy, 1979; McDermott and Bonen, 1992; Palmer and Fournier, 1997), indicating that, contrary to the long standing concept, muscle is a gluconeogenic tissue. Some of the enzyme activities required to carry out this process have been identified in muscle tissue, but others have not (Crabtree *et al.*, 1972; Newsholme and Williams, 1978; Zammit *et al.*, 1978; Newsholme and Leech, 1983). It is not clear how the energetically unfavourable reversal of the pyruvate kinase reaction (pyruvate-phosphoenolpyruvate) is accomplished. However, Dyson *et al.* (1975) suggested that it may be possible to form PEP directly from pyruvate via pyruvate kinase. The reversibility of these reactions is included in the modified metabolic pathway shown in Fig. 1.5.

Clearly the long-held view that the Cori cycle is the sole mechanism for removal of muscle lactate, and that the blood stream is the sole source of the free glucose within muscle tissue, is not entirely correct. Lactate can be reconverted to hexose-phosphate and incorporated into glycogen. Glucose can arise from hydrolytic degradation of glycogen. The revised pathway (Fig. 1.5) is a more accurate description of the metabolic events.

The disturbance of liver lysosomal function causes disturbance to overall cellular carbohydrate metabolism. This has been found in studies where hepatic lysosomal glycogen metabolism was inhibited by antibodies to acid α–(1,4) glucosidase (Geddes *et al.*, 1983; Taylor, 1985), by castanospermine (Saul *et al.*, 1985) and by acarbose (Geddes and Taylor, 1985a,b). The coupling of an increase in lysosomal glycogen with an overall decrease in cellular glycogen content indicates that a feed back control mechanism exists in normal tissue. Such a mechanism seems to control overall glycogen synthesis, and has an effect upon the mechanism by which glycogen is incorporated into lysosomes.

Skeletal muscle glycogen metabolism appears not to be affected by acarbose. However, a study indicated that cardiac muscle glycogen metabolism can be perturbed by acarbose (Haugaard *et al.*, 1984), although no induced lysosomal glycogen storage in cardiac muscle was noted by Lullmann-Rauch (1982).

1.1.8 **Glycogen Turnover**

There is evidence that glycogen synthase and glycogen phosphorylase can be simultaneously active in rat liver during net glycogen synthesis (Katz *et al.*, 1979; Newman and Armstrong, 1978; Van de Werve and Jeanrenaud, 1987). The simultaneous occurrence of both reactions is an energy-requiring process referred to as glycogen cycling (Fig 1.6). However, the experimental reports regarding simultaneous glycogen synthesis and degradation (mostly in liver), are conflicting. Glycogen cycling is usually defined as the flux through phosphorylase relative to the flux through synthase (represented by V4/V3; Fig. 1.6). The relative rate of glycogen cycling is complicated to measure, and different approaches have yielded different results, from minimal to extensive cycling (Katz *et al.*, 1979; Postle and Bloxham, 1980; Youn *et al.*, 1986; Shulman *et al.*, 1988; David *et al.*, 1990; Wajngot *et al.*, 1991; Magnusson *et al.*, 1994). Glycogen turnover is indicated in the liver by ^{13}C NMR studies which show a decline in the ^{13}C resonance from glycogen previously labelled from [^{13}C]glucose, despite continued glycogen synthesis (Shulman *et al.*, 1987; David *et al.*, 1990; Magnusson *et al.*, 1994). Other isotopic studies either support (Wajngot *et al.*, 1991; Barrett *et al.*, 1994) or refute (Katz *et al.*, 1979; Postle and Bloxham, 1980) the occurrence of hepatic glycogen cycling. In perfused livers from 24-h-fasted rats, the rate of glycogen breakdown was measured to be as much as 60% of the rate of net glycogen synthesis (Shulman *et al.*, 1988). The liver glycogen was monitored with ^{13}C nuclear magnetic resonance (NMR) spectroscopy. Using a similar method, David *et al.*, (1990) demonstrated that in liver of both fed and fasted anaesthetised rats glycogen synthesis and degradation occurred simultaneously. The rate of degradation was estimated to be about

15% of the rate of net synthesis in the fasted animals and to be higher in the fed animals. Wajngot *et al.*, (1991) recently measured liver glycogen cycling in normal and insulin-dependent diabetic subjects, and in both normal and diabetic subjects was found to range between 2 and 12% hepatic glycogen cycling. Liver glycogen synthesis and degradation occur simultaneously in humans under conditions of net glycogen synthesis. The relative turnover rate was significantly higher in fed than in the fasted state. These results indicate that glycogen may regulate its own breakdown and that glycogen turnover may be an important factor in limiting the accumulation of liver glycogen in humans (Magnusson *et al.*, 1994). There is a report of significant glycogen turnover in isolated skeletal muscle, and changes upon hormonal addition (Challiss *et al.*, 1987). In addition, glycogen turnover was investigated in rat heart (Laughlin *et al.*, 1988; Goodwin *et al.*, 1995). The isolated working rat heart was adapted for simultaneous determination of glycogen synthesis and degradation using a dual isotope technique . In the absence of added hormones, hearts were predominantly glycogenolytic and there was simultaneous synthesis (11% of the rate of glycogenolysis) and glycogen turnover was 5% (Goodwin *et al.*, 1995).

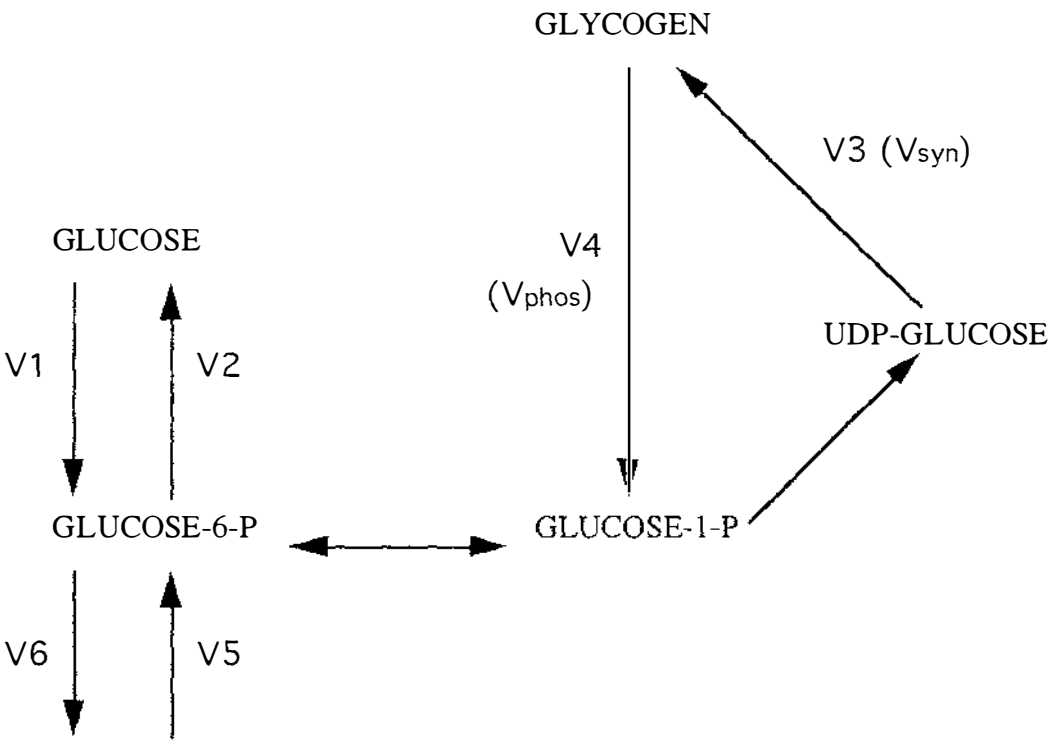

Figure 1.6 Scheme of glucose and glycogen metabolism in liver.

V1, glucokinase; V2, glucose-6-phosphatase; V3, glycogen synthase; V4, glycogen phosphorylase; V5, gluconeogenesis; V6, glycolysis. V1 and V2 represent glucose-glucose-6-phosphate (G-6-P) cycle, and V3 and V4 represent glycogen cycle. (Reproduced from Magnusson *et al.*, 1994).

1.1.9 Diurnal variations in the Content of Glycogen and the Levels of Blood Metabolites and Hormones in Rats

Glycogen content exhibits diurnal variation in both liver and skeletal muscle (Cohn and Joseph, 1971; Conlee *et al.*, 1976; Bouillon and Berdanier, 1981). The peak in food consumption is followed some hours later by a peak in the liver glycogen concentration (Conlee *et al.*, 1976; Geddes, 1985). The hepatic glycogen levels in the study by Conlee *et al.* (1976) reached a peak at 1200 h of 53.7 ± 7.8 mg/g tissue wet weight, and at 2000 h the levels decreased to a nadir of 29.3 ± 3.5 mg/g tissue wet weight at the end of the light period. Kaminsky and Kosenko (1987) compared a number of investigations into the diurnal variation of rat hepatic glycogen contents from different authors and concluded that the absolute amounts of glycogen and the amplitude of the diurnal rhythm showed significant variation. Geddes (1985) has reported some variation in the molecular size distribution of hepatic glycogen during the 24h period, with a decrease in low molecular weight glycogen (< 2000S) and a subsequent increase in high molecular weight glycogen (> 2000S) from 1200 to 2100 h.

The activities of key hepatic enzymes which regulate glycogen also exhibit diurnal variations. Glycogen synthetase activity in rats is maximum at the beginning of the dark period and declines to a minimum during the light period (Kaminsky and Kosenko, 1987). Glycogen phosphorylase also demonstrates diurnal variation, primarily depending on the pattern of feeding with activity reaching a maximum during the dark period and a minimum during the light period (Kaminsky and Kosenko, 1987). Significant diurnal rhythms of phosphorylase kinase and phosphatase have also been observed and appear to play a role in regulating the diurnal rhythm of phosphorylase a activity (Kaminsky and Kosenko, 1987).

Blood glucose levels are relatively stable throughout the diurnal period, with a small rise at 1600 h (Conlee *et al.*, 1976). The levels of lactate in the blood are stable throughout the 24 hours. Plasma free fatty acid levels exhibit diurnal variation. The concentration is lowest in the early morning (0400 h) at 0.11 mM and then rises during the light period to reach 0.43 mM at 1600 h (Conlee *et al.*, 1976). Furthermore, plasma insulin and corticosterone levels have been shown to vary in a circadian fashion (Conlee *et al.*, 1976). Diurnal variations are the result of interactions arising from the feeding habits, light-dark cycle, and variations in hormone levels.

1.1.10 *Postmortem* Glycogenolysis

It has long been known that after death there is very rapid loss of glycogen from liver (Sharp, 1935a, Morrione and Mamelock, 1952; Geddes and Rapson, 1973) and skeletal muscle (Sharp, 1935a, Lawrie *et al.*, 1959; Newbold and Lee, 1965; Newbold and Scopes, 1967; Bendall, 1973; Fischer and Hamm, 1980; Calder and Geddes, 1990a). Insufficient generation of ATP, due largely to the shortage of oxygen is the major cause of the rapid glycogenolysis (Levine, 1965; Burton and Ishida, 1965; Glinsmann *et al.*, 1969; Woods and Krebs, 1971; Bendall and Taylor, 1972). It has now been reported that glycogen degradation also takes place via a hydrolytic pathway catalysed by the lysosomal acid α-glucosidase (Devos and Hers, 1980; Bollen *et al.*, 1983; Lutaya *et al.*, 1983; Conaglen *et al.*, 1984; Calder and Geddes, 1990a).

Geddes and Rapson (1973) reported that, in the liver, glycogen content decreased 20% within the first 4 minutes after death; 40% of the glycogen is lost in the first 60 minutes and more than 60% of the glycogen is lost after 120 minutes. Liver glycogen loss *post mortem* has been shown to be a metabolically inhomogeneous process (Geddes and Rapson, 1973). In the first 15 minutes after death 39% of the high molecular weight (> 500 x 10^6) and 27% of the low molecular weight (< 500 x 10^6) glycogen was lost. At 60 and 120 minutes these figures were 69% and 37%, and 78% and 61% respectively (Geddes and Rapson, 1973). Similar studies have been performed upon mammalian skeletal muscle glycogen (Calder and Geddes, 1990a) and it was concluded that *post mortem* glycogenolysis is a combination of phosphorolysis and hydrolysis. Tissue sampling for measurement of glycogen content must be carried out using methods that avoid significant changes due to post mortem glycogenolysis, or to pre-death glycogen loss due to lack of oxygenation of tissues.

1.2 ETHANOL METABOLISM

1.2.1 Introduction

Beer, wines, and spirits and other alcoholic drinks contain the aliphatic alcohol, ethanol, (ethyl alcohol ; referred to as alcohol in this thesis). Ethanol abuse can have serious metabolic consequence for the liver (Crow and Greenway, 1989; Fraser, 1989), nervous system (Charness *et al.*, 1989; Saunders and Whitfield, 1994), the gastrointestinal tract (Seitz *et al.*, 1994), the cardiovascular system (Segel *et al.*, 1984; Kaysen and Noth, 1984), haematopoiesis (Larkin and Watson-Williams, 1984), muscle (Haller and Knochel, 1984: Palmer *et al.*, 1991) and the endocrine system (Noth and Walter, 1984).

Many recent investigations have shown that genetic variations in alcohol and acetaldehyde metabolism may be responsible for individual and racial differences in acute reactions to ethanol, alcohol drinking habits and vulnerability to alcohol-related organ damage (Agarwal and Goedde, 1990; Saunders and Whitfield, 1994).

1.2.2 Ethanol Metabolism

Ethanol is eliminated from the body mainly by oxidation. Non-oxidative elimination, i.e. through lungs and kidneys, is considered to account for less than 10% of total elimination (Israel *et al.*, 1970; Plapp *et al.*, 1984; Lieber, 1985). In most animals, including man, ethanol is primarily metabolized in the liver. Approximately 75% of total ethanol metabolism in man and 90% or more in other species occurs in the liver. The liver parenchymal cell (hepatocyte) contains three pathways for the oxidative metabolism of alcohol.

The major pathway which accounts for 80-90% of ethanol oxidation (Li, 1977; Crow 1985; Crabb *et al.*, 1987; Crow and Hardman, 1989), involves the enzymes alcohol dehydrogenase (ADH; EC 1.1.1.1) and aldehyde dehydrogenase (AlDH; EC 1.2.1.3.). These two enzymes oxidize alcohol, via the intermediate acetaldehyde, to acetate with the concomitant reduction of the cofactor NAD^+ (nicotinamide adenine dinucleotide) to its reduced form, NADH (Fig. 1.7). Acetate is readily metabolized in the body, the initial reaction in the pathway being its conversion to acetyl CoA. ADH is present in the cell cytosol, whereas AlDH activity is found both in the cytosol and in the mitochondria. The

reduction of NAD^+ to NADH associated with alcohol metabolism via this pathway generates reducing equivalents as NADH in the cytosolic and mitochondrial compartments. This redox change is one important route by which alcohol perturbs liver metabolism.

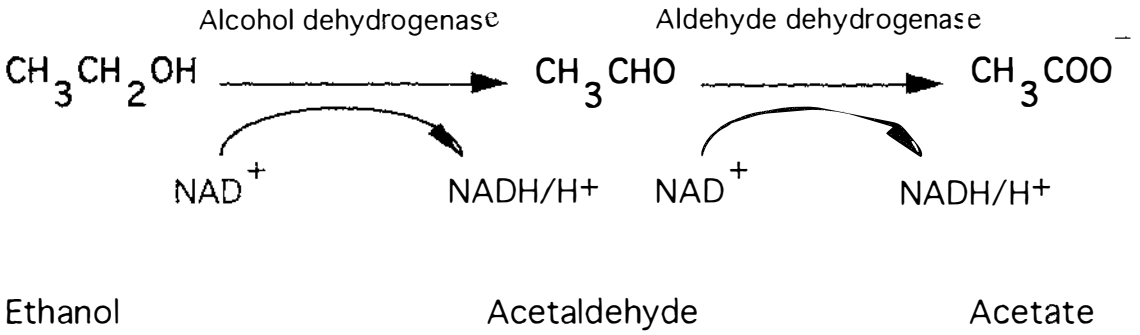

Figure 1.7 Conversion of Ethanol to Acetaldehyde and Acetate by ADH and ALDH

The second pathway of hepatic alcohol oxidation is located on internal membranes (endoplasmic reticulum), the microsomal ethanol oxidizing system (MEOS), (Fig. 1.8). This system is dependent on cytochrome P_{450} and involves the cofactor NADPH and molecular oxygen. This pathway also generates acetaldehyde, the further oxidative metabolism of which involves AlDH. The MEOS activity is increased by chronic exposure to alcohol and this induction is associated with proliferation of the endoplasmic reticulum. Heavy drinkers may have an accelerated capacity to metabolize alcohol. MEOS has a higher K_M than ADH and is therefore considered to play an increasing role in ethanol oxidation at higher ethanol levels (Grunnet *et al.*, 1973; Lieber, 1988). However, this view of the role of MEOS in ethanol metabolism *in vivo* is not shared by all investigators (Crow, 1985; Crow and Hardman, 1989).

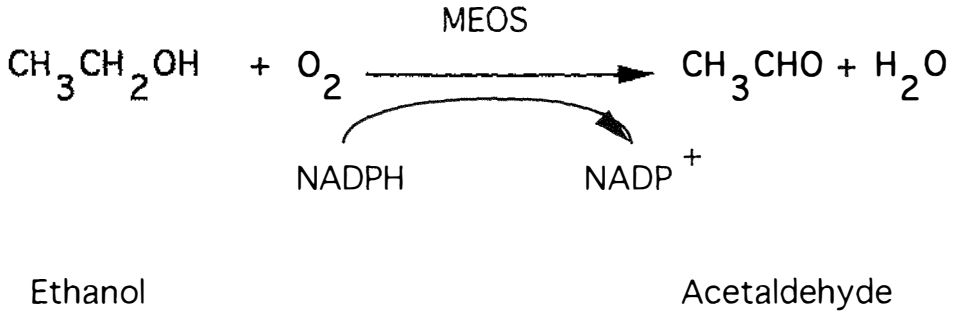

Figure 1.8 Converson of Ethanol to Acetaldehyde via MEOS

The third minor pathway of hepatic alcohol oxidation is associated with specialized cell organelles termed peroxisomes and involves the enzyme catalase in the presence of an independent oxidizing system which generates hydrogen peroxide (H_2O_2) (Fig 1.9). Catalase has been shown to convert ethanol to acetaldehyde *in vitro* but it probably does not play a significant role in ethanol oxidation *in vivo* (Crow, 1985; Lieber,1985; Crabb *et al.*, 1987).

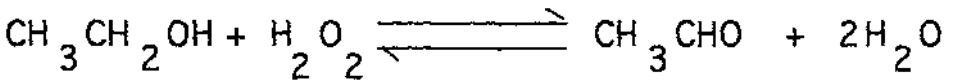

Figure 1.9 Conversion of Ethanol to Acetaldehyde via Catalase

1.2.3 Rates of Ethanol Elimination

The rate at which ethanol is eliminated from the body may be determined by measuring the decrease in its concentration in blood or breath. There is a variation between individuals, and factors such as nutrient status, previous drinking and smoking habits can affect the observed rate (Kopun and Popping, 1977; Holford, 1987). Above blood ethanol concentrations of 10-15 mM (45-70mg / 100 ml) the rate of ethanol elimination is independent of its concentration in the blood, and is therefore a zero-order process (Wagner *et al.*, 1976; Holford,1987). At very high blood ethanol concentrations (200 mg/100ml or more), the rate of elimination can approximate to a first - order process (Hammond *et al.*, 1973; O'Neill *et al.*, 1984). It is very much faster than would be expected from the rates observed at lower concentrations. At a blood concentration of

200 - 400 mg/100 ml the rate of elimination was about 50% higher than the apparently zero-order rates observed at lower concentrations (Kielding *et al.*, 1983). These observations suggest that in some individuals additional pathways of ethanol elimination operate at very high concentrations (Holford, 1987).

1.2.4 **Regulation of Ethanol Metabolism**

Factors that control the rate of alcohol metabolism in mammals have been the subject of debate (for detailed reviews, see Higgins, 1979; Rongstad and Grunnet, 1979; Khanna and Israel, 1980; Dawson, 1983; Crow, 1985; Crow and Hardman, 1989; Hardman *et al.*, 1991). There have been two contradicting views as to how the rate of ethanol metabolism is regulated. One hypothesis is that the rate of ethanol oxidation is determined by the rate at which NADH is reoxidised to NAD^+ (Hawkins and Kalant, 1972; Khanna and Israel, 1980; Thurman, *et al.*, 1989). This view is based on data showing increased rates of ethanol clearance in the presence of substrates that increased rates of NADH reoxidation (Meijer *et al.*, 1975; Krebs and Stubbs, 1975). It was concluded that the decrease in ratio of free $[NAD^+]$ / [NADH] in liver cytosol during ethanol metabolism indicates that either ADH activity was becoming limited by the supply of NAD^+ (Khanna and Israel, 1980) or ADH was being inhibited by NADH (Thurman *et al.*, 1989).

The second hypothesis is that the amount of alcohol dehydrogenase is the important rate determining factor in the regulation of ethanol metabolism (Crow *et al.*, 1977a,b; Cornell *et al.*, 1979; Braggins and Crow, 1981; Cornell, 1983; Bosron *et al.*, 1983). This hypothesis is based on the following findings:

(1) Calculations of the total ADH activity have indicated that the enzyme in rat liver is working at 50 - 80% of maximum velocity in ethanol elimination *in vivo* (Crow *et al.*, 1977 a,b) which opposes the idea the alcohol dehydrogenase is present in excess, as believed in the first theory (Hawkins and Kalant, 1972).

(2) The liver cell does not become depleted of NAD^+ during ethanol metabolism (Crow *et al.*, 1983a); therefore, the activity of ADH is not restricted by the availability of NAD^+.

(3) The concentrations of NADH occurring during ethanol oxidation *in vivo* inhibit rat liver ADH by approximately 10 - 20% (Cornell *et al.*, 1979; Cornell, 1983; Bosron *et al.*, 1983), indicating that the rate of ethanol metabolism could only be increased by a maximum of 20% if the rate of NADH reoxidation was increased (Crow and Hardman, 1989; Page *et al.*, 1991).

(4) The cytosolic redox change may be explained by the kinetic properties of cytosolic malate dehydrogenase (MDH) (Crow *et al.*, 1982;1983b). MDH is not saturated with NADH at physiological concentrations and therefore the rate of oxidation of NADH by

MDH rises with increasing [NADH] until it equals the rate of production of NADH and a new steady state is achieved.

1.2.5 **Ethanol Oxidation and Exercise**

Ingested ethanol has an energy content (7.377 kcal/g), and this has received some attention in relation to exercise in the early nineties (Lide, 1991). Earlier studies had focused on the effects of ethanol on performance (Hebbelink, 1959; Nelson, 1959), and on respiratory (Bobo, 1970; Bond *et al.*, 1983), cardiovascular (Blomqvist *et al.*, 1970; Kelbaek *et al.*, 1985), metabolic (Blomqvist *et al.*, 1970; Bobo, 1970), and endocrine responses (Hadley *et al.*, 1988; Schürch *et al.*, 1982; Juhlin-Dannfelt *et al.*, 1977). More recently experiments have been carried out on oxidation of ethanol and its contribution to energy yield at rest or during exercise (Massicotte *et al.*, 1993). These researchers measured the oxidation of ethanol at rest and during prolonged moderate exercise with use of ^{13}C labelling. Five healthy young males performed three exercises on a cycle ergometer with ingestion of 0.4 (trial A) and 0.8 (trial B) g/kg body wt. of ^{13}C ethanol or water only (trial C). The subjects were also studied during a 90 min rest period after the ingestion of 0.8 g/kg body wt. of ^{13}C ethanol (trial D). At rest, over the 90 min observation period, only 2.1g of the 64.6 g of ethanol ingested were oxidized, providing 11% of the total energy expenditure. Over the 90 min of exercise, the amounts of ethanol oxidized were similar in trial A and B. The contribution of ethanol represented about 5 % of the total energy expenditure, which is much lower than that previously reported for exogenous carbohydrates (8-18%). They concluded that the small contribution of ethanol to energy metabolism did not significantly modify endogeneous substrate oxidation.

In terms of the effect of exercise on rates of ethanol metabolism, Canzanelli *et al.*, (1934) and Pawan (1968) reported no effect of exercise on the metabolism of ethanol. Other studies showed that exercise increased ethanol clearance in rats (Ardies *et al.*, 1989) and in humans (Schürch *et al.*, 1982). For example, after ingestion of 0.66g/kg body weight of ethanol, Schürch *et al.*, (1982) reported that the blood ethanol concentration was 43% lower at the end of a 90 min exercise period than a 90 minute rest period. The authors concluded that the faster ethanol clearance during exercise was due mainly to the loss of ethanol in sweat and expired gas, because skeletal muscles are not able to utilise ethanol directly (Schürch *et al.*, 1982). It appears that during exercise the amount of ethanol metabolized in the liver is not influenced by the amount ingested but could be limited by the capacity of the liver to metabolize ethanol (Kashiwaji *et al.*, 1982; Dawson, 1983). The end product of ethanol oxidation in the liver, through the alcohol dehydrogenase pathway (Fig 1.7) or through the less important microsomal ethanol-oxidizing system

(Fig 1.8), is acetate. This intermediate can enter the TCA cycle in the hepatocyte or, alternatively, can be released into the circulation to be oxidized in peripheral tissues, including working muscles during exercise. In fact, Juhlin-Dannfelt *et al.*, (1977) observed that the uptake of acetate and lactate across an exercising leg was actually increased when exercise was performed with administration of 50 g of ethanol (0.7g/kg body wt). At rest ethanol is known to reduce gluconeogenesis from lactate and glycerol (Krebs, 1968) and could be responsible for the development of hypoglycemia in the fasting condition (Arky and Freinkel, 1966). However, the plasma glucose level has been shown to remain normal during prolonged exercise after ethanol administration (Juhlin-Dannfelt *et al.*, 1977). This is probably because, although liver glucose output is reduced, uptake of circulating glucose by working skeletal muscles is also reduced, as demonstrated by Juhlin-Dannfelt *et al.*, (1977).

1.3 ETHANOL AND CARBOHYDRATE METABOLISM

1.3.1 Effects on Carbohydrate Digestion and Absorption

The effect of ethanol on carbohydrate metabolism was comprehensively reviewed by Tejwani and Duruibe (1985). These authors described some of the effects of ethanol on carbohydrate digestion and absorption. Generally, ethanol may have two effects on digestion and absorption. Firstly, chronic ethanol decreases pancreatic amylase activity (Chariot *et al.*, 1981). Secondly, ethanol severely (up to 80%) decreases active transport of glucose in the small intestine of fasted animals (De Castellarnau and Bolufer, 1979), and this inhibition of active transport is thought to be due to alteration of intestinal membrane permeability by ethanol.

1.3.2 The effect of ethanol on blood glucose concentrations and liver glycogen content in the fed state.

This subject has previously been reviewed (Marks, 1978; Sneyd, 1989; Patel, 1989). The effect of ethanol on blood glucose concentrations varies with the nutritional state. In the fed state, when liver glycogen stores are high, the consumption of ethanol either produces hyperglycemia (see section 3.11 and 3.12) or fails to alter the blood glucose concentration. When glycogen stores are low, ethanol produces hypoglycemia (see section 1.3.3).

Generally, glycogenolysis and gluconeogenesis are responsible for the maintenance of blood glucose concentrations. In fact, glycogenolysis is an important pathway for hepatic glucose production in the post-absorptive state. In the fed state, liver contains about 50-75 g of glycogen. This glycogen can serve as a short term fuel store. Glycogenolysis can maintain a normal blood glucose for several hours.

Some early results, as reviewed by Tejwani and Duruibe (1985), have suggested that ethanol can reduce liver glycogen considerably (by more than half) in well fed animals (Senior and Wolfsdorf, 1979; Potter and Morris, 1980). This suggests that ethanol-induced hyperglycemia may be explained, at least in part, by increased glycogenolysis. However, in experiments using perfused liver, it has been observed that an ethanol-induced increase in glucose output is not necessarily accompanied by a decrease in the liver glycogen content (Topping *et al.*, 1979). Therefore the extent of the possible contribution of glycogen breakdown to ethanol-induced hyperglycemia remains unresolved.

1.3.3 **Effect of Ethanol on Blood Glucose Concentration in the Starved State.**

This topic has been reviewed previously by (Marks, 1978; Patel, 1989). There are four clinically distinct types of alcohol-induced hypoglycaemia, namely:

(1) Alcohol-induced fasting hypoglycaemia.
(2) The reactive hypoglycaemia of chronic alcoholism.
(3) The alcohol potentiation of drug (or exercise) - induced hypoglycaemia.
(4) Alcohol-promoted reactive hypoglycaemia.

1.3.3.1 **Alcohol-induced fasting hypoglycaemia**

Alcohol-induced fasting hypoglycaemia is a dangerous disorder carrying a 10 per cent mortality rate in adults and a 25 per cent mortality rate in children (Madison, 1968). This is the best known and most thoroughly investigated, but possibly the least common variety of alcohol-induced hypoglycaemia. It usually develops in chronically malnourished or more acutely food deprived individuals within 6 to 36 hours after ingesting a moderate-to-large amount of alcohol. However, this condition may occur in weekend binge drinkers and even in occasional drinkers who have missed a meal or two (Marks, 1978; Patel, 1989).

Alcohol-induced fasting hypoglycaemia can be demonstrated experimentally in normal healthy volunteers by fasting them for 36 to 72 hours before administering alcohol either orally or intravenously. Normal people regularly consuming high protein low carbohydrate diets and children on inadequate or even adequate diets, are sensitive to the

hypoglycaemic effects of alcohol (McLaughlan *et al.,* 1973). Under normal fasting conditions, liver glycogen stores are considerably depleted, and regulation of blood glucose levels is dependent upon gluconeogenesis. Since alcohol inhibits hepatic gluconeogenesis, alcohol induced hypoglycemia occurs only after glycogen reserves in the liver are largely depleted (Marks, 1978). It is possible that a combination of exercise, alcohol and a low carbohydrate diet or low total food intake would produce a dangerous state of hypoglycemia.

1.3.3.2 **Alcohol potentiation of drug-induced hypoglycaemia**

The development of an extremely severe form of hypoglycaemia following the ingestion of alcohol by insulin-treated diabetics was first described by Arky *et al.,* (1968). Experimentally, modest amounts of alcohol delay recovery from hypoglycaemia produced by intravenously administered insulin (0.1 unit / kg body weight) in normal healthy subjects and reduce the growth hormone response (Arky, *et al.*, 1968; Priem *et al.*, 1976). The hypoglycaemia resulting from the combined effects of alcohol and sulphonylureas is seemingly less severe than with insulin (Seltzer, 1972).

1.3.3.3 **Essential reactive hypoglycaemia in alcoholics**

It has been shown that chronic alcoholics are more likely than normal healthy subjects to develop symptomatic reactive hypoglycaemia following ingestion of an oral glucose load (Tintera and Lovell, 1949; Tintera, 1966; Farmer *et al.*, 1971; Cohen, 1976). The mechanism for this reactive hypoglycaemia is poorly understood.

1.3.3.4 **Alcohol-induced reactive hypoglycaemia**

Reactive hypoglycemia is a condition of low blood sugar that occurs after eating. In the presence of carbohydrates, alcohol stimulates the insulin response, resulting the development of frank reactive hypoglycemia. O'Keefe and Marks (1977) have clearly demonstrated that in human subjects a combination of sucrose and alcohol induces a more severe hypoglycemic response than does sugar alone.

Reactive hypoglycemia can be demonstrated in healthy individuals who have consumed a mix of alcohol and sugar, such as vodka with orange juice or beer with bar nibbles. Patel, (1989) observed that proteins and fats protectagainstalcohol-induced hypoglycemia by blocking intestinal absorption of alcohol. On the other hand, a carbohydrate rich food such as bread does not necessarily protect against alcohol-induced reactive hypoglycemia,

indeed, it may exacerbate the condition. Larue-Achagiotis *et al.* (1990) reported that in rat the ingestion of alcohol together with an oral glucose load could trigger a reactive hypoglycemia. Stress greatly enhanced the phenomenon. Alcohol can induce hypoglycemia, even in previously well fed individuals, during periods of increased peripheral glucose utilization, such as exercise.

1.3.4 **Ethanol and Glucose Tolerance**

The relationship between ethanol and glucose tolerance is not clear. There are conflicting reports on this topic. According to some authors, improved glucose tolerance is seen (Metz *et al.*, 1969; Nikkilä and Taskinen, 1975; McMonagle and Felig, 1975). Others do not observe this (Walsh and O'Sullivan, 1974; Marks, 1978), and still others see an impairment of glucose tolerance with ethanol consumption (Dornhorst and Quyang, 1971; Phillips and Safrit, 1971). One of the reasons for contradictory findings concerns the methodology used to determine the effect of ethanol on glucose tolerance (Nikkilä and Taskinen, 1975; reviewed by Marks, 1978). Those studies reporting an improved glucose tolerance generally administered ethanol to healthy individuals prior to administration of glucose. In some studies, insulin and/or glucose were administered directly into the bloodstream and the impact of ethanol on glucose levels was then evaluated. It appears that the timing of measurement of the ethanol effect is crucial. This methodology represents an artificial situation and thus may not accurately reflect the true physiological response (Metz *et al.*, 1969; Nikkilä and Taskinen, 1975; Huttunen and Kortelainen, 1990).

It has been reported that ethanol induces insulin resistance (Yki-Järvinen and Nikkilä, 1985; Yki-Järvinen *et al.*, 1988). In humans, 70-80% of the ethanol oxidised appears as free acetate in the hepatic vein. Oxidation of acetate in extrahepatic tissues could suppress uptake of other substrates including glucose, and this might slow the glucose disappearance rate. Glucose intolerance could also be explained by diminished insulin responses: catecholamines released in response to alcohol could suppress glucose-induced insulin secretion (Klingman and Hagg, 1958). Moreover, it has been suggested that ethanol impairs the integrity of the microtubular system of β--cells (Malaisse *et al.*, 1971). Subsequently, the hyperinsulinemia provoked by alcohol has been attributed to a priming effect of alcohol on the β-cells (Friedenberg *et al.*, 1971; Nikkila and Taskinen, 1975).

1.3.5 **Endocrine Effects of Ethanol**

1.3.5.1 **Ethanol and Insulin**

The relationship between glucose, ethanol and insulin seems to be complex. Excess blood glucose is normally stored as glycogen while there is no storage form for ethanol. Insulin is produced by the β-cells in the pancreatic islets of Langerhans in response to increased blood glucose. Insulin stimulates glucose uptake (Itaya, 1979) and glycogenesis in the liver. It appears that when both glucose and ethanol are present, ethanol may to be used in preference to glucose.

Numerous studies have shown that ethanol affects glucose metabolism by increasing the glucose effect on insulin secretion (Field *et al.*, 1963; Freinkel *et al.*, 1965; Metz, 1969; McMonagle and Felig, 1975; Nikkilä and Taskinen, 1975; Marks, 1978; Yki-Järvinen and Nikkila, 1985; Shelmet *et al.*, 1988; Larue-Achagiotis *et al.,* 1990; Adner and Nygren, 1992; Kendrick *et al.*, 1993; Boyd and Moss, 1993). Metz *et al.*, (1969) reported that ethanol in the presence of glucose resulted in the release of a higher insulin concentration than would had occured with glucose alone. In the absence of glucose, ethanol shows no effect on insulin levels. Therefore, ethanol augments a glucose signal in the β-cells but does not generate a signal of its own (Metz *et al.*, 1969).

Ethanol plays a vital role in the clearance of glucose from the bloodstream. The increase in insulin causes greater clearance of glucose from the bloodstream than would occur in the absence of ethanol. This leads to apparent hypoglycemia or lower than normal blood glucose levels (McMonagle and Felig, 1975; Nikkilä and Taskinen, 1975; Yki-Järvinen and Nikkilä, 1985; Kallner and Blomquist, 1991).

In contrast, the presence of excessive ethanol in the diet has been considered to lead to insulin resistance, which has been equated with a role of ethanol in initiation of NIDDM (Non-insulin dependent diabetes mellitus). (Yki-Järvinen and Nikkilä, 1985; Balkau *et al.*, 1992). The link between alcohol and type II diabetes is not fully understood. However, recent studies suggest that moderate ethanol consumption reduced the risk of NIDDM (Perry *et al.*, 1995; Rimm *et al.*, 1995). Unlike true NIDDM or type 2 diabetes, ethanol-induced diabetes appears to be fully reversible (Phillips and Safrit, 1971; Balkau *et al.*, 1992). In the presence of ethanol, more dietary glucose can be stored as glycogen as less is needed for energy. Ethanol increases the glucose signal for insulin secretion resulting in a stronger insulin response and greater storage of glucose as glycogen.

Some studies have reported that high alcohol consumption leads to hyperglycemia and not hypoglycemia. Ethanol appears to enhance absorption of dietary carbohydrate and delays glucose uptake into tissue leaving plasma glucose levels high (Dornhorst and Quyang, 1971; Nikkilä and Taskinen, 1975; Yki-Järvinen and Nikkila, 1985).

In contrast to the above findings which largely show that ethanol increases glucose effects on insulin secretion, Tejwani and Duruibe (1985) have summarised a number of studies in which ethanol appeared to decrease plasma insulin. They indicated that Potter and Morris (1980) observed that the administration of ethanol (4.5 g/kg) decreased plasma insulin. The plasma insulin decreasing effect of ethanol was dose dependent. Potter and Morris also found that plasma glucose was elevated only in fed rats.

Some researchers (Nikkilä and Taskinen, 1975; McMonagle and Felig, 1975) observed improved glucose tolerance in fed animals. They administered lower doses than 4.5 g/kg and found that the insulin response was increased in fed animals and decreased in fasted animals. Tejwani and Duruibe (1985) also reported that Singh and Patel (1976) had observed a lack of effect of ethanol on insulin on blood glucose, but this was at low ethanol doses (0.4 g/kg). However, later studies (Patel and Singh, 1979) showed that insulin release from pancreatic islets in response to glucose was inhibited by ethanol administered by different routes. The effects were enhanced by pyrazole and did not require ethanol metabolites (Potter *et al.*, 1979) so were presumably direct effects of ethanol.

1.3.5.2 **Effects of Ethanol on Glucacon and Catecholamines**

Glucagon is produced by the α-cells in the pancreatic islets of Langerhans in response to decreased blood glucose levels (Palmer and Ensinck, 1975). Glucagon plays a vital role controlling the blood glucose by enhancing both glycogenolysis and gluconeogenesis. Tejwani and Duruibe reported that Potter and Morris (1980) had observed that ethanol induced hyperglycemia was related to elevation of plasma glucagon levels and to suppression of plasma insulin levels. They also reported that Forssel (1981) had observed an increase in urinary excretion of catecholamines with ethanol in animals but that Leppaluoto *et al.* (1975) found no such change in humans.

1.3.6 **Aims of this thesis**

This thesis attempts to investigate the effects of alcohol on glycogen structure and its metabolism in fed, starved and starved-fed animals, taking into account factors such as:
(a) post mortem degradation
(b) careful isolation
(c) the separate structures and metabolism of low (cytosolic) and high (lysosomal) molecular weight glycogen.

Glycogen is heterogeneous with respect to molecular size and is found to be compartmentalized within both the cytosol and the lysosome (see reviews by Geddes, 1985, 1986 and Calder, 1991). In this thesis, the effects of alcohol (ethanol) on the molecular size and content of tissue glycogens were determined in fed and starved rats and during the fed to starved and starved to fed transition. Molecular weight profiles of liver and hindlimb skeletal muscle glycogen were determined using the method of sucrose density gradient ultracentrifugation.

The effect of ethanol on the molecular weight distribution of glycogen has not been investigated before. Chapter 3 was designed to investigate the effect of ethanol on the glycogen content and glycogen molecular weight distribution of liver from fed rats.

Until recently, little has been known about the effect of ethanol on glycogen synthesis in skeletal muscle and liver, following refeeding after starvation in the rat. Although a few studies have been performed recently in which ethanol was demonstrated to inhibit hepatic glycogen resynthesis (Cook *et al.*, 1988; Palmer *et al.*, 1991), and muscle glycogen resynthesis (Xu *et al.*, 1992) with glucose refeeding following starvation, there have been no chow refeeding studies following prolonged starvation. In addition, there are no data available about the effects of ethanol on tissue content of glycogen in the starved state. Chapter 4 addresses the question of whether ethanol inhibits tissue glycogen deposition in response to chow refeeding after starvation, taking into account the separate structures and metabolism of low (cytosolic) and high (lysosomal) molecular weight glycogens. The effects of ethanol on tissue glycogen content in starved rats is also examined.

All the above mentioned studies were performed with a single dose of ethanol. Although there have been some studies which investigate the effect of a chronic ethanol liquid diet on tissue glycogen content and metabolism, there have been no previous studies on effects of repeated doses of ethanol on different tissue (such as liver, muscle, heart and kidney) glycogen content. The objectives of the work described in Chapter 5 were to investigate the effects of repeated doses of ethanol on tissue glycogen content.

CHAPTER 2

GENERAL METHODS

2.1 Materials and Equipment

2.1.1 Sources of Materials

Table 2.1 Sources of Materials

The following sources of chemicals and other materials were used

Source	Item
Ajax Chemicals, Sydney, Australia	Urea, alcium chloride, TCA
Beckman, Palo Alto, Ca, U.S.A.	Polyallomer centrifuge tubes
Chelsea Sugar Co., Auckland, NZ	Sucrose
Hopkin and Williams Ltd, Essex, England	Iodine
May and Baker Ltd, Dagenham, England	Mercuric calcium powder, Phenol detached crystals, Silver sulphate, Sodium citrate
New Zealand Industrial Gases Ltd., New Zealand	Liquid Nitrogen
Rhone - Poulenc, Chemicals Ltd., Bristol, U.K.	Sulphuric Acid, Acetic Acid, Methanol, Citric acid
Scientific Instruments Co., London, U.K.	Visking Dialysis Tubing
Scientific Supplies Ltd., Auckland, New Zealand	Potassium Iodide
Sorvall Instuments, DuPont Company, Wilmington, U.S.A.	Centrifuge tubes

2.1.2 Water

All water used was purified to a conductivity of less than 10 mΩ cm by a Millipore RO 4 filtration system (Millipore Corporation, Bedford, Massachusetts, U.S.A.).

2.1.3 Centrifuges

Unless otherwise indicated all centrifugations were performed using a Sorvall RC-2B or RC 5B Refrigerated Superspeed Centrifuge (Du Pont Instruments, Newport, Connecticut, U.S.A.). All sucrose density gradients were centrifuged (29,300 g, 30 min, 20 ^{0}C) in a Beckman L2-65B Ultracentrifuge (Beckman, Palo Alto, California, U.S.A.) using a SW 27 (6 bucket) rotor.

2.1.4 Absorbance Measurements

All absorbances were measured using a Hitachi U-1100 spectrophotometer.

2.1.5 Freeze Dryer

Tissue glycogen solutions were lyophilised in a Unitrap II Freeze Drier (Virtis, Gardiner, New York, U.S.A.).

2.1.6 Homogeniser

The liver and muscle tissue samples for glycogen analysis were homogenised with an Ultra-Turrax T45 homogeniser (Janke and Kunkel, Staufen, Germany).

2.2 Animals

2.2.1 Feed and Maintenance

All animals were New Zealand bred male Sprague-Dawley rats weighing 230-260 g, maintained at 25 ^{0}C on a 14 hour light 10 hour dark cycle. The animals were supplied with water *ad libitum* and fed on standard pellets (moisture 10-13%, protein 18%, fat 5.5%, carbohydrate 55-60%, fibre 3.5%). In some studies, starved rats were used (Chapter 4).

2.2.2 Ethanol Treatment

Rats were administered a single dose of ethanol in 4 ml of aqueous solution. The specific doses used are indicated in relevant sections. Control animals received water. Ethanol administration was carried out using intragastic intubation, as this is less stressful for the animals than intraperitoneal injection. As far as possible, all experiments were started at the same time of day (9.00 am ± 1 h) to minimise any diurnal changes in tissue glycogen content and metabolism.

2.2.3 Tissue Sampling

Rats were killed by cervical dislocation and the liver and other tissues were removed. This was carried out within a few seconds (in order to minimise *post mortem* degradation of glycogen) without the use of anaesthetic. The use of anaesthetic also can affect liver glycogen content, so this was avoided. The liver was removed within 10-15 seconds, freeze-clamped between aluminium blocks approximately 10 cm diameter x 2 cm thickness that had been precooled in liquid nitrogen and then placed in liquid nitrogen. Hindlimb muscle was removed next, within 30-40 seconds, and where necessary kidney and heart. All tissues were clamped and placed in liquid nitrogen. For liver and muscle, where glycogen was to be purified, small samples were retained for quantitative glycogen analysis (section 2.3). These samples were ground to a fine powder in a chilled morter and pestle, then stored at -70 ^{0}C. The remainder of the sample was then used for glycogen purification as described in sections 2.4.2.1 (liver) and 2.4.2.2 (muscle). Heart and kidney tissue samples were powdered and stored for quantitative analysis.

2.3 Quantitative Analysis of Tissue Glycogen

2.3.1 Extraction of Tissue Glycogen Using Methanol

The micro-method of Kemp and van Heijningen (1954) was used to determine glycogen content in liver, muscle, heart and kidney tissue. A small tissue sample weighing between 25 and 150 mg was placed in a chilled, hand-held glass homogeniser. The tissue was ground with 5.0 ml of 80% methanol at 4 ^{0}C. A further 2.0 ml of 80% methanol was used to wash this solution into a centrifuge tube. The suspension was centrifuged (910 x g, 4 ^{0}C, 5 min, SM 24 rotor). Whilst glucose is soluble in 80% methanol and remains in the supernatant, glycogen is insoluble and is pelleted. The pellet was resuspended in 15 ml of deproteinsing solution, which consisted of trichloroacetic

acid (5 g, AR) and Ag_2SO_4 (100 mg, AR) dissolved in water and made up to 100 ml. The solution is stored in an amber bottle in the cold. The resuspended pellet was then heated at 100 ^{0}C for 15min, cooled and centrifuged (960 x g, 10 min, 20 ^{0}C, SS 34 rotor). The pellet was discarded and 1.0 ml of the supernatant (diluted) was used to determine the glycogen content (section 2.3.2).

2.3.2 **Hydrolysis of Glycogen and Determination of Glucose**

The colorimetric method of Hodge and Hofreiter (1962) was routinely used to determine the concentration of glucose formed from hydrolysed glycogen in supernatants prepared as described in section 2.3.1. This is very sensitive method and may be used to determine concentrations of up to 0.1 mg/ml with a deviation of ± 2%.

A 1.0 ml sample containing glucose (up to 0.1 mg/ml), or water as a blank, was mixed with 1.0 ml of 5% (w/v) phenol and 5.0 ml of concentrated sulphuric acid (96%). The solution was incubated for 10 min at room temperature and then remixed, and incubated for 20 min in a 25 ^{0}C water bath. The absorbance at 490 nm was measured using glucose as a standard, with glucose at a concentration of 0.1 mg/ml giving an absorbance of between 0.95 and 1.0 (Fig. 2.1).

Although this method is regularly used to determine glucose or glycogen concentrations, especially for tissue determination of these compounds, it is not glucose-specific. Rather it is hexose-specific and capable of measuring a number of hexose metabolites, including glucose and fructose, with equal efficiency. However, since the carbohydrate portion of glycogen is 99% glucose, this method provides a sensitive analytical measure of glycogen after glycogen extraction from tissue, as described in section 2.3.1.

Two mg of commercial glycogen (SIGMA) was added to 40 mg liver tissue (wet weight). Both commercial glycogen and liver tissue were ground with 80% methanol. The results showed good recoveries (mean value > 95%), for extraction and determination of glycogen as described in section 2.3.2.

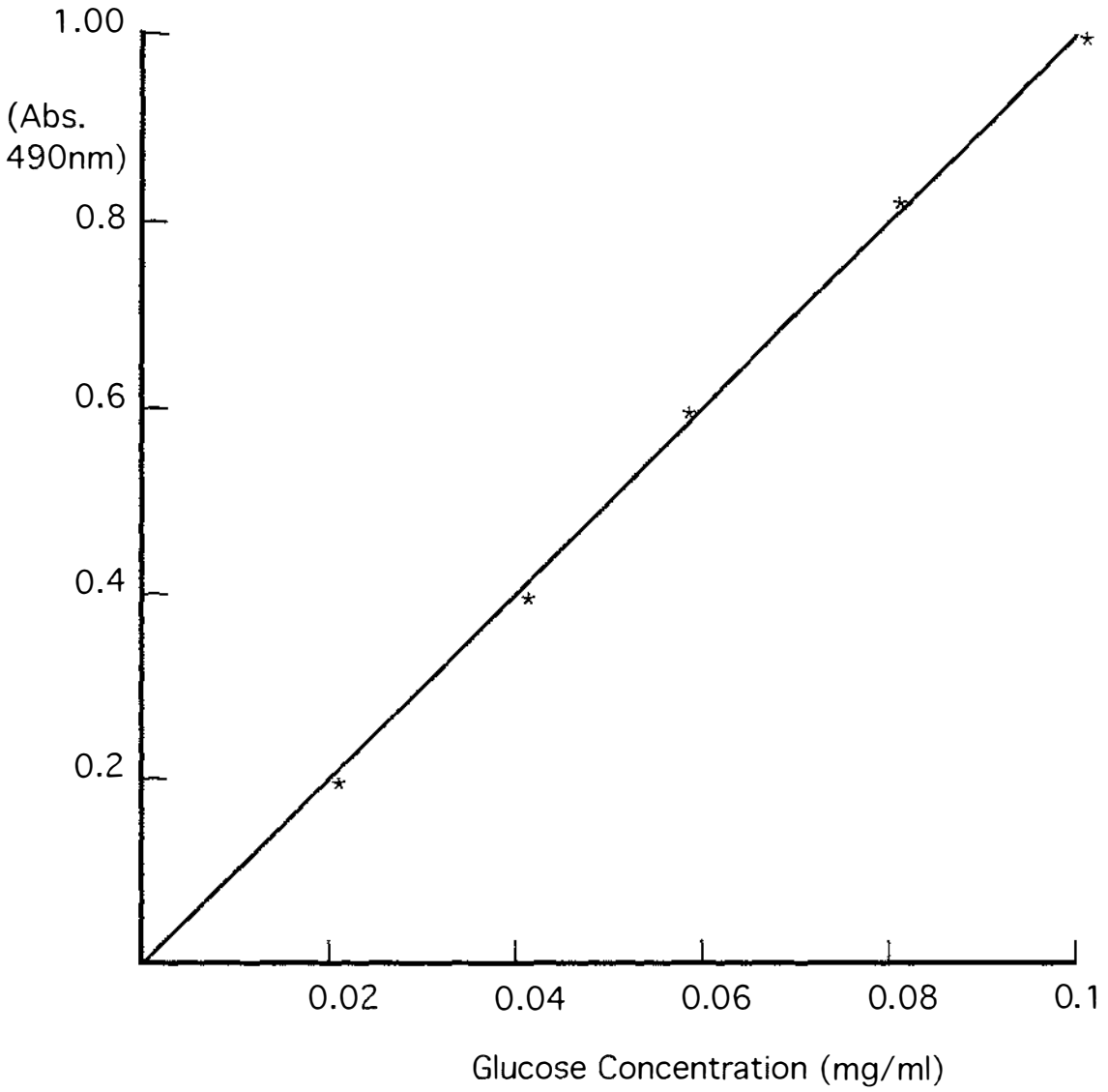

Figure 2.1 Standard Curve for Detection of Glucose by the Phenol Sulphuric Acid Assay

Standard glucose solutions containing up to0.1 mg/ml glucose were assayed using the method of Hodge and Hofreiter (1962), as decribed in section 2.3.2

2.4 Determination of Molecular Weight Distribution of Tissue Glycogen

2.4.1 Background

2.4.1.1 The Structure of Liver Glycogen Extracted Using 45% Phenol

The extraction of liver glycogen using 45% phenol was first performed by Laskov and co-workers (Laskov and Margoliash, 1963; Laskov and Gross, 1965). Later the molecular size of glycogen extracted from bovine liver by several methods was compared (Geddes and Greenwood, 1965; Geddes, 1969) and it was concluded that the phenol extraction technique provides high yields (85-90%), a high level of purity (it was free of other carbohydrates), and is highly reproducible, as well as yielding a product closest to the native structure of glycogen. Also the pH range of 5.9-6.1 avoids extreme pH changes.

Phenol-extracted liver glycogen was comprised of a mixture of single, spherical β-particles as well as aggregates of these of varying size, up to 200 nm in diameter, the α–particles, which represent the highest level of glycogen organisation. For glycogen prepared in this way, it has been reported that no changes in characteristics were observed upon prolonged storage (Orrell and Bueding, 1964; Calder, 1987).

2.4.1.2 The Extraction and Purification of Rat Muscle Glycogen

Mercuric chloride has been used previously to extract skeletal muscle glycogen (Mordoh *et al.*, 1966; see reviews Geddes, 1985; Calder,1991; Geddes and Chow, 1994). This method was developed to extract "native" skeletal muscle glycogen. Calder (1987) reported that approximately 50% (yield) of rat muscle glycogen was routinely isolated in pure form and this had a protein content of approximately 9 mg per 100 mg of glycogen. Mercuric chloride must be used at a concentration no more than 3% and pH 5.0 to be effective in preventing the activity of degradative enzymes (Calder, 1987). Calder (1987) reported over the entire procedure 63% of tissue glycogen was extracted. The greatest loss (35%) was during centrifugation of the tissue homogenate, possibly due to physical entrapment of glycogen by the sedimenting protein and cell debris. Upon dialysis of the final preparation, a water insoluble fraction was evident. This accounted for approximately 20% of the glycogen present (approximately 12 % of tissue glycogen).

Thus the yield of soluble glycogen purified was lowered to 51% of tissue glycogen. The properties of the insoluble portion of glycogen were investigated by Calder (1987). The molecular weight distribution of rat muscle glycogen was determined before and after removal of the insoluble material and its removal did not alter the size distribution (Calder, 1987). This is because the insoluble material travelled through the gradient and was pelleted at the bottom of the tube.

During the purification carbohydrate other than glycogen was effectively removed. This occurred chiefly at the first cycle of the ethanol precipitation and water resuspension cycle. Most of the tissue protein was also removed during the purification. There was progressive loss of protein at later steps and the final glycogen preparation.

Calder (1987) reported that the most likely reason that high molecular weight skeletal muscle glycogen remained undetected is that most other preparations have suffered extensive *post mortem* glycogen degradation or inhomogeneous losses of material during purification. It is shown in section 3.2.3 that both liver and muscle glycogen undergo rapid degradation *post mortem*. It was shown that the high molecular weight material is lost most rapidly (Geddes and Rapson, 1973; Calder and Geddes, 1990a). Within 5 minutes of death more than 50% of skeletal muscle glycogen was lost, and much of this was the high molecular weight component.

2.4.1.3 **Determination of the Molecular Weight of Glycogen**

The molecular weight of glycogen has been analysed by a variety of techniques including measurement of osmotic pressure (Oakley and Young, 1936), light scattering (Harrap and Manners, 1952), turbidity (Kjolberg *et al.*, 1963), sedimentation coefficient (Bridgeman,1942: Bell *et al.*, 1948), size under the electron microscope (Laskov and Gross, 1965), sucrose density gradients (see reviews Geddes, 1985; Calder, 1991) and newly developed citrate gradients (Geddes and Chow, 1994a).

The sedimentation coefficient (S) of a macromolecule is indirectly related to its molecular weight (Tanford, 1961) and can be obtained by fractionating the molecule upon a sucrose density gradient (Geddes *et al.*, 1977a,b) followed by calculation of S for each fraction using the formula of Martin and Ames (1961). In conjunction with an accurate determination of the diffusion coefficient (D), S can then be used to accurately determine the molecular weight of each fraction (Geddes *et al.*, 1977a) by applying the Svedberg equation.

Division into molecular weight ranges has proved useful in the investigation of the effects upon glycogen structure of metabolic perturbations such as fasting (Geddes and Taylor, 1985a; Calder and Geddes, 1990b,c; Geddes and Chow, 1994b), refeeding following fasting (Geddes and Stratton, 1977a; Calder and Geddes, 1990b,c; Geddes and Chow, 1994b), drug administration (Geddes *et al.*, 1983; Ching *et al.*, 1985; Geddes and Taylor, 1985a,b; Calder and Geddes, 1989a), and glycogen storage diseases (Geddes and Taylor, 1985a; Calder and Geddes, 1989b) and in investigating the structure of the glycogen located within lysosomes (Geddes and Stratton, 1977b; Geddes and Taylor, 1985a,b; Calder and Geddes, 1989c; Geddes and Chow, 1994). The molecular weight distribution of glycogens from different tissues has been arbitrarily divided for comparative purposes into low (< 250 x 10^6 Da), medium (250 - 500 x 10^6 Da) and high (> 500 x 10^6 Da) molecular weight material (Geddes, 1985). The proportions of glycogen in each of these size ranges is shown for glycogens from several sources in Table 2.2 (Data from Calder, 1991). The combination of mild extraction procedures and accurate determination of molecular weight distributions has revealed that native glycogen may attain a size in excess of 1000 x 10^6 Da (Geddes *et al.*, 1977a,b). At least 95% of glycogen is larger in size than 10 x 10^6 Da. Since this is the estimated size of a single β-particle, it is apparent that almost all glycogen molecules are composed of more than a single β-particle. Electron microscopic studies of such glycogen preparations revealed that the average glycogen particle, even in the lowest molecular weight fractions, was a dimer or a trimer of β-particles (Geddes *et al.*, 1977a).

Throughout this thesis glycogen molecular weight distributions are arbitrarily divided into low (< 250 x 10^6 daltons) and high (> 250 x 10^6 daltons) molecular weight regions for comparative purposes (see section 2.4.2.5). A similar division has been used previously (Geddes and Rapson, 1973; Calder and Geddes, 1992).

Liver tissue was homogenised in an efficient Ultra Turrax T 45 homogeniser. The mechanical force of the Ultra-Turrax did not alter the molecular weight distribution of the purified glycogen (Calder, 1987). Similarly, there was no difference between the molecular weight distribution of rat liver glycogen before or after lyophilisation indicating that the freeze-drying process did not change glycogen structure (Calder, 1987).

More recently, the separation of low (cytosolic) and high (lysosomal) molecular weight glycogen on small citrate gradients has been compared with the conventional sucrose gradient separation. The citrate method was shown to be vastly more sensitive and it can be used with as little as 0.1 mg of glycogen, compared with 40 mg which is the normal loading on a sucrose gradient. Although the new citrate technique is an easy, rapid

method and more sensitive, it lacks some of the precision of the sucrose density gradient method, and has a higher degree of error, when compared with the conventional method (Geddes and Chow, 1994a). Therefore, for the purposes of the present study, the use of sucrose density gradients was retained.

Glycogen source	Low Mol.Wt.	Medium Mol.Wt	High mol.Wt	Reference
Rabbit Liver	64	18	18	Geddes *et al.*, 1977a.b
Rat Liver	65	15	21	Calder and Geddes, 1985a,b
Mouse Liver	59	14	27	Calder, 1987
Rat Skeletal Muscle	65	16	18	Calder and Geddes, 1985a,b
Mouse Skeletal Muscle	59	13	28	Calder, 1987
Human Placenta	84	5	11	Blows *et al.*, 1988
Rabbit Brain	100	0	0	Chee *et al.*, 1983
Rabbit Liver	100	0	0	Calder 1987

Table 2.2 Molecular Weight Distribution of Glycogens from Various Sources (From Calder, 1991)

Liver, placenta and brain glycogens were isolated by phenol-cold water extraction. Muscle glycogen was isolated by mercuric chloride extraction. Molecular weight distributions were determined by fractionation of the glycogen on sucrose density gradients followed by calculation of the sedimentation coefficient of each fraction and application of Svedberg equation.

2.4.1.4 **Determination of Glycogen Concentrations in Sucrose Gradients** (iodine-iodide)

In order to use this method to measure accurately glycogen concentrations in fractions from sucrose density gradients, it must be shown that sucrose does not significantly interfere with the colour reaction (Hartfield *et al.*, 1989). This has been done previously by Calder, (1987) and the data are reproduced in Table 2.3 which demonstrates that sucrose, several other mono and disaccharides, and protein did not have any significant effect upon absorbance at 460 nm in the presence of iodine-iodide and saturated calcium chloride.

It has been known that, particularly for extensive studies on the components of starch (Greenwood, 1965), the colour of the complex (starch-iodine-iodide) formed depends upon the degree of branching and has been used to detect starch (Barger, 1930; Bailey and Whelan, 1961) and measure its components.

Archibald *et al.*, (1961) showed that there is a relationship between the absorbance spectra of glycogen-or amylopection iodine complexes and the structure of the polysaccharide. Glycogens showed an absorbance maximum in the range 420-490 nm and amylopections in the range 530-555 nm).

It had been shown that addition of salts increased the absorbancy of the polysaccharide - iodine complexes. This study was extended to ammonium sulphate (Archibald *et al.*, 1961) and calcium chloride (Krisman, 1962; Bobrova, 1986) was shown to be effective in increasing the colour of the polysaccharide-iodine complex.

This was investigated further (Drochmans, 1966) and comparisons were made of the absorbance spectrum of various glycogen-iodine complexes in the absence and presence of ammonium sulphate or calcium chloride. Both salts enhance the colour, and the maximum absorbance in the presence of ammonium sulphate is 470-490 nm. In the presence of calcium chloride glycogen shows a maximum absorbance of 460-470 nm or 400-410 nm depending upon its source. It was concluded that the former maximum is a characteristic of undegraded glycogen and the latter of degraded glycogen.

sample	concentration (mg/ml)	OD 460
Rat liver glycogen	1.00	1.520
	0.75	1.120
	0.50	0.740
	0.25	0.380
Glucose	10.00	0.029
	2.00	0.029
Lactose	10.00	0.010
	2.00	0
Maltose	10.00	0.049
	2.00	0.037
Maltotrisoe	10.00	0.036
	2.00	0.011
Sucrose	100.00	0.026
	10.00	0.002
	2.00	0
BSA	0.10	0.040
	0.05	0.024
	0.01	0
Phosphorylase b	0.10	0
	0.05	0

Table 2.3 Interference of Sugars and Protein with Glycogen Determination (From Calder,1987).

Sample (0.4 ml) dissolved in water was mixed with 2.6 ml of the iodine-iodide reagent (section 2.4.2.4) and the absorbance at 460 nm measured.

2.4.2 Methods Used in This Study

2.4.2.1 Liver Glycogen Extraction Using 45%_Phenol

Rat liver glycogen was prepared by the cold water extraction method of Laskov and Margoliash (1963), this being a modification of the RNA isolation method of Kirby (1956). This method is thought to produce glycogen with properties similar to native liver glycogen. It relies upon phenol denaturing and extracting enzymes, thereby eliminating sources of glycogen degradation (see section 2.4.1.1).

The whole liver was removed from liquid nitrogen, weighed and broken into small pieces with a chilled mortar and pestle. The liver tissue was then homogenised with 45% (w/v) phenol (approx. 1:25 (w/v) tissue: 45% phenol) in an Ultra-Turrax homogeniser at 3/4 speed for 1 min, or until no further lumps could be detected. Homogenisation was carried out in short bursts to minimise thermal degradation of glycogen (Geddes and Rapson, 1973).

The homogenate was gently stirred for 45 min at room temperature and centrifuged (380 x g, 15 min, 20 ^{0}C). This resulted in the formation of two distinct layers. The upper aqueous layer, which contained glycogen and RNA, was gently removed by aspiration without disturbing the lower phenolic layer, which contained glycogen, denatured protein and DNA.

Glycogen was precipitated from the aqueous layer by addition of 3 volumes of 95% (v/v) ethanol followed by centrifugation (1300 x g, 15 min, 4 ^{0}C). It was then redissolved in water and a further 3 volumes of 95% (v/v) ethanol was added to again precipitate the glycogen. This was usually completed within 24 h at 4 ^{0}C. The precipitated glycogen was collected by centrifugation (1300 x g, 15 min, 20 ^{0}C) and redissolved in a minimum volume of water. Any debris was removed by low speed centrifugation (380 x g, 15 min, 20 ^{0}C). A few crystals of lithium chloride were added to the solution to facilitate precipitation (Chow, 1992). Any debris was removed by low speed centrifugation (380 x g, 15 min, 20 ^{0}C) and then the glycogen solution was dialysed overnight against running water. Finally the solution was lyophilised. The dry product was weighed, and stored in sealed plastic tubes in a desiccator at room temperature.

2.4.2.2 **Muscle Glycogen Extraction Using 3% Mercuric Chloride**

Once the muscle sample had been removed from the liquid nitrogen, it was crushed and weighed and then added to 4-5 volumes of 3% mercuric chloride dissolved in citrate buffer (pH 5.0; 205 ml of 0.1 M solution of citric acid + 295 ml of 0.1 M solution of sodium citrate, diluted to a total of 1000 ml) and homogenised in a Ultra-Turrax homogeniser for bursts of up to 45 sec. The homogenate was then centrifuged (380 x g, 15 min, 4 ^{0}C) and the upper layer was strained through two layers of cheese cloth, to remove any fatty tissue that did not deposit in the lower cell debris layer.

The glycogen from the upper strained layer was precipitated with the addition of 3 volumes of ethanol, and centrifuged (1300 g, 15 min, 4 ^{0}C) into a pellet which was then dissolved in a minimum volume of water. For samples prepared by this extraction

method the glycogen purification procedure was the same as described above (section 2.4.2.1).

2.4.2.3 **Sucrose Density Gradient Ultracentrifugation**

Sucrose gradients were prepared in bulk by the method of Baxter-Gabbard (1972). Beckman polyallomer tubes (25 mm x 89 mm, No. 326823, Beckman instruments Inc., Palo Alto, California, U.S.A.) were filled with 30.6 ml of 30% (w/w) sucrose. They were twice frozen at -20 ^{0}C and thawed at 4 ^{0}C, and then frozen a third time. They were stored frozen until required. Prior to ultracentrifugation the gradients were thawed overnight at 4 ^{0}C. In this process a nearly linear gradient of sucrose is formed, the gradient becoming steeper with each successive freezing and thawing. Gradients with a reproducible sucrose range of 5-50% are finally formed. Immediately prior to use gradients were allowed to equilibrate to room temperature for 1 h. Four ml of glycogen dissolved in water, usually at a concentration of 10 mg/ml, was gently layered on top of a gradient with a Pasteur pipette.

The gradients were then centrifuged (29,300 g, 30 min, 20 ^{0}C) in a Beckman L2-65B Ultracentrifuge. The gradient was then fractionated using a 1 ml auto pipettor. Each gradient yielded 20 fractions of 1.7 ml. The glycogen content in each fraction was then determined as in section 2.4.2.4.

Previous experiments (Taylor, 1985) have shown that native glycogen concentrations within a range of 0.5 - 12 mg/ml can be applied to this type of gradient without any detectable changes in the molecular weight profiles of the glycogens. Therefore the arbitrary concentration of 10 mg/ml was chosen and used for all experiments unless otherwise stated.

2.4.2.4 **Determination of Glycogen Concentrations in Sucrose Gradients**

This was carried out using the iodine-iodide method. The iodine-iodide solution was prepared by dissolving 2.64 g of iodine and 26.4 g of potassium iodide in 100 ml of water. This can be kept for a period of several months, but must be stored without light. An 0.5 ml aliquot of this solution was then mixed with 130 ml of saturated calcium chloride to form the iodine-iodide reagent. The final reagent cannot be stored due to the unstable nature of the complex. An 0.4 ml sample containing glycogen, or water as a blank, was added to 2.6 ml of freshly prepared iodine-iodide reagent, mixed thoroughly

(Fig. 2.2)

and the absorbance at 460 nm was measured. The glycogen content in each gradient fraction was determined in triplicate. The amount of glycogen in each fraction was calculated as a percentage of the total glycogen in the gradient.

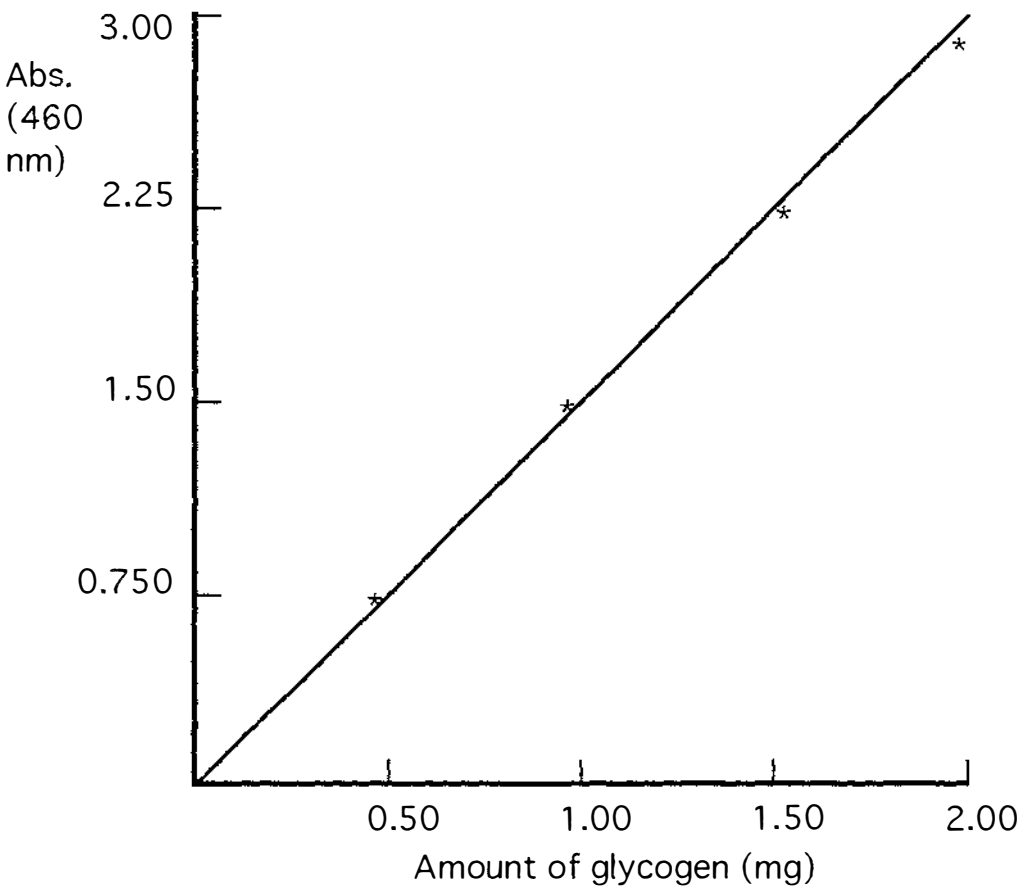

Figure 2.2 Standard Curve for Detection of Glycogen by the Iodine-Iodide Method

An 0.4 ml sample containing glycogen was added to 2.6 ml of freshly prepared iodine - iodide reagent, mixed and absorbance at 460 nm measured (Section 2.4.2.4).

CHAPTER 3

3.1 THE EFFECTS OF ETHANOL ON LIVER GLYCOGEN IN FED RATS.

3.1.1 Contrasting Effects of Ethanol on Blood Glucose Concentrations in Fed and Starved Rats.

The hypoglycemic effect of ethanol in the starved state was first described by Brown and Harvey in 1941. Subsequently, it was confirmed that ethanol induces hypoglycemia in 48 hours fasted rats (Forsander *et al.*, 1965; Krebs *et al.*, 1969) and humans (Field *et al.*, 1963; Kreisberg *et al.*, 1971; Wilson *et al.*, 1981). Hypoglycemia results from a decrease in the cytosolic free [NAD^+] to [NADH] ratio which is due to oxidation of ethanol by alcohol dehydrogenase and aldehyde dehydrogenase (Krebs *et al.*, 1969). This change in the redox state decreases the concentration of pyruvate and of other gluconeogenic intermediates (Krebs *et al.*, 1969; Guynn and Pieklik, 1975; Zakim, 1968), and inhibits gluconeogenesis from substrates such as lactate (Krebs *et al.*, 1969; Kreisberg *et al.*, 1971; Crow *et al.*, 1978; Puhakainen *et al.*, 1991), glycerol (Krebs *et al.*, 1969; Puhakainen *et al.*, 1991), and gluconeogenic amino acids (Krebs *et al.*, 1969).

In contrast, the effects of ethanol on carbohydrate metabolism in the fed state are poorly understood (Shelmet *et al,* 1988; Sneyd, 1989). It has long been known that ethanol does not produce hypoglycemia in fed animals (Forsander *et al.*, 1965; Dittmar and Hetenyi, 1978; Sneyd, 1989) or humans (Field *et al.,* 1963; Kreisberg *et al.*, 1971). In some studies, an ethanol induced hyperglycemic response has been observed in fed animals (Sneyd, 1989). Hyperglycemia may result from inhibition of glycolysis, stimulation of gluconeogenesis, stimulation of glycogenolysis, inhibition of glucose uptake by muscle tissue or combinations of some or all of these factors.

Potter and Morris (1980) observed that ethanol-induced hyperglycemia was related to elevation of plasma glucagon levels and to suppression of plasma insulin levels.

Subsequently, Erwin and Towell (1983) found a marked difference in ethanol-induced hyperglycemia in two lines of mice (LS/Ibg and SS/Ibg) with differential central nervous system sensitivity to ethanol. They performed experiments to determine if ethanol induced hyperglycemia was primarily a consequence of enhanced glycogenolysis. Ethanol (3.6 g/kg, IP) produced a 2.5-fold increase in plasma glucose in control LS fed mice, whereas no increase in plasma glucose was observed in animals fasted for 24 hours. In SS mice a significant increase in plasma glucose was observed only at the highest dose (4 g/kg) of ethanol. They concluded from their finding that the ethanol-induced hyperglycemia in LS mice is produced by an adrenergically mediated increase in glycogenolysis. Fasting, adrenalectomy and pretreatment with α- and ß-adrenergic antagonists eliminated the ethanol-induced hyperglycemic response suggesting that the ethanol-induced hyperglycemia was mediated by the central nervous system (Erwin and Towell 1983).

3.1.2 **The Mechanism of Autoregulation of Hepatic Glucose Production**

Glycogenolysis and gluconeogenesis are chiefly responsible for hepatic glucose output and it has been estimated that glycogenolysis normally accounts for more than 70% of hepatic glucose output in the post-absorptive state (Nilsson and Hultman, 1973; Consoli *et al.*, 1987; 1990). In other physiological situations, such as post-prandial hepatic glycogen repletion (McGarry *et al.,* 1987) and recovery from hypoglycemia (Frizzell *et al.*, 1988; Lecavalier *et al.*, 1989), and in non-insulin- dependent diabetes mellitus (Consoli *et al.*, 1990), gluconeogenesis may be the dominant pathway for the production of glucose.

Gluconeogenic precursors such as lactate (Jenssen *et al.*, 1990; Tappy *et al.*, 1995), glycerol (Jahoor *et al.*, 1990), or fructose (Tounian *et al.*, 1994) stimulate gluconeogenesis but leave total endogeneous glucose output unchanged (reviewed by Tappy *et al.*, 1997). The factors that determine the relative contributions of gluconeogenesis and glycogenolysis to overall hepatic glucose output in these situations are unclear. There are several mechanisms by which gluconeogenesis from precursors such as lactate could occur without overall hepatic glucose output. These are (1) inhibition of gluconeogenesis from other precursors; (2) inhibition of glycogenolysis; and (3) stimulation of glycogen synthesis. All of these mechanisms may be involved in this autoregulation (Jenssen *et al.*, 1990; Jahoor *et al.*, 1990; Tappy *et al.*, 1995). Tappy *et al.* (1995) have demonstrated that in healthy humans the autoregulation of hepatic glucose production during lactate infusion is still operative when glycogenolysis is suppressed by an infusion of exogeneous glucose, suggesting that stimulation of glycogen synthesis is involved in the autoregulation of hepatic glucose production.

This indicates that synthesis of glucose 6-phosphate from lactate is matched by an equivalent glycogen synthesis within liver. This view is supported by the report that lactate inhibits glycogen phosphorylase and stimulates the activity of glycogen synthase in rat hepatocytes (Rossetti *et al.*, 1993).

3.1.3 Possible Mechanisms to Account for a Hyperglycemic Effect of Ethanol in Fed Animals

(a) Stimulation of Glycogenolysis

Some studies have shown that ethanol causes a decrease in liver glycogen content in fed animals, which could explain the occurrence of hyperglycemia. Tennet (1941) and Klingman and Haag (1958) first suggested that ethanol produces hyperglycemia by enhancing the release of catecholamines from the adrenal gland. Subsequent studies revealed that in mammalian species including the dog, cat, rat and man, ethanol induced the release of adrenal epinephrine (Perman, 1960; Anton, 1965; Davis *et al.*, 1967). The work of Forsander *et al.* (1965) confirmed that glucose was released from livers of fed rats into the perfusion medium. This release was slightly greater when ethanol was present. With livers from starved rats no release of glucose was observed. Some researchers have shown that ethanol-induced hyperglycemia is a consequence of increased glycogenolysis and this effect is most likely a result of the action of epinephrine (Ammon and Estler, 1968). The effect of ethanol (5 g/kg) administration on liver glycogen content was studied by Nelson *et al.* (1969), who observed that glycogen concentration decreased steadily and reached a minimum level at 24 hours after ethanol administration. However, as discussed later (section 3.1.4), there are some problems with the data reported in this paper.

More recently, Kubota *et al.* (1992) demonstrated that plasma glucose concentration and hepatic glucose production remained unchanged in fed rats during ethanol infusion, but liver glycogen decreased by 75%. These results demonstrate that the lack of a hypoglycemic effect of ethanol in the fed state can be attributed to increased glycogenolysis. However, although Kubota *et al.* (1992) observed that ethanol stimulated glycogenolysis in livers from the fed rats, they did not find hyperglycemia in the fed rats (see section 3.1.4).

The mechanism by which glycogenolysis is stimulated by ethanol is not fully understood. Glucose 6-phosphate is a well-known inhibitor of glycogen phosphorylase, and an allosteric activator of glycogen synthase (Huijing, 1975; Hems and Whitton,

1980; Minassian *et al.*, 1994). Therefore a decrease in glucose 6-phosphate concentration could provide one mechanism by which glycogenolysis is stimulated. Changes in other known regulators of glycogen synthase or phosphorylase, such as UDP-glucose, AMP, inorganic phosphate, and cAMP, might also be involved (Kubota *et al.*, 1992).

(b) Stimulation of Gluconeogenesis

Krebs and co-workers (1968;1969) examined the effect of ethanol on gluconeogenesis in perfused livers from rats starved for 48 hours and found that ethanol inhibited gluconeogenesis from lactate by 66%. Inhibition of gluconeogenesis from lactate has also been observed in isolated rat hepatocytes (Cornell *et al.*, 1974; Crow *et al.*, 1978; Phillips *et al.*, 1985) but gluconeogenesis from pyruvate is actually increased by ethanol (Crow *et al.*, 1978). Thus it seems that the effects of ethanol on rates of gluconeogenesis depend on the substrate being used.

Although catecholamines (Perman, 1961; Erwin and Towell, 1983) or glucagon (Potter and Morris, 1980) may be partly responsible for the hyperglycemic effect of ethanol, ethanol also has direct effects on the liver. When ethanol is added to the perfusion medium of livers from fed rats it stimulates glucose output (Topping *et al.*, 1979; 1982). Ethanol decreases the concentration of fructose 2,6-bisphosphate in the liver of fed rats and hepatocytes from either fed or fasted rats (van Schaftingen *et al.*, 1980; Claus *et al.*, 1982). Ethanol stimulates fructose 1,6-bisphosphatase, and inactivates phosphofructo-1-kinase. The net effect is a stimulation of gluconeogenesis and glucose output by the liver.

In one study on human subjects, ethanol inhibited gluconeogenesis from lactate by about 70% and from glycerol by about 65%, but failed to alter hepatic glucose production in non-insulin-dependent diabetes mellitus patients (Puhakainen *et al.*, 1991). These data indirectly suggest that the inhibition of gluconeogenesis leads to a compensatory increase in glycogenolysis which allows hepatic glucose production to remain constant.

It therefore appears that while ethanol might increase rates of gluconeogenesis from some substrates in experiments using liver perfusion or isolated hepatocytes, the primary effect *in vivo* is inhibition of gluconeogenesis. Hyperglycemia induced by ethanol is therefore unlikely to be caused by stimulation of gluconeogenesis.

(c) Inhibition of glycolysis

Inhibition of glycolysis by ethanol could lead to the observed elevation of blood glucose concentration. Although there is little evidence that ethanol inhibits glycolysis *in vivo*, it seems that under certain experimental conditions ethanol would inhibit glycolysis *in vitro* (Sneyd 1989). Thurman and Scholz (1977) demonstrated that ethanol inhibits glycolysis in perfused livers from fed rats and suggested that this inhibition caused the increase in oxygen uptake observed in the presence of ethanol. However, the relevance of this to the situation *in vivo* is unclear.

(d) Inhibition of glucose uptake

There is little or no direct evidence that ethanol inhibits glucose uptake (for a review see Sneyd, 1989). *In vivo,* ethanol inhibits glucose utilization in muscle during stimulation by insulin in dogs (Lochner *et al.,* 1967) and humans (Yki-Järvinen and Nikkila, 1985; Yki-Järvinen *et al.*, 1988; Shelmet *et al.*, 1988). This suggests that ethanol inhibits glucose utilisation by peripheral tissues which are insulin sensitive, and this action may promote hyperglycemia.

(e) Combined mechanisms

As mentioned above, some results suggest that ethanol enhances glycogenolysis in the livers of fed animals, perhaps via the release of catecholamines or glucagon. Other results show that, depending on experimental conditions, ethanol may either inhibit or enhance rates of gluconeogenesis, may inhibit glycolysis, and may inhibit glucose utilisation by peripheral tissues. Kubota *et al.* (1992) raised three possibilities to explain the lack of a hypoglycemic effect of ethanol in the fed state. First, a decrease in hepatic glucose output could be counterbalanced by a decrease in peripheral glucose utilization. Second, inhibition of gluconeogenesis could be compensated by an increase in glycogenolysis resulting in unchanged overall hepatic output. Third, ethanol might not inhibit gluconeogenesis in the fed state. In fact, the effects of ethanol on blood glucose concentration may result from a combination of some or all of the above factors, and will clearly vary depending on the experimental approaches used.

Carefully controlled experiments need to be carried out to determine how ethanol affects tissue glycogen content, glycogenesis, glycogenolysis, gluconeogenesis, and glucose uptake by muscle tissue and glycolysis, in relation to the autoregulation of

hepatic glucose production. The experiments in this section of this thesis were designed to clarify whether ethanol decreases tissue glycogen content.

3.1.4 Effects of Ethanol on Liver Glycogen Content in Fed Rats

As indicated above, a few studies have produced results suggesting that ethanol causes a decrease in liver glycogen content. The effect of ethanol (5 g/kg) administration on glycogen content was studied by Nelson *et al.* (1969) and their data are summarized in Table 3.1. The control values for liver glycogen content obtained by these researchers are very low compared to other investigators (usually 200-400 μmol/g wet weight; see Table 3.2) and this introduces some doubt as to the validity of the experiments. Glycogen was isolated by the method of Good *et al.* (1933), which is an old method that is not widely used.

The results show that glycogen concentration decreased steadily and reached a minimum level at 24 hours after ethanol administration. These investigators did not use control animals for their experiments and they failed to report whether the rats were fed or starved, after ethanol treatment. Therefore, it is unclear whether the glycogen depletion was due to ethanol treatment or to starvation.

Table 3.1 Liver Glycogen at Various Time Periods After 5 g/kg Ethanol Administration (Data from Nelson *et al.*, 1969)

Time after ethanol		No. of animals	Glycogen (μmol//g)
Control	0	5	138 ± 5.2
Ethanol	10 min	3	125 ± 7.1
	12 hours	3	50 ± 6.8
	24 hours	3	12 ± 6.8
	48 hours	3	78 ± 2.2

Male albino rats of Wistar strain (fed *ad libitum*) were injected intraperitoneally with ethanol (5 g/kg of body weight), diluted with isotonic NaCl. Blood alcohol concentration reached a maximum level in 60 min and there was no detectable alcohol after 12 hours. After various time intervals the animals were killed by stunning and decapitation.

In another study, designed to determine the reason for the lack of a hypoglycemic effect of ethanol in the fed state, Kubota *et al.* (1992) studied the effect of ethanol on glucose turnover, liver glycogenolysis and concentrations of glucose metabolites. They

performed experiments on two groups of rats. Three days before the experiments, catheters were surgically inserted for blood sampling and infusions. On the experimental day, the rats received 4-hour infusions of [3-^{3}H] glucose for measurement of glucose turnover, and either ethanol or saline. Kubota *et al.* administered ethanol as a bolus at 0 min (100 µl of 20% ethanol) and then as a continuous infusion (20% ethanol) over 4 hours at a rate of 10 µl/min. They observed very high fed liver glycogen content (control value 242 mmol/kg of dry weight or 897 µmol/g wet weight), in comparison with most of the other researchers (see Table 3.2). This high glycogen content could be due to either the 4-hour glucose infusion or to errors in calculation. However, they showed that liver glycogen content was 75% lower at 240 min in the ethanol-infused (61 mmol/kg of dry weight) than in the saline-infused rats (242 mmol/kg of dry weight), indicating that glycogen content had fallen by 181 µmol/g of dry weight or 628 µmol/g wet liver weight (the dry-wet ratio is 3.47). The control data of Kubota *et al.* would be compatible with other researchers if they had reported 242 µmol/g wet weight of liver. It is possible that they might have confused dry weight and wet weight in their calculations. Alternatively, the application of glucose by intragastric bolus in starvation refeeding experiments with rats might explain the high blood glucose levels induced. In studies using rats (Niewöhner *et al.*, 1984, Niewöhner and Nuttall, 1995; Cook *et al.*, 1988) or mice (Cox and Palmer, 1987) in which glucose was administered by intragastric bolus, blood glucose concentrations of greater than 15 mmol/L were observed, whereas normal blood glucose concentrations are 4-6 mmol/L (Calder and Geddes, 1992; Sugden *et al.*, 1992). Very high blood glucose concentrations favour direct hepatic deposition of glucose (Newgard *et al.*, 1984) (see section 4.3.9). Kubota *et al.* (1992) observed a plasma glucose concentration of about 8 mM shortly after glucose infusion. There is a possibility that the liver could have extracted some glucose from blood plasma and this could have resulted in a very high liver glycogen content.

3.1.5 **Normal Liver Glycogen Content.**

There is considerable variation in the literature in the content of glycogen reported from liver of fed rats. As shown in Table 3.2, values range from as low as 138 µmol per g wet weight of liver (Nelson *et al,* 1969) to as high as about 500 µmol per g wet weight (Freminet *et al,* 1984) (excluding the extremely high value reported by Kubota *et al.* (1992) about which there is some doubt; see discussion above). To some extent the variation probably reflects individual variation between animals, but it is probably also due to differences in experimental procedures, such as differences in methods used to kill the animals and sample the tissue (see Table 3.2), different strains of animals and their maintenance, and different analytical methods.

Table 3.2 Comparison of Rat Liver Glycogen Contents Determined by Various Investigators.

Rat liver glycogen content (μmol/g wet weight)	Method	Mode of killing, Anaesthesia used	Reference
304		Ether	Kalkoff *et al.*, 1966
229		Nembutal	Rawat, 1968
218		Ether air	Start and Newsholme, 1968
138		Decapitation	Nelson *et al.*, 1969
382	Enzymatic (Keppler and Decker, 1974)	Ethyl ether	Freminet and Leclerc, 1980
339		Pentobarbital	Boyd *et al.*, 1981
493	Enzymatic (Keppler and Decker, 1974)	Ethyl ether	Freminet *et al.*, 1984
292	Enzymatic (Keppler and Decker, 1974)	Sodium pentobarbital 6 mg/100 g body wt.	Sugden *et al.* (1992)
400	Enzymatic (Keppler and Decker, 1974)	Sodium pentobarbital 6 mg/100 g body wt.	Holness *et al.* 1988a
198	Enzymatic (Keppler and Decker, 1974)	Pentobarbital 60 mg/kg body wt.	Pallardo and Williamson, 1989
428	Micro method (Kemp and van Heijningen, 1954)	Cervical dislocation	Calder and Geddes, 1990a
403	Micro method (Kemp and van Heijningen, 1954)	Cervical dislocation	Calder and Geddes, 1992
897	Enzymatic (Keppler and Decker, 1974)	Pentobarbital 50 mg/100 g body wt.	Kubota *et al.*, 1992
271		Pentobarbital 7 mg/100 g body wt.	Minassian *et al.*, 1994
362	Anthrone (1957)	Pentobarbital	Winder *et al.*, 1995

The values are expressed in μmol/g wet weight. All the animals were fed. Blanks indicate that data are not available.

It has long been known that the method of sacrifice of the animal influences tissue glycogen content. It is particularly important that animals are killed without anaesthetic if possible, and that the livers are removed rapidly to minimise the possibility of *post-mortem* degradation of glycogen (Geddes and Rapson, 1973; Calder and Geddes, 1990a), since it is well established that the delay between killing and freezing represents an important factor which influences values of glycogen content in liver and muscle. Results from studies in which these criteria have been satisfied suggest that the normal values for liver glycogen in fed rats range from about 300-400 μmol per g wet weight (or 50-64 mg/g wet weight) (Calder and Geddes, 1990a; Calder and Geddes, 1992; Holness *et al.*, 1988a; Sugden *et al.*, 1992). In a study such as the current one, some decrease in liver glycogen in control animals may result from the stress of handling and stomach-tubing, as stress may cause release of hormones that will initiate glycogenolysis. For this reason, control animals handled the same way as treated animals were included with each experiment. Control experiments were also carried out at exactly the same time as the ethanol treatment, as liver glycogen content varies with the time of day (Sugden *et al,* 1992).

3.1.6 **Aims of the present study**

As indicated above, some evidence in the literature suggests that a single large dose of ethanol can cause depletion of liver glycogen content in fed animals (Nelson *et al.*, 1969; Kubota *et al.,* 1992), but the results of these studies are of questionable significance due to problems with the values reported for liver glycogen content and the experimental approaches used. A primary aim of this study was to reinvestigate the possibility that a single large dose of ethanol depletes liver glycogen content. In recent years, molecular weight distributions of glycogen have been determined, using sucrose density gradient centrifugation, for many tissues and species (see reviews by Geddes, 1985; Calder, 1991), but the effect of ethanol on the molecular weight distribution of glycogen has not been investigated. A further aim of this study was to investigate whether ethanol treatment caused any change in the molecular weight distribution of liver glycogen stores.

In many acute studies, ethanol is given to animals by intraperitoneal injection. The advantage is the simplicity of the technique; the disadvantages are that the route is unphysiological, animals may suffer stress and internal injury, and the blood alcohol concentration rises rapidly. To avoid these problems, oral or intragastric ethanol administration is more desirable (Li, 1991). In this study, ethanol was administered using intragastic intubation. This method retains the oral route, but ensures that each animal receives a consistent dose of ethanol.

3.2 RESULTS AND DISCUSSION

3.2.1 Dose-Response Experiments.

3.2.1.1 Experimental Approach

Rats were administered a single dose of ethanol, in 4 ml of aqueous solution, and sacrificed 90 minutes later. The amounts of ethanol given are specified for each experiment. Control animals received an equivalent volume of water and were also killed 90 minutes after dosing. Ethanol dosing was always carried out between 9 and 10 am.

3.2.1.2 Preliminary Dose-Response Experiments: Quantitative Analysis of the Effects of Ethanol on Glycogen Content in Liver and Muscle.

3.2.1.2 a. Liver

The effects of a single dose of ethanol of either 5 or 6 g/kg on liver glycogen are presented in Figure 3.1 and Table 3.3. Both doses markedly decreased liver glycogen content in fed animals. A single dose of ethanol of 5 g/kg depleted liver glycogen content by 39%, and a dose of 6 g/kg by 56%. These results clearly indicate that a single large dose of ethanol produces a dramatic decrease in liver glycogen content, soon (90 minutes) after ethanol treatment. The liver glycogen content measured in control rats (345 ± 19 μmol/g wet weight) agrees with recent, reliable values reported in the literature, for example 362 μmol/g wet weight (Winder *et al.*, 1995.; see Table 3.2). Since there appeared to be a clear dose-response relationship for liver glycogen depletion by ethanol in the present experiment, a further experiment was designed to include lower ethanol doses (Section 3.2.1.3).

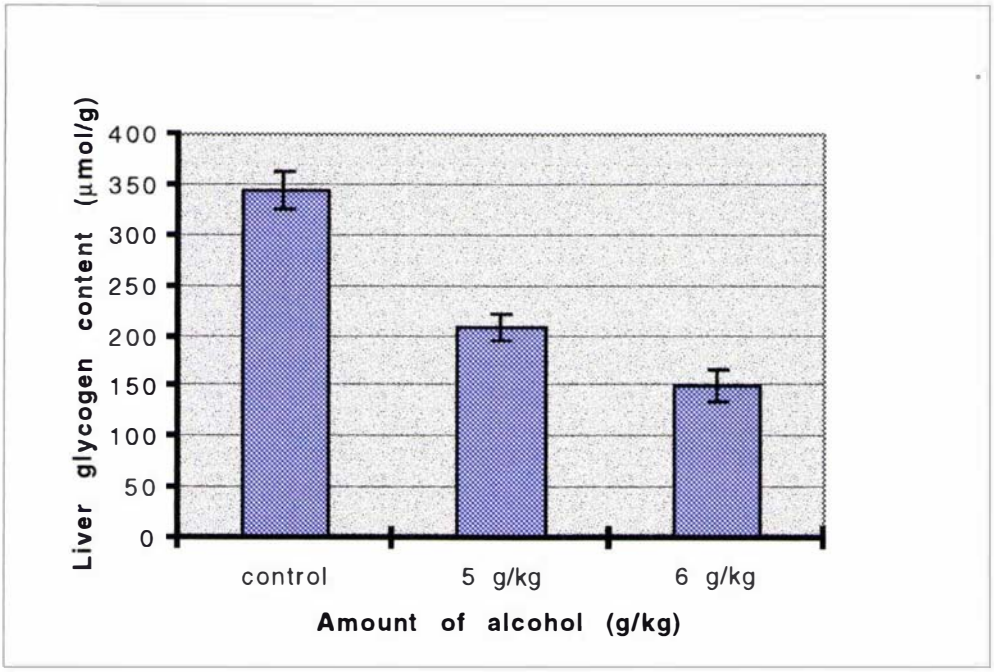

Figure 3.1 Effects of Ethanol on Liver Glycogen Content in Fed Rats

For conditions see Table 3.3.

Table 3.3 Effects of Ethanol on Liver Glycogen Content in Fed Rats

Control (µmol/g) (n=3)	**Treated** (5 g/kg) (n=3)	**Treated** (6 g/kg) (n=3)
345 ± 19	209 ± 13	152 ± 16

Rats were administered a single dose of ethanol of either 5 or 6 g/kg. Control animals received an equivalent amount of water. The animals were sacrificed 90 minutes after treatment, and the glycogen content of liver was determined as described in section 2.3. Results shown are means (± S.E.M.) for three rats.

3.2.1.2.b **Muscle**

The effects of a single dose of ethanol of either 5 or 6 g/kg on rat hindlimb muscle glycogen are shown in Table 3.4. Neither a 5 g/kg nor a 6 g/kg dose of ethanol caused any significant glycogen depletion in muscle in fed animals. Therefore, we did not perform any further experiments on the effects of ethanol on muscle glycogen from fed rats.

Table 3.4 Effects of Ethanol on Muscle Glycogen Content in Fed Rats

Control (μmol/g) (n=3)	**Treated** (5 g/kg) (n=3)	**Treated** (6 g/kg) (n=3)
28 ± 4	30 ± 4	26 ± 3

Rats were administered a single dose of ethanol of either 5 or 6 g/kg. Control animals received an equivalent amount of water. The animals were sacrificed 90 minutes after treatment, and the glycogen content of muscle was determined as described in section 2.3. Results are shown (± S.E.M.) for three rats.

3.2.1.3 **Further Dose-Response Experiments: Effects of Different Ethanol Doses on Liver Glycogen Content and Molecular Weight Distribution.**

3.2.1.3 a **Quantitative Analysis of Liver Glycogen Content**.

The results in Figure 3.2 and Table 3.5 show that liver glycogen content is decreased by 7-27% by a low (2.0 g/kg) dose of ethanol. With increasing amounts of ethanol liver glycogen content progressively decreased, by up to 61-78% with a dose of 6 g/kg. The latter dose of ethanol is sufficient to cause about 1-2 hours of sleep in most rats. The content of liver glycogen decreased to a consistently low level in all three experiments, although the percentage decrease is more in the first experiment because of high glycogen content in liver from control animals. The lowest liver glycogen content observed, 87 μmol per g wet weight, is about 5-fold higher than the liver content observed after 40 hours of starvation (16 μmol/g wet weight; 95% loss; Calder and Geddes, 1992).

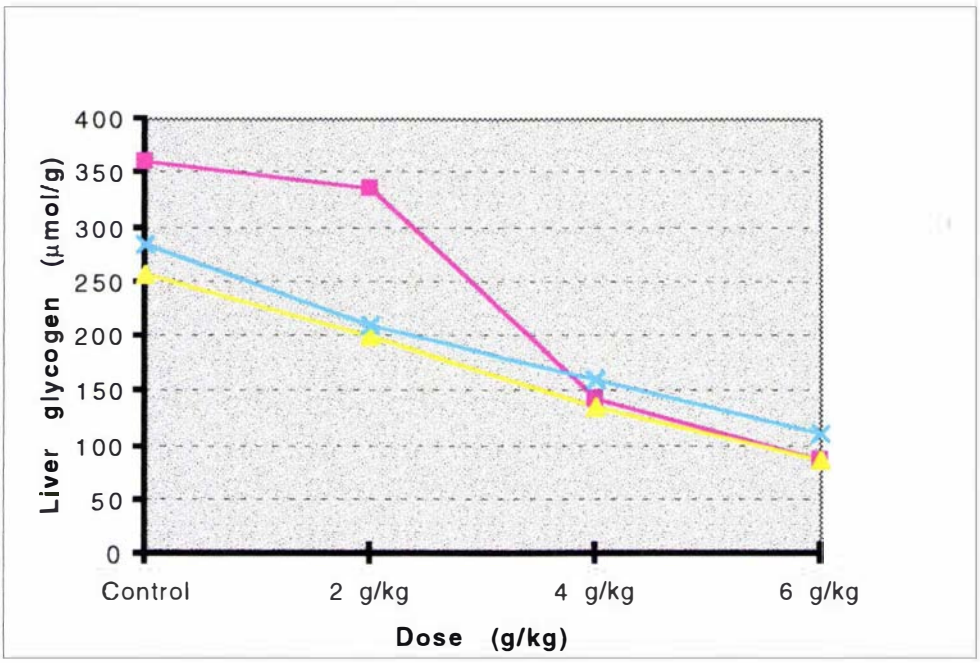

Figure 3.2 Effects of Ethanol on Liver Glycogen in Fed Rats

Dose respose of the effect of ethanol on fed liver glycogen content. Rats were treated with ethanol or water (controls) as described in section 3.2.1. The animals were sacrificed 90 minutes after treatment, and the glycogen content of liver was determined as described in section 2.3. Results are shown for three independent experiments.

In contrast to our results, Nelson *et al.*, (1969) found different results and drew different conclusions. They reported a control value of 138 µmol/g (see Table 3.2), which was approximately two and a half times lower than our control values. They showed that in ethanol treated animals liver glycogen content had depleted by 10% within 10 minutes and reached a minimum level (65% depletion) 24 hours after a 5 g/kg ethanol dose. In our dose response experiments, we observed approximately 40% depletion at 90 minutes after 5 g/kg ethanol administration (Table 3.3). We also showed that liver glycogen content had decreased by 61-71% with a dose of 6 g/kg (Table 3.5). The 10% depletion in liver glycogen content observed by Nelson *et al*. 10 minutes after a 5 g/kg ethanol dose is not inconsistent with the 40-78% depletion we observed 90 minutes after ethanol dosing. However, the next time interval monitored in their study was 12 hours and it is difficult to make any further comparison of their results with those in Table 3.5 because of the large difference in time involved.

Table 3.5 Dose Response of the Effect of Ethanol on Liver Glycogen Content

Experiment No	Ethanol Dose (g/kg rat)	Liver glycogen content (μmol /g wet weight)	(% relative to control)
1	0	360 ± 11	100
	2	335 ± 08	93
	4	143 ± 08	40
	6	87 ± 14	24
2	0	257 ± 04	100
	2	199 ± 21	77
	4	136 ± 15	53
	6	87 ± 17	34
3	0	285 ± 02	100
	2	210 ± 02	73
	4	160 ± 09	56
	6	111 ± 23	39

Rats were administered a single dose of ethanol of 2, 4 or 6 g/kg, and sacrificed 90 minutes later. Control animals received an equivalent volume of water. The glycogen content of liver was determined, in each case, on a pooled, powdered, liver sample from two animals. Each pooled liver sampled was assayed in triplicate, and results are shown (±S.E.M) for the three determinations. The whole experiment was repeated three times and 1, 2, and 3 refer to the three independent experiments.

Kubota *et al.* (1992) found that liver glycogen content was 75% lower at 240 min in ethanol-infused than in saline-infused rats. This is a similar level of depletion to that seen at 90 minutes with a 6 g/kg ethanol dose in our experiments, but again the time difference (90 minutes compared with 240 minutes) and the different mode of ethanol administration (intragastric single dose vs infusion) makes direct comparision of these results difficult.

Although there are differences in the amount of decrease, it is important to note that all three studies (our study, Nelson *et al.* and Kubota *et al.*) found a decrease in liver glycogen content with ethanol treatment. The differences in results between these experiments may be related to differences in experimental procedures such as (a) amount of ethanol, (b) route of ethanol administration, (c) duration of administration, (d) amount of the glucose load, (e) the dietary state of the animal and (f) the analytical methods used. Therefore to resolve clearly the time-course of the effects of ethanol on liver glycogen content, further experiments were conducted in fed animals, using an ethanol dose of 6 g/kg, to determine at what time after dosing the effect of ethanol was maximal, and over what time the liver glycogen content returned to normal (see section 3.2.2, Time course experiments.)

3.2.1.3.b **Molecular Weight Distribution of Liver Glycogen.**

Animals were administered a single dose of 2, 4 or 6 g/kg, and sacrificed 90 minutes later. The molecular weight distributions of the purified glycogen extracted from treated and control livers are summarised in Figure 3.3 and Table 3.6. The glycogen isolated from the livers of control animals shows a distribution pattern similar to that observed by other researchers for liver glycogen from normal, well-fed animals (Calder and Geddes, 1992; Geddes *et al,* 1992). Ethanol decreases the content of both high (fractions 9-20) and low (fractions 1-8) molecular weight glycogen (Figure 3.3).

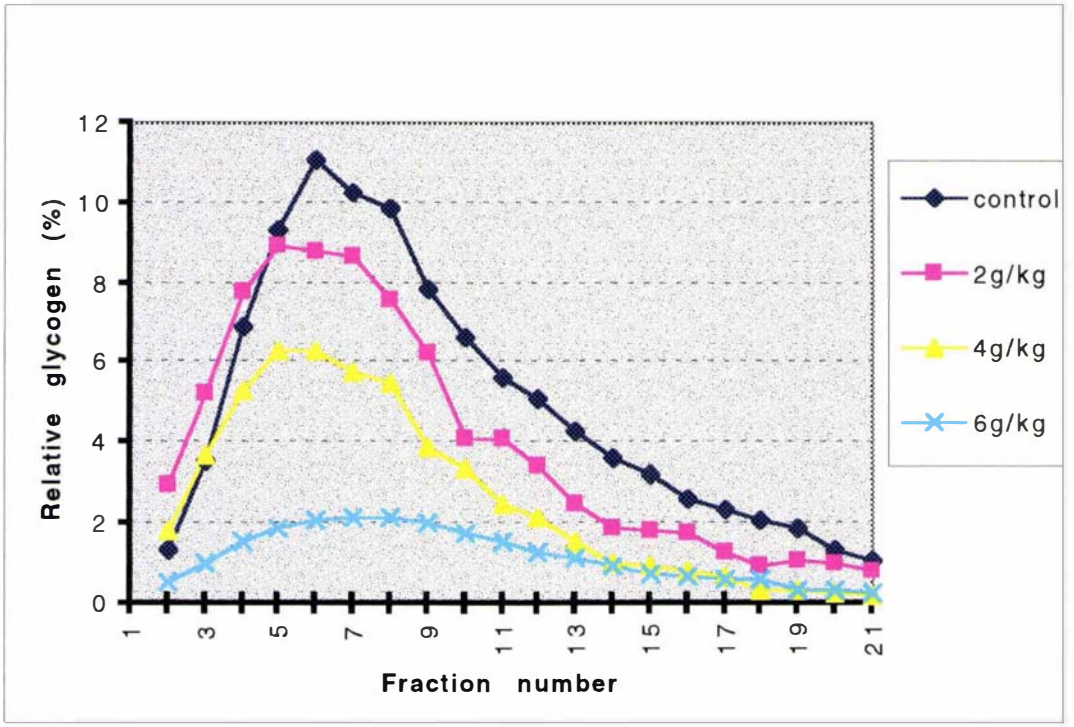

Figure 3.3 Effects of Ethanol on Molecular Weight Distribution of Glycogen in Liver of Fed Rats

Rats were administered a single dose of ethanol of 2, 4 or 6 g/kg, and sacrificed 90 minutes later. Control animals received an equivalent volume of water. The amount of glycogen in each fraction is expressed as a percentage of the total amount of glycogen present in the fed liver. Results are the average of three independent gradients.

This is confirmed by the data shown in Table 3.6, where the amounts of high and low molecular weight glycogen have been calculated for each ethanol dose, relative to the glycogen content in the control. Control ratios for molecular weight distribution (low:high) ranged from 50:50 to 65:35, with a mean of 58:42 as shown in Table 3.6. This is a similar range to that reported previously for liver glycogen (Calder and Geddes, 1992; Geddes *et al*., 1992). At the lowest ethanol dose (2 g/kg) there appears to be slightly greater loss of low than of high molecular weight glycogen and a total liver glycogen level of approximately 0.78 times (average for experiments 1, 2 and 3) the control level was seen (Table 3.5).

Table 3.6 Molecular Weight Distribution of Glycogen in Liver of Fed, Ethanol Treated-Rats

	Amount of glycogen as % of total glycogen in fed control		Amount of glycogen in molecular size range as % of that in fed control	
Ethanol dose (g/kg rat)	**Low MW < 250 x 10^6**	**High MW > 250 x 10^6**	**Low**	**High**
0 (control)	58 ± 4	42 ± 4	100	100
2	41 ± 6	36 ± 1	71	86
4	28 ± 2	19 ± 7	48	45
6	19 ± 3	13 ± 2	33	31

Rats were administered a single dose of ethanol of 2, 4 or 6 g/kg, and sacrificed 90 minutes later. Control animals received an equivalent volume of water. The glycogen in each gradient centrifugation has been divided into low (<250 x 10^6) and high >250 x 10^6 molecular weight classes. The dividing line was taken between fractions density 8 and 9 from the gradient (Fig. 3.3). The amount of glycogen in each size range is expressed as a percentage of the total amount of glycogen present in the fed liver. Results are the mean of three independent experiments.

However, with an ethanol dose of 4 g/kg, there was a significant fall in total hepatic glycogen concentration (about 50%), and the high and low molecular weight glycogen concentrations were 45% and 48% of their respective control values. At an ethanol dose of 6 g/kg, glycogen levels had decreased to about 30% of the fed level and low and high molecular weight glycogen pools had dropped to 33% and 31% of the fed levels respectively. It can be seen from Table 3.6 that ethanol doses of 4 g/kg and 6 g/kg had decreased both low and high molecular weight glycogen almost evenly. Since there has been no previous study of the effects of ethanol on tissue glycogen molecular weight distribution, we have not been able to compare these results with any other studies. These results suggest that ethanol may have two effects. Firstly, ethanol may have accelerated glycogen breakdown of both high and low molecular weight to

the same extent. Secondly, ethanol may have impaired glycogen synthesis both low (primarily cytosolic) and high molecular weight (primarily lysosomal) glycogen to a very similar extent.

3.2.2 **Time-Course Experiments.**

3.2.2.1 **Experimental Approach**

Rats were administered a single 4 ml dose of either ethanol (6 g/kg) or water, and were sacrificed 0.33, 3, 12, 24 or 48 hours afterwards. After treatment, the animals were permitted free access to food and water.

3.2.2.2 **Quantitative Analysis of Liver Glycogen**

For the dose-response experiments described in section 3.2.1, all animals were killed 90 minutes after ethanol administration. Subsequently, time-course experiments were carried out, using an ethanol dose of 6 g/kg, to investigate at what time after dosing the effect of ethanol was maximal, and over what time the liver glycogen content returned to normal.

The results shown in Figure 3.4 and Table 3.7 indicate that the decrease in liver glycogen content caused by ethanol was maximal at 90 minutes, when glycogen levels had been depleted by about 68%. At 20 minutes a small decrease in content was observed, and by 3 hours after dosing the content had begun to increase. By 12 hours after dosing, the liver glycogen content was again very close to control values. At 24 hours, the liver glycogen content in treated animals had increased to well above that in the controls, (163%; mean of 2 independent experiments). This is the first report of over-production of glycogen caused by ethanol in fed animals. By 48 hours the liver glycogen content had returned to normal values. Over-synthesis of liver glycogen has been previously reported after a period of mild starvation in rabbits (Geddes and Stratton, 1977a) and rats (Holness*et al.*, 1988a; Calder and Geddes, 1992).

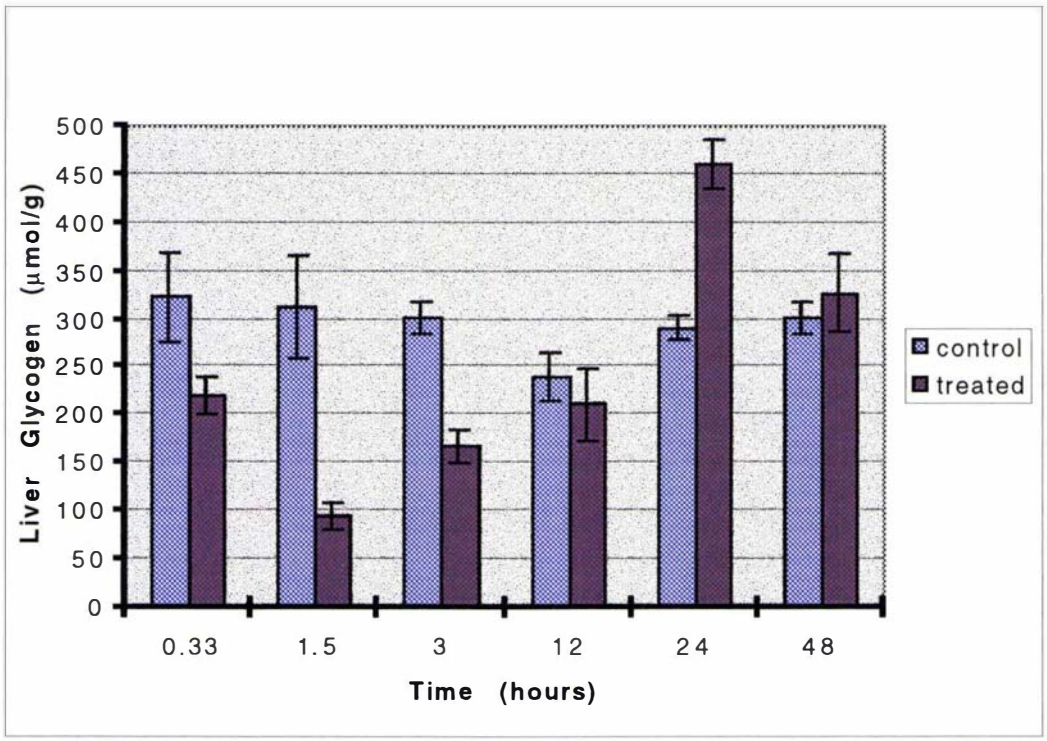

Figure 3.4 Time course of the Effect of Ethanol on Liver Glycogen Content

Rats were treated with ethanol (6 g/kg) or water (controls) as described in section 3.2.2. The animals were sacrificed at the times indicated after treatment and the glycogen content of the liver was determined as described in section 2.3. The 1.5 hours results are an average of the three experiments described in section 3.2.1.3. Two experiments were carried out, one including 0.33, 3, 12, and 24 hour time intervals and the second omitting 0.33 hour and including 48 hours. Results at 3, 12 and 24 hours are an average of the two experiments and error bars indicate ± S.E.M. for triplicate determinations on two pooled livers from each of two experiments. For 0.33 and 48 hour experiments, error bars indicate ± S.E.M. for triplicate determinations on two pooled livers.

The extent of over-synthesis in rats reported by Calder and Geddes (1992) was 165%, which falls within the range of the over-synthesis observed after 24 hours in this study (187% and 138%; Table 3.7). There have also been reports of over-synthesis of muscle glycogen following its depletion by starvation (Calder and Geddes, 1992; Holness *et al.*,1988a), exercise (Hultman, 1967) or electrical stimulation (Hamilton *et al.*, 1984). The depletion of liver glycogen caused by ethanol leads to a marked over-synthesis as the animals recover from the effects of the ethanol. The extent of this over-synthesis is similar to that observed after starvation and refeeding (see Chapter 4). The observation raises some interesting possibilities in terms of using controlled ethanol doses for liver glycogen loading for athletes prior to endurance events.

However, more experimental work is required to determine whether the over-synthesis effect would occur with lower ethanol doses, as a dose of ethanol equivalent to 6 g/kg would produce severe intoxication in human subjects.

Table 3.7 Time Course of the Effect of Ethanol on Liver Glycogen Content

Time (hours)	Liver glycogen content (μmol/g) wet weight)		% glycogen in treated livers relative to controls
	Control	Treated	
0.33	330 ± 45	221 ± 20	67
1.5	301 ± 53	95 ± 14	32
3.0	311 ± 16	145 ± 17	47
	295 ± 14	190 ± 09	64
12.0	229 ± 25	212 ± 38	93
	250 ± 07	211 ± 09	84
24.0	243 ± 13	455 ± 26	187
	338 ± 26	467 ± 23	138
48.0	301 ± 17	328 ± 40	109

For conditions see Figure 3.4. Results are shown as mean ± S.E.M. for the three determinations. Two experiments were carried out, one including 0.33, 3, 12, and 24 hour time intervals (upper results) and the second omitting 0.33 hour and including 48 hours (lower results). The 1.5 hours results are an average of the three experiments shown in Table 3.5.

Time course experiments revealed a marked difference between our results and those of Nelson *et al.* (1969). They showed glycogen content decreased progressively and reached a minimum level (12 μmol/g) at 24 hours after ethanol (5 g/kg) administration. This value is approximately 9% of their control liver glycogen content. Our control liver glycogen content was about 300 μmol/g (average) and in the time course experiments, we observed a minimum level of 95 μmol/g, which is about 32% of our control values, at 90 minutes after 6 g/kg ethanol administration.

Another major difference between our results and those of Nelson *et al.* is that in their experiments, glycogen reaccumulation started after 12 hours and reached about 60% of their fed liver glycogen content after 48 hours. In our experiments, by 3 hours after dosing glycogen content had begun to increase, and by 12 hours after dosing the liver glycogen content was very close to our control value (about 88%). At 24 hours the liver glycogen content in ethanol treated rats in our experiments had increased to 163%, and by 48 hours the liver glycogen content had returned to normal. In our experiment, the glycogen level had started to increase much earlier and returned to the control value again more rapidly than observed by Nelson *et al.* It is important to note that in their experiment, the glycogen level never returned to their control value. Since Nelson *et al.* failed to show control data, and to indicate the nutritional status of their animals, it is unclear whether the glycogen depletion seen in their experiments was due to ethanol or to starvation. They failed to observe the oversynthesis of liver glycogen that we observed in ethanol treated rats, but this is not surprising if the animals were not fed following ethanol treatment.

Glycogen reaccumulation in our experiments is reasonably linear from the lowest glycogen content at 1.5 hours to the highest at 24 hours in ethanol treated rats. At 12 hours, glycogen content from control rats was low because the rats did not eat much food in the day time. In the normal experimental animal fed *ad ibitum*, food consumption usually peaks shortly after the start of the dark period. The content of liver glycogen was also low at 24 hours in one experiment. The possible reasons are that these rats might have been agitated, or that the decreased content reflects individual variation, or both.

3.2.2.3 **Molecular Weight Distribution of Glycogen.**

For each time interval after ethanol dosing, purified glycogen extracted from treated and control livers was subjected to density gradient centrifugation. Figure 3.5 shows plots of one curve for each time interval for ethanol-treated rats, and Table 3.8 shows the proportion of glycogen calculated to be in the low and high molecular weight ranges for both treated and control animals.

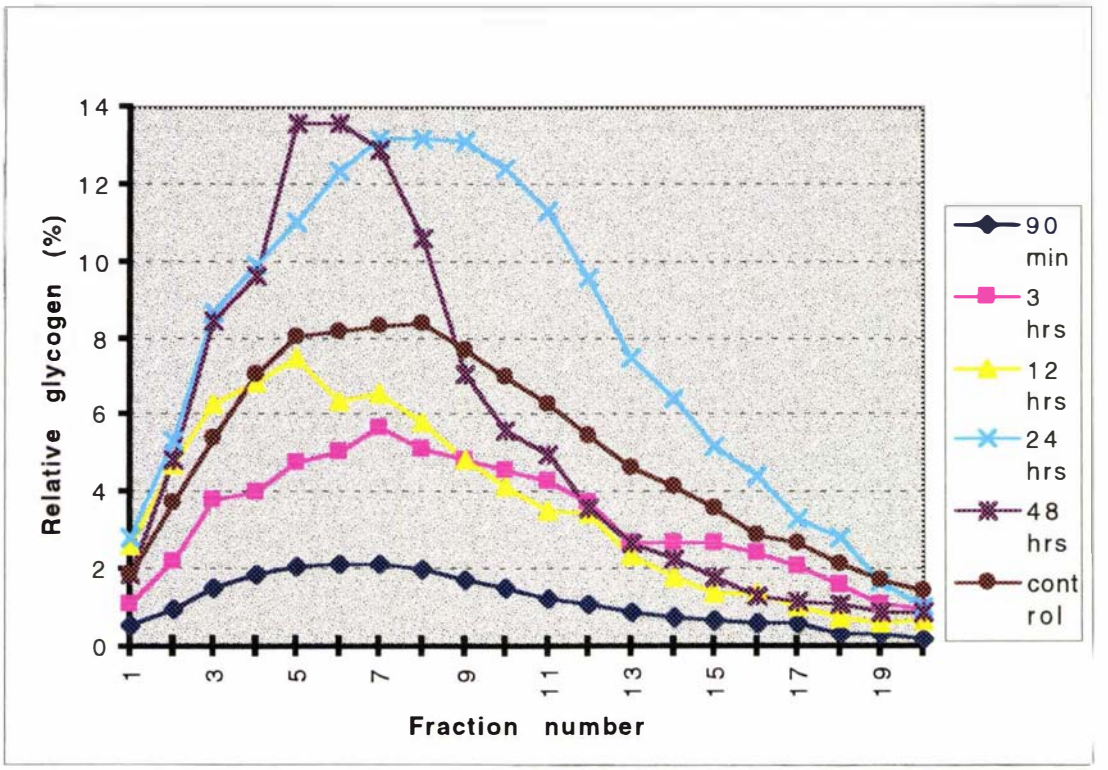

Figure 3.5 Molecular Weight Distribution of Liver Glycogen at Different Time Intervals Following a 6 g/kg Ethanol Dose

Rats were administered a single 4 ml dose of either ethanol (6 g/kg) or water, and were sacrificed 90 min, 3, 12, 24 or 48 hours afterwards. After treatment, the animals were permitted free access to food and water. The amount of glycogen in each fraction is expressed as percentage of the total amount of glycogen present in fed liver. Results are the mean of the three gradients (± S.E.M.).

Table 3.8 Molecular Weight Distribution of Glycogen at Different Time Intervals Following a 6 g/kg Ethanol Dose

Time (hrs)	Relative amount of glycogen in molecular size range (%)					
	Control (1)		Treated (2)		Treated glycogen (% of the relevant control) (3)	
	low	high	low	high	LMW	HMW
0.33	61	39	35	32	58	80
1.5	58	42	19	13	33	31
3.0	51	49	24	22	47	45
	47	53	32	33	68	62
12.0	50	50	46	47	92	94
	57	43	54	30	95	70
24.0	53	47	115	70	217	148
	62	38	69	69	111	181
48.0	58	42	75	33	129	78

For conditions see Figure 3.5. The glycogen in each gradient centrifugation was divided into low and high molecular weight classes as described for Table 3.6. Treated samples were corrected for the quantitative changes observed see Table 3.7. The results for 1.5 hours are the average of three experiments (see Table 3.7). Other results shown are for individual experiments. Controls represent data for glycogen purified from animals which received a dose of water rather than ethanol, and were sacrificed at the same time intervals as treated animals. The glycogen content in control and treated animals is expressed as a % of the total glycogen in the control animals at that time. Column 3 indicates the percentage of treated glycogen in each molecular weight range relative to the appropriate control (Column 1).

All the controls fall within the range of 47-62% of glycogen in the low molecular weight range, and 38-53% in the high molecular weight range. These are similar to results from controls in both the dose response experiments in Table 3.6 and those published elsewhere (Calder and Geddes, 1992; Geddes *et al.*, 1992). Also in Table 3.8, the percentage of glycogen in each molecular weight range relative to the appropriate control is shown. It is apparent that the depletion of liver glycogen seen at

20 minutes occurs slightly more in the low molecular weight range. At 3 hours almost equal depletion has occurred in both the low and high molecular weight glycogen, as was also apparent at 90 minutes in the dose-response experiments (the average 90 minute results are included in Table 3.5 for comparison with other time intervals). Similarly the over-synthesis of glycogen that is apparent at 24 hours is observed in both low and high molecular weight ranges, although to different extents in the two experiments shown. This variation may indicate that individual animals vary in the extent of over-synthesis of both high and low molecular weight glycogen, or that there is a switch from over-synthesis of high to over-synthesis of low molecular weight glycogen (as has been observed after starvation and refeeding, Calder and Geddes, 1992) and that this switch occurs at different times in different animals.

By 48 hours, the overall glycogen content in treated animals has returned close to the control value (1.09 times of the fed level). The level of low molecular weight glycogen in treated animals was 1.29 times the fed level, while the level of high molecular weight glycogen was 0.78 times of the fed level. This imbalance is much less at 12 hours (before over-synthesis occurred) when the liver glycogen content in treated animals is also close to that in controls. It thus appears possible that over-synthesis of glycogen after depletion by ethanol may lead to an increase in low molecular weight glycogen relative to high molecular weight at a time interval as long as 48 hours after the initial ethanol dose. This is in contrast to results after starvation and refeeding where molecular weight distribution of glycogen had returned to normal after 48 hours (Calder and Geddes, 1992). The longer-term after effects of an ethanol dose (for example at 24-96 hours) on the molecular weight distribution of glycogen warrant further investigation.

The results in this chapter demonstrate that ethanol causes little or no effect on molecular weight distributions and levels are depleted almost evenly across the molecular weight range. It is unclear whether ethanol depletes both low and high molecular weight glycogen simultaneously or one pool of glycogen affects other stores of glycogen. Geddes (1985) reported that a feedback mechanism, which may control the metabolism of both pools of glycogen simultaneously, appears to operate. Clearly, this question needs to be addressed in future. However, over the last 30 years, molecular size distribution has been studied in different physiological situations such as fasting (Geddes and Taylor,1985a; Calder and Geddes, 1990b,c), refeeding following fasting (Geddes and Stratton, 1977a; Calder and Geddes, 1990b,c; Geddes and Chow, 1994b) and *post mortem* degradation (Geddes and Rapson, 1973; Calder and Geddes, 1990a). These studies confirmed that glycogen synthesis and degradation is usually

inhomogeneous in both liver and muscle in these situations; this contrasts with the generally similar degree of depletion seen in each molecular weight range with ethanol.

These results are the first attempt to investigate the effects of ethanol on low (cytosolic) and high (lysosomal) molecular weight glycogen distribution in fed liver. The fact that both pools of glycogen are depleted indicates that ethanol must affect synthesis or breakdown of lysosomal as well as cytosolic glycogen. Further experiments are required to explore the mechanisms by which ethanol interferes with lysosomal and cytosolic glycogen metabolism. Most glycogen researchers have not given great attention to lysosomal carbohydrate metabolism, but this should be considered as approximately 20% of the glycogen (mostly high molecular weight) may be located in the lysosomal compartment. (Geddes, 1985; Calder, 1991; Geddes and Chow, 1994b).

3.2.3 **The Post Mortem Degradation of Liver Glycogen**

The results of the previous section illustrate that acute (6 g/kg) treatment with ethanol extensively depleted liver glycogen content in fed rats. The depletion of liver glycogen was maximal at 90 minutes after an ethanol dose of 6 g/kg. After death, there is very rapid loss of glycogen from liver (Geddes and Rapson, 1973) and muscle (Calder and Geddes, 1990a; see section 1.17). We needed to establish that decreased liver glycogen after ethanol dosing is not caused by an increase in *post mortem* glycogen breakdown. Therefore, experiments were conducted to determine whether ethanol treatment influences *post mortem* glycogen degradation.

3.2.3.1 **Experimental Procedure**

Rats were treated with ethanol (6 g/kg in 4 ml of aqueous solution) or water (controls) then killed 10 minutes later. The animals were left intact after death for various times up to 2 hours, after which the liver was freeze clamped and analysed for glycogen as described in Sections 2.2.3 and 2.3 respectively. Ethanol administration was carried out using intragastic intubation. All the experiments were carried out at the same time of the day (9 am).

3.2.3.2 Liver Glycogen Content after *post mortem* Degradation

Figure 3.6 and Table 3.9 show the liver glycogen content at various times after death. There was a rapid fall in hepatic glycogen concentration in both control and treated rats after death. We designed the experiments to test whether a single dose of ethanol accelerates *post mortem* glycogen degradation. The liver glycogen content was degraded a similar to extent of in both control and treated rats. It appears that ethanol does not have a significant effect on *post mortem* liver glycogen degradation. If anything, the *post mortem* degradation rate is slightly decreased by ethanol. The decreased liver glycogen observed after ethanol dosing is clearly not caused by an increase in *post mortem* glycogen breakdown. Results with control animals are in accordance with those of Calder (1987), and Geddes and Rapson (1973). Calder reported that glycogen levels had fallen by 35% at 45 min *post mortem* and by 60% at 120 min *post mortem,* and Geddes and Rapson (1973) observed a 50% drop in liver glycogen over the first 90 min *post mortem.*

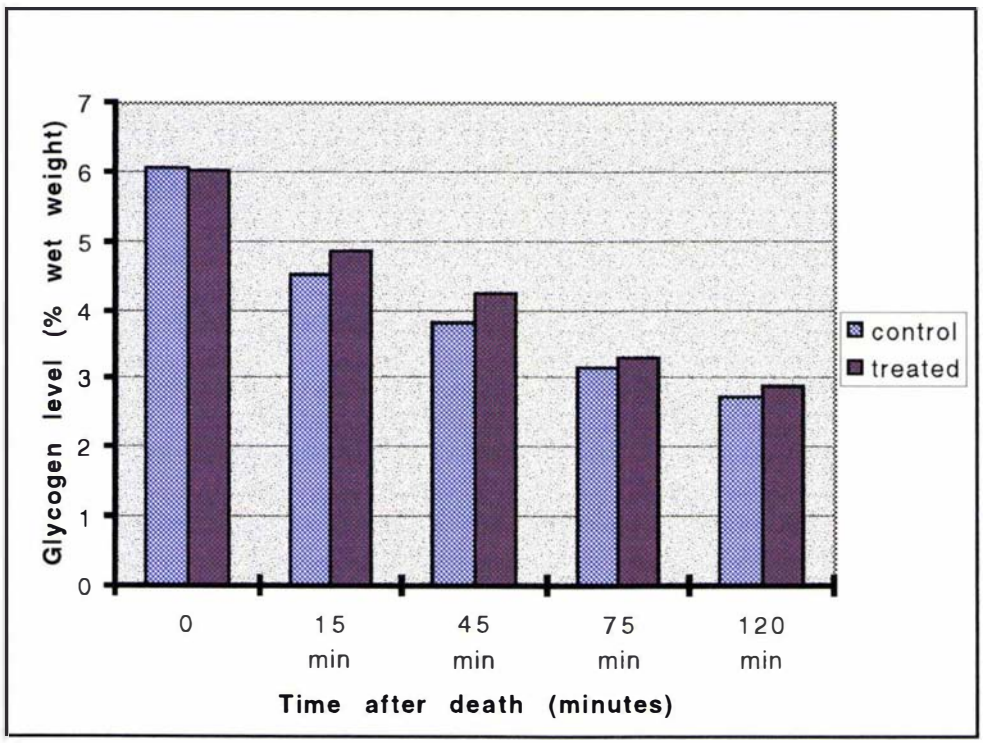

Figure 3.6 Effects of Ethanol on Post Mortem Liver Glycogen Levels

Rats were killed and left intact for various times before removal of the liver. Liver glycogen content was determined as described in section 2.3 and is expressed as g of glycogen per 100 g liver wet weight (%). Data are average results for two animals.

Table 3.9 Post Mortem Liver Glycogen Content

	Liver Glycogen Content			
Time after death	**Control** (% and μ mol/g wet weight)	**Depletion** (%)	**Treated** (% and μ mol/g wet weight)	**Depletion** (%)
0	6.06 (374)		6.02 (370)	
15	4.54 (280)	25	4.89 (301)	18
45	3.84 (237)	37	4.25 (262)	29
75	3.18 (196)	47.5	3.33 (206)	44.5
120	2.76 (170)	55	2.91 (180)	51

Rats were killed and left intact for various times before removal of the liver. Liver glycogen content was determined as described in section 2.3 and expressed as g of glycogen per 100 g liver wet weight (%) and μmol/g tissue wet weight (in parentheses). Data are average of results from two animals.

3.2.3.3 Muscle Glycogen Level After *post mortem* Degradation

The *post mortem* concentrations of skeletal muscle glycogen are shown in Figure 3.7 and Table 3.10. There was a rapid loss of glycogen. In control rats, the loss of glycogen was so rapid that within 15 min of death approximately 58% had been degraded, and two hours after death only 23% of muscle glycogen remained. Similar results were observed in treated animals, confirming that ethanol has no effect on *post mortem* glycogen degradation in muscle. These results are in good agreement with those of previous investigators (Calder and Geddes, 1990a).

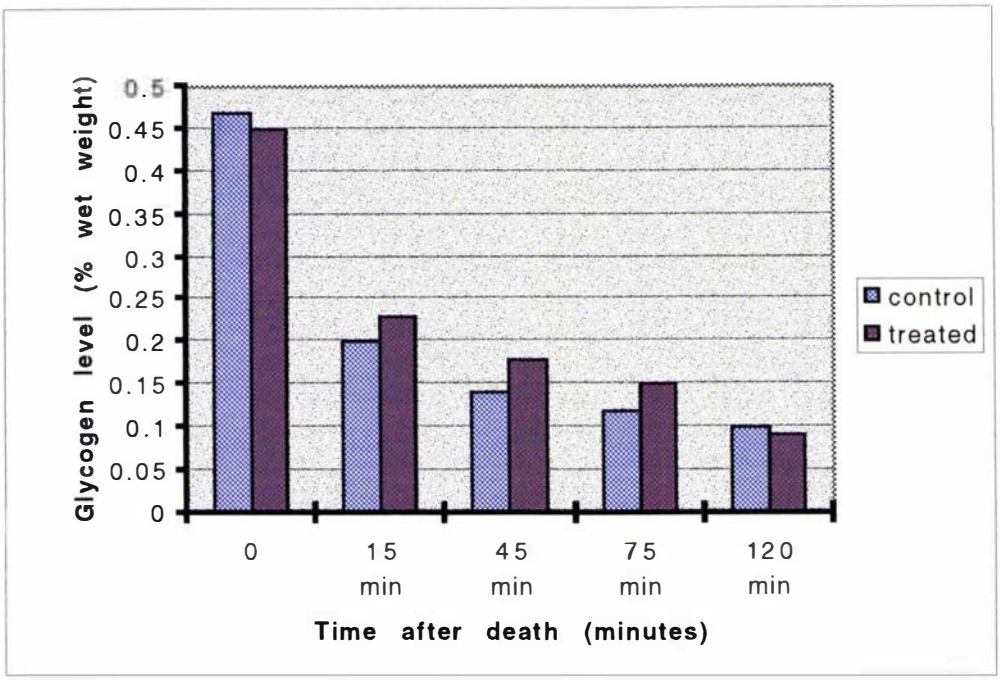

Figure 3.7 Effects of Ethanol on Post mortem Muscle Glycogen Levels

Rats were killed and left intact for various times before removal of the muscle. Muscle glycogen content was determined as described in section 2.3 and is expressed as g of glycogen per 100 g muscle wet weight (%). Data are average results for two animals.

Table 3.10 Post Mortem Muscle Glycogen Content

	Muscle Glycogen Content			
Time after death	**Control** (% and μ mol/g wet weight)	**Depletion** (%)	**Treated** (% and μ mol/g wet weight)	**Depletion** (%)
0	0.47 (29.4)		0.45 (28.2)	
15	0.20 (12.1)	58.5	0.23 (14.4)	49.0
45	0.14 (8.8)	70.0	0.18 (11.1)	61.0
75	0.12 (7.7)	73.5	0.15 (9.4)	66.5
120	0.10 (6.6)	77.0	0.09 (5.5)	88.5

Rats were killed and left intact for various times before removal of the muscle. Muscle glycogen content was determined as described in section 2.3 and expressed as g of glycogen per 100 g muscle wet weight (%) and μmol/g tissue wet weight (in parentheses). Data are average of results from two animals.

CONCLUSIONS

This chapter was designed to investigate effects of ethanol on glycogen content and molecular weight distribution in fed animals, taking into account *post mortem* glycogen degradation, metabolic inhomogeneity and separate low (cytosol) and high (lysosomal) molecular weight distribution.

It is well established that ethanol intake causes a hypoglycemic response in starved animals which is believed to be caused by ethanol-induced inhibition of gluconeogenesis. This is in opposition to an expected hyperglycemic response in fed animals. The reason for this is not well understood. We designed these experiments on the assumption that a single dose of ethanol would deplete liver glycogen which might lead to hyperglycemia. In the first part of the work described in this chapter, rats were given doses of ethanol of either 2, 4, or 6 g/kg. The lowest ethanol dose (2 g/kg) depleted liver glycogen content by 7-27% while the highest dose (6 g/kg) showed 60-78% depletion. Ethanol doses of 4 g/kg and 6 g/kg decreased both low and high molecular weight glycogen almost evenly. In the second part of the work described in this chapter, time course experiments were undertaken. Maximal glycogen depletion was observed at 90 minutes after an ethanol dose of 6 g/kg. After 24 hours, an increase in glycogen content was seen in ethanol treated animals. However, after 48 hours, liver glycogen content had returned to normal values in ethanol treated animals, although the content of low molecular weight was elevated relative to high molecular weight. These results demonstrate that acute treatment with ethanol causes significant changes in the liver glycogen content in fed animals. The depletion of liver glycogen by ethanol is a possible explanation of reduced stamina in sports performance due to high alcohol intake. Finally, experiments were conducted which showed that ethanol did not affect the rate of *post-mortem* glycogen degradation.

CHAPTER 4

THE EFFECTS OF ETHANOL ON GLYCOGEN CONTENT IN LIVER AND MUSCLE FROM STARVED AND STARVED - REFED RATS

4.1 Introduction

4.1.1 Liver Glycogen Content for Different Species in the Fed State

Normal liver glycogen levels in well-fed rats are in the range 5-8% of liver fresh weight, or approximately 300-500 μmol/g of tissue wet weight. Typical values obtained for different species are for rats 8% or 493 μmol/g (Freminet *et al.*, 1984), 5.4% or 333 μmol/g (Holness *et al.*, 1986), 4.7% or 290 μmol/g (Konishi and Fuwa, 1983), 6.6% or 407 μmol/g (Geddes and Taylor, 1985b), for dogs 6% or 370 μmol/g (de Bruijne and de Koster, 1983), for humans 5.4% or 333 μmol/kg (Hultman, 1978); for guinea pigs 9.6% or 592 μmol/g (Freminet *et al.*, 1984); and for rabbits 5.5% or 339 μmol/g (Geddes and Stratton, 1977b).

4.1.2 The Metabolic Response to Starvation

The metabolic response to starvation has been divided into various categories according to the length of time since the last intake of food:
(1) The interprandial phase, which is the period between meals in a normal day.
(2) The postabsorptive phase, which is the overnight fast period of 12 hours. This period may extend to 24 hours.
(3) The prolonged fast lasting longer than 24 hours, which may extend to several days or weeks.

An early response to carbohydrate deprivation is to maintain the blood glucose concentration in order to meet the energy requirements of the brain. In order to minimise the loss of glucose and maintain blood glucose levels, both glucose utilisation (uptake and phosphorylation) and oxidation in mainly oxidative tissues are reduced.

This metabolic response is supplemented by glucose production by hepatic gluconeogenesis and glycogen mobilisation mainly in the liver and skeletal muscle.

4.1.2.1 **The Fed-Fasting Transition**

When animals eat, glycogen synthesis is rapid, glycogen breakdown is slow, glycolysis is active, gluconeogenesis is minimal, fat synthesis is high, and fatty acid oxidation / ketone body production is switched off. After only a few hours of fasting, these situations are reversed. Glycogen synthesis ceases, glycogen breakdown is active, glycolysis is inactive, gluconeogenesis is activated, lipogenesis stops, and fatty acid oxidation and ketogenesis are switched on.

Figure 4.1 illustrates relative changes in metabolic parameters during the first 24 hours in the fed-to-starved transition. This period includes both the interprandial phase and post absorptive phase (see section 4.1.2). Transition from the fed to the fasted state is characterised by a sequence of metabolic alterations from gastrointestinal absorption (1-6 hours), to glycogenolysis (12-16 hours), then to gluconeogenesis (from 12 hours up to 1 week), and ketosis (3 days onwards). The length of the first phase (absorption phase) of fasting largely depends on the carbohydrate concentration of the preceding meal. After a predominantly carbohydrate-containing meal the liver will actively remove glucose in response to increased insulin secretion and suppression of glucagon release. Some of this glucose is incorporated into glycogen and some undergoes glycolysis to pyruvate and lactate. Insulin and glucagon regulate the concentration of cyclic AMP (Claus and Pilkis, 1981) which, in turn, initiates complex enzyme changes to inhibit glycogen synthesis and simultaneously activate glycogen breakdown and gluconeogenesis.

The liver in the fed state contains about 7% by weight of glycogen, about 150 g. Since the rate of carbohydrate utilisation in an average subject leading a normal life is between 100 and 200 g per day, liver glycogen cannot serve as a long-term fuel store but merely buffers variations for up to 24 hours. Glycogenolysis maintains a normal blood glucose for the first 12-16 hours of fast, then is largely superseded by gluconeogenesis (Felig, 1975).

A full description of the metabolic events occurring throughout starvation may be found elsewhere (Cahill, 1976; Szepesi, 1976; Owen *et al.*, 1976; Newsholme and Leech, 1983; White *et al.*, 1984; Sugden *et al.*, 1989).

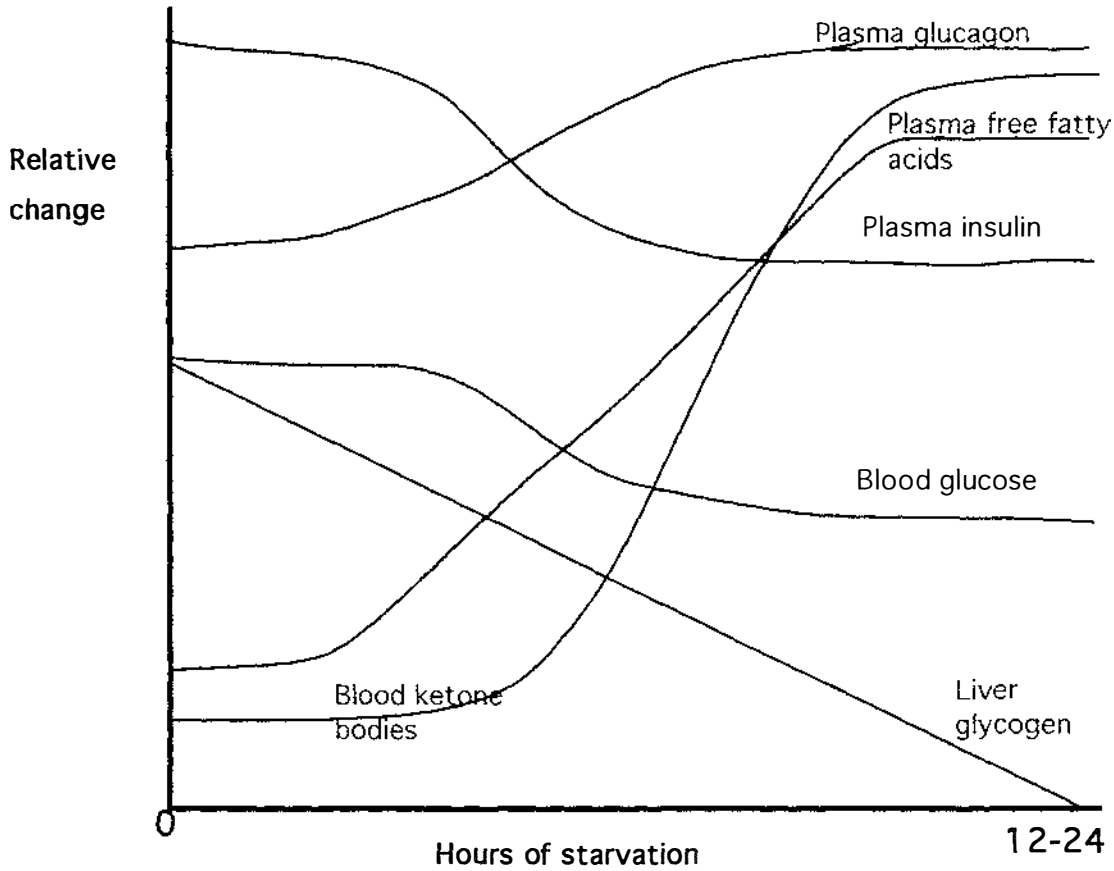

Figure 4.1 Relative Changes in Metabolic Parameters During the Onset of Starvation

As the animal passes from the fed to the fasting condition, glucose availability from food becomes less, and liver glycogen is drawn upon in an attempt to maintain the blood glucose. The concentration of insulin in the blood decreases, and glucagon increases. When glucose utilization diminishes in adipose tissue and the inhibitory effect of insulin on lipolysis becomes less, fat is mobilized as free fatty acids and glycerol. As the liver glycogen stores become depleted, blood glucose tends to fall. (Adapted from Harper's Biochemistry, 1990).

4.1.2.2 Quantitation of Hepatic Glycogenolysis and Gluconeogenesis in Fasting Humans

Hepatic gluconeogenesis and glycogenolysis are essential processes for maintaining plasma glucose during fasting. The relative contributions of these processes to glucose production have always been difficult to quantify in humans. Until recently, methods for measuring metabolic turnover depended largely on isotopic labelling of constituents or measurement of arterial-venous differences. Magnetic resonance spectroscopy is a newer technique with some advantages over other methods; the main advantage is that

hepatic glycogen concentration can be measured noninvasively at multiple time points (Iles and Cohen, 1987).

The rate of net hepatic glycogenolysis was assessed in humans by serially measuring hepatic glycogen concentration using ^{13}C NMR spectroscopy at 3 to 12 hour intervals during 68 hours starvation (Rothman *et al.*, 1991). The net rate of gluconeogenesis was calculated by subtracting the rate of net hepatic glycogenolysis from the rate of glucose production in the whole body. The volume of the liver was measured with magnetic resonance imaging on all subjects. Rates of total glucose production were assessed after administration of a primed continuous infusion of (6-^{13}H) glucose at 22, 43, and 67 hours starvation.

The surprising observation in this study was that the calculated net rate of hepatic glycogenolysis for the first 22 hours of fasting accounted for only 36% of total glucose production, while gluconeogenesis accounted for 64%. Such a finding conflicts sharply with previous calculations derived from isotopic uptake or splanchnic arteriovenous differences, which indicate a figure for the contribution to glucose production by glycogenolysis of greater than 65% after 12-14 hours of fasting (Wahren *et al.*, 1972; Consoli *et al.*, 1987).

The first measurement of glucose production in the study by Rothman *et al.* (1991) was at 22 hours, when glycogen stores are known to be depleted. They also used a relatively small sized evening meal (650 kcal) before the fast, which may have contributed to the higher gluconeogenic estimation. Additionally, to the extent that glucose cycling occurs in the liver and kidney, their measurements may overestimate rates of net hepatic and renal glucose production.

Other attempts have been made to determine the contribution of gluconeogenesis to glucose production in healthy subjects after an overnight fast and values have ranged from 25 to 70% (Consoli *et al.*, 1987; Björkman *et al.*, 1989; Magnusson *et al.*, 1992; Lee *et al.*, 1994; Gay *et al.*, 1994; Pimenta *et al.*, 1994; Hellerstein *et al.*, 1995; Landau *et al.*, 1996; Petersen *et al.* 1996). This variation is partly due to dietary preparation, but there is considerable variation in glycogen stores at the beginning of fasting even with the same dietary preparation (Magnusson *et al.*, 1992). Landau *et al.* (1996) have recently developed a new method for estimating contributions of gluconeogenesis to glucose production.

They found that after an overnight fast gluconeogenesis accounts for about 50%, and after 42 hours of fasting for almost 100%, of glucose production in healthy subjects. Similarly, Petersen *et al.* (1996) estimated that gluconeogenesis contributes approximately 50% to glucose production during the first 12 hours of fasting.

4.1.2.3 Glycogen Mobilization in Respose to Starvation

A. Liver

Glycogen depletion

Starvation for a time period of 6 hours leaves a hepatic glycogen reserve of approximately 40% of the fed value (Holness and Sugden, 1989). A period of starvation greater than 12 hours largely depletes the hepatic glycogen store (McGarry and Foster, 1980), leaving a reserve of 5% of the fed value after 24 hours (Holness and Sudgen, 1989). The most rapid rate of hepatic glycogen depletion in rats has been shown to occur at four to six hours into a fast (Holness and Sugden, 1989). After prolonged starvation (40 hours) Calder and Geddes (1992) observed a decrease of 11% in body weight and 42% in the weight of the liver of fasted rats. The level of glycogen storage can be depleted by up to 95% but does not seem to be depleted further by more prolonged starvation (see below).

Glycogen Rebound After 72 hours Starvation in Rats

After the depletion of glycogen during the first 2 days of starvation, glycogen re-synthesis occurs on day 3 of starvation and a small amount of reaccumulation is observed in the liver (Sasse, 1975; Bois-Joyeux *et al.*, 1987; Mouterde *et al.*, 1992; Minassian *et al.*, 1994). Despite the probable importance of this phenomenon, little is known about the gluconeogenic capacity and glycogen synthesis in the liver after 72 hours starvation. Glutamine is a good substrate for glycogen synthesis in hepatocytes from 72 hours starved rats, but not in those from 24 hours starved rats. This phenomenon seems to be related to an increased intracellular metabolism of glutamine which induces increased cell swelling and, in turn, an increase in the glycogen synthase activation after 3 days of starvation (Mouterde *et al.*, 1992). This increased synthase activation may explain why glutamine is a good substrate for glycogen synthesis in hepatocytes from 72 hours starved rats. Furthermore, it was proposed that glutamine may play a key role for the glycogen synthesis observed *in vivo* after 3 days of starvation, as a potential substrate for glycogen synthesis (Mouterde *et al.*, 1992).

Minassian *et al.*, (1994) have attempted to understand the mechanism of the rebound of glycogen stores in the situation *in vivo*. They concluded that rebound of glycogen does not involve activation of glycogen synthase or inhibition of glycogen phosphorylase. It could be dependent on the concentration of the precursor substrate of glycogenesis, glucose 6-phosphate, which is higher by about 45% in the liver of 72 and 96 hours fasted rats than in the liver of 48 hours fasted rats.

B. Muscle

Muscle is usually classified as skeletal, cardiac, or smooth muscle. Skeletal muscle makes up 40% of the body mass but can account for much more than 40% of the energy metabolism during exercise. Smooth muscle has very low metabolic activity while cardiac muscle preferentially oxidizes fatty acids and depends on aerobic metabolism.

Skeletal muscle is composed of several types of fibres and the biochemical properties of muscle fibres have important bearings on function. Type I or slow twitch fibres have a large number of mitochondria and highly active oxidative pathways such as ß-oxidation of fatty acids and the citric acid cycle. They have a very good blood supply and relatively lower levels of glycogen. Type II or fast twitch fibres are divided into two classes. Both classes have fewer mitochondria compared to Type 1. Type IIA fibres are oxidative and Type IIB are glycolytic. Oxidative metabolism in type IIB fibres is limited by poor blood supply. Type II fibres contain higher amounts of glycogen than Type I (Holness *et al.*, 1988c).

Skeletal muscle glycogen is mobilized in response to starvation. The normal fed glycogen levels of skeletal muscle are 40-50 μmol/g, but are dependent on a number of factors, including fibre composition (Newsholme and Leech, 1983). During starvation the full effect on the liver glycogen seems to occur within 15 hours, but the level of stored glycogen in muscle continues to drop even after 120 hours (Geddes and Chow, 1994). They reported that after 120 hours of starvation, muscle glycogen levels fell to 41% of their value in the well-fed animal. Both Conlee *et al.* (1976) and Holness *et al.* (1988b) have demonstrated glycogen depletion of between 23 and 49% for the different muscle groups after 48 hours of starvation. Calder and Geddes (1992) found depletion of 55% after 40 hours of starvation for hindlimb muscle.

Holness *et al.* (1988c) reported skeletal muscle glycogen levels ranging from 6.24 - 7.71 μg/mg wet weight (34-43 μmol/g) for various muscles types (Table 4.1). The most marked glycogen depletion after prolonged starvation (48 hours) was observed in

extensor digitorum longus and soleus compared with those muscles where depletion was more modest, plantaris, tibialis-anterior and gastrocnemius (Holness *et al.*, 1988c).

Skeletal muscle	Content of oxidative fibres (%)	Glycogen content (μg/mg wet weight)		Depletion of glycogen (%)
		Fed	Starved	
Type I Soleus	100	6.24	4.11	34.0
Type II				
Extensor digitorum longus	62	7.62	4.38	42.5
Plantaris	59	7.17	5.35	25.0
Tibialis anterior	68	7.36	5.66	23.0
Gastrocnemius	42	7.71	6.27	19.0

Table 4.1 Skeletal Muscle Glycogen Concentrations in Starvation (Data reproduced from Holness *et al.*, 1988c).

4.1.3 The Metabolic Response to Refeeding After Starvation

4.1.3.1 The Refeeding Transition in Liver

On refeeding, metabolism is quickly restored to the condition of the fed state. One of the characteristics of the hepatic response to carbohydrate refeeding after starvation is the suppression of net hepatic glucose output, and stimulation of glycogen deposition (Shikama and Ui, 1978; Sugden *et al.*, 1983; Newgard *et al.*, 1983; Claus *et al.*, 1984; Niewöehner *et al.*, 1984; Holness and Sugden, 1987; Shulman and Landau, 1992). After chow refeeding, portal glucose concentrations in rats increased nearly threefold (3.2 to 8.2 mM) within 1 hour of refeeding. The portal glucose levels peaked at 10 mM after 4 hours, remained elevated for a further 6 hours and subsequently declined to 7.2 mM 24 hours after refeeding (Holness *et al.*, 1988a).

The increased concentration of blood glucose on refeeding causes an increase in the release of insulin. This elevated insulin level causes the immediate cessation of hepatic gluconeogenesis in hepatic tissue and lipolysis in adipose tissue. The transition from the fasted to the fed state involves a reversal of the metabolic response to starvation, including alterations in phosphorylation states and concentrations of enzymes, as well as changes in the concentrations of key regulatory molecules (reviewed by Goodridge *et al.,* 1986; Granner *et al.*, 1986; Hod *et al.*, 1986; Tao and Towle, 1986; Pilkis *et al.,* 1988; Sugden *et al.*, 1989).

The response of the liver to a glucose load normally involves the sequential inactivation of glycogen phosphorylase and activation of glycogen synthase (Stalmans *et al.*, 1974; Halimi *et al.*, 1987; reviewed by Stalmans *et al.*, 1987). When the concentration of phosphorylase a has fallen below a critical level (approximately 10% of total activity), glycogen synthase phosphatase becomes active, with concomitant conversion of glycogen synthase b to glycogen synthase a. The threshold value of phosphorylase a for glycogen synthase activation is affected by the pre-existing glycogen concentration. It appears that in normal overnight fasted rats re-fed chow *ad libitum*, neither activation of glycogen synthase (Van de Werve and Jeanrenaud, 1984) nor inactivation of glycogen phosphorylase (Ciudad *et al.*, 1979) is obligatory for glycogen synthesis, which may be driven by an increase in substrate availability (a 'push mechanism'). For example, when fructose is provided as a glycogenic substrate, activation of glycogen synthase occurs in parallel with activation of phosphorylase (Stalmans *et al.*, 1987; Ciudad *et al.*, 1979).

4.1.3.2 **The Refeeding Transition in Muscle**

There is convincing evidence that muscle glycolysis and carbohydrate oxidation may not be immediately restored after refeeding. As in liver, the changes in enzyme activities and metabolic concentrations were slow and this may be attributed to the continued preferential utilization of lipid fuels (Sugden *et al.,* 1989). The repletion of skeletal muscle glycogen after refeeding is a quantitatively important aspect of carbohydrate disposal during the starved-to-fed transition.

There is no evidence for any delay in restoration of high rates of skeletal muscle glucose uptake assessed on the basis of rates of glycogen deposition after refeeding (Holness *et al.,* 1988c). Holness *et al.,* (1988c) examined the rates of glycogen deposition in some skeletal muscles in rats during the starved-to-refed transition. They reported that glycogen concentrations steadily increased during the first 8 hours after chow refeeding and the normal fed value was exceeded in all muscles. It appears that

rates of glycogen deposition after refeeding are directly proportional to the extent to which glycogen depletion has occurred during starvation (rather than being related to fibre type) (Holness *et al.*, 1988c). The relative contributions of glycogen synthesis and glycolysis/oxidation to total glucose utilisation following refeeding remain to be established.

4.1.3.3 **Effect on Glycogen Molecular Weight Distribution of Refeeding After Prolonged Starvation**

In the liver, prolonged starvation (40 hours) resulted in the mobilization of approximately 95% of the high molecular weight glycogen and approximately 98% of the low molecular weight glycogen (Calder and Geddes 1990b, 1992). Upon refeeding there was rapid resynthesis of glycogen of all size ranges, but there was initial oversynthesis of the high molecular weight glycogen. After 4 hours of refeeding, the total glycogen content of liver was about half of the fed level, whereas the level of high molecular weight glycogen had returned to the fed level. In contrast, at this stage of refeeding, low molecular mass glycogen was present at only 25% of the fed level. At this stage high molecular weight glycogen made up two-thirds of the glycogen present, compared with about 35% in the fed state. The oversynthesis of high molecular weight glycogen continued with further refeeding and at 13 hours and 14 hours the level was twice that observed in the fed state. After 13 hours of refeeding the low molecular weight glycogen had returned to the fed level. With further refeeding the low molecular glycogen also demonstrated oversynthesis and reached 1.5 times the fed level after 24 hours. By 48 hours of refeeding both high and low molecular weight glycogen contents had returned to those found in the fed state.

In skeletal muscle, prolonged starvation (40 hours) resulted in the mobilization of approximately 20% of the high molecular weight glycogen and 73% of the low molecular weight glycogen (Calder and Geddes, 1990b,1992). This resulted in a 2 fold increase in the ratio of high molecular weight to low molecular weight glycogen. After 30 minutes of refeeding, the glycogen content had returned to fed levels. However, as observed in the liver the high molecular weight glycogen was oversynthesised, resulting in levels 1.3 times that observed in the fed state. The high molecular weight at this time of refeeding represented 50% of the glycogen present, in comparison to approximately 35% in the fed state. After 4 and 13 hours of refeeding, the high molecular weight glycogen content had increased to 1.6 and 1.3 times the fed level respectively.

The low molecular weight glycogen of the skeletal muscles also exhibited oversynthesis. The fed level was reached after 4 hours of refeeding and 1.3 times the fed level was attained after 13 hours of refeeding. As observed in the liver, by 48 hours of refeeding the glycogen level and molecular weight distribution had returned to those of the fed state (Calder and Geddes, 1990b, 1992).

The inhomogeneity of glycogen synthesis with respect to molecular weight distribution upon refeeding may indicate the possibility of more than one site of synthesis within both liver and muscle cells (Calder and Geddes, 1990b, 1992).

4.1.3.4 **The Pathway of Glycogen Resynthesis After Refeeding**

Recent studies have cast doubt on the longstanding concept (Hers, 1955) that upon termination of a fasting period, dietary glucose is converted into glycogen, primarily via a direct pathway (Fig. 4.2) involving the following sequence: glucose ----> glucose-6-P ----> glucose-1-P ----> UDP-glucose----> glycogen. McGarry and co workers, in particular, propose that the bulk of liver glycogen is formed by an indirect pathway involving the sequence: glucose----> lactate----> glucose-6-P----> glycogen (Newgard *et al.* 1983, 1984b). In the indirect pathway, dietary glucose is taken up by peripheral tissues and metabolized to lactate. Lactate is transported to liver and converted to glucose 6-phosphate by the gluconeogenic pathway, and glucose 6-phosphate is then incorporated into hepatic glycogen (Fig. 4.3; see reviews by Foster, 1984; Pilkis *et al.*, 1985; Katz *et al.*, 1986; McGarry *et al.*, 1987; Landau and Wahren, 1988; Sugden *et al.*, 1989; Shulman and Landau, 1992). Therefore gluconeogenic flux contributes not only to hepatic glucose production but also to hepatic glycogen repletion in response to dietary carbohydrate intake after prolonged starvation.

The extent to which glucose (via direct pathway) and C3 derivatives of glucose (via the indirect pathway) are used as precursors for hepatic glycogen synthesis after refeeding was controversial (Huang and Veech, 1988; Lang *et al.*, 1986; Landau and Wahren, 1988; Rognstad, 1989; Sugden *et al.*, 1989; Shulman and Landau, 1992). However, it is now generally recognised that a substantial proportion (up to 70%) of the liver glycogen deposited in response to carbohydrate feeding is synthesised via the indirect pathway. A major question remaining unanswered is the location of synthesis of the C3 precursor (presumably lactate).

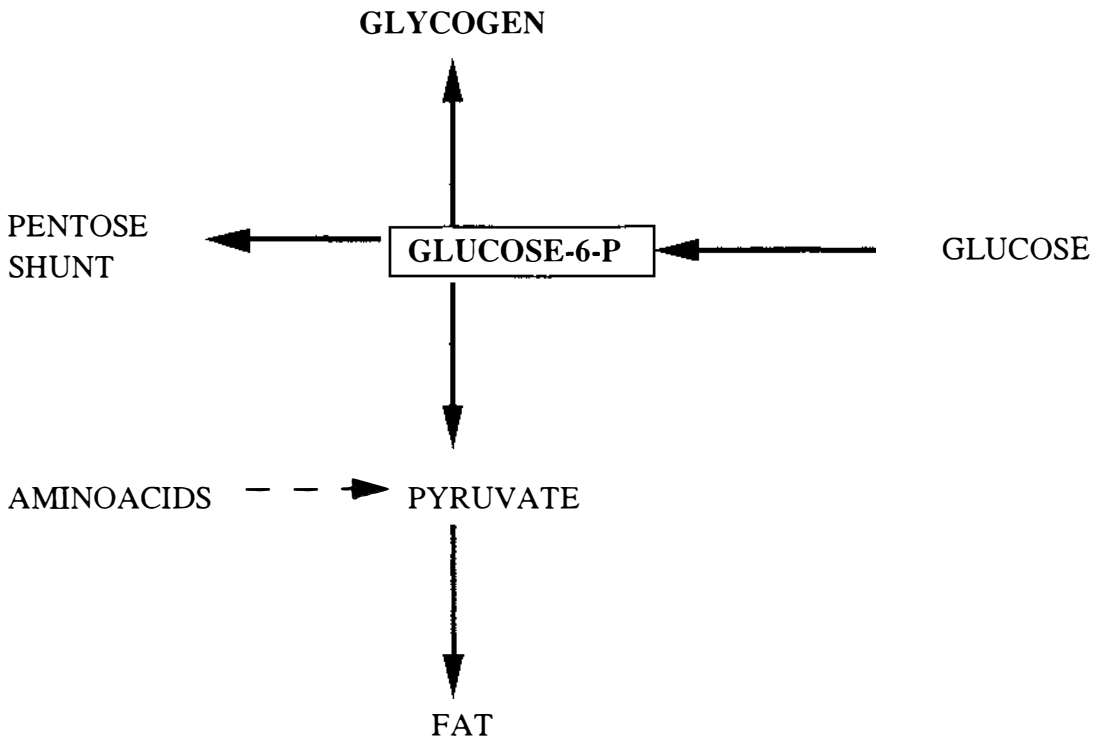

Figure 4.2 Hepatic Glucose Metabolism After Refeeding-Classic view or direct pathway) (Redrawn from Foster, 1984; McGarry *et al.*, 1987).

The traditional interpretation is that refeeding of glucose is accompanied by uptake of glucose by the liver, followed by phosphorylation, and incorporation into glycogen.

The primary site of disposal of a dietary glucose load is controversial and the conversion of dietary glucose to lactate and related 3-carbon metabolites is presumed to occur at a number of sites (see reviews by McGarry *et al.*, 1987; Shulman and Landau, 1992), including the gastrointestinal tract (Windmueller, 1980; Smadje *et al.*, 1988), muscle, skin (Kelley *et al.*, 1988; Cox and Palmer, 1988), central nervous system and erythrocytes (Palmer *et al.*, 1991).

However, there is considerable evidence that much glucose is taken up by the liver (Huang and Veech, 1988; Kelley *et al.*, 1988) and that hepatic lactate production occurs in the post-prandial period. It has been proposed that dietary glucose is metabolised by the liver, skeletal muscle and other tissues (mainly brain) in approximately equal proportions (Huang and Veech, 1988; Elia *et al.*, 1988).

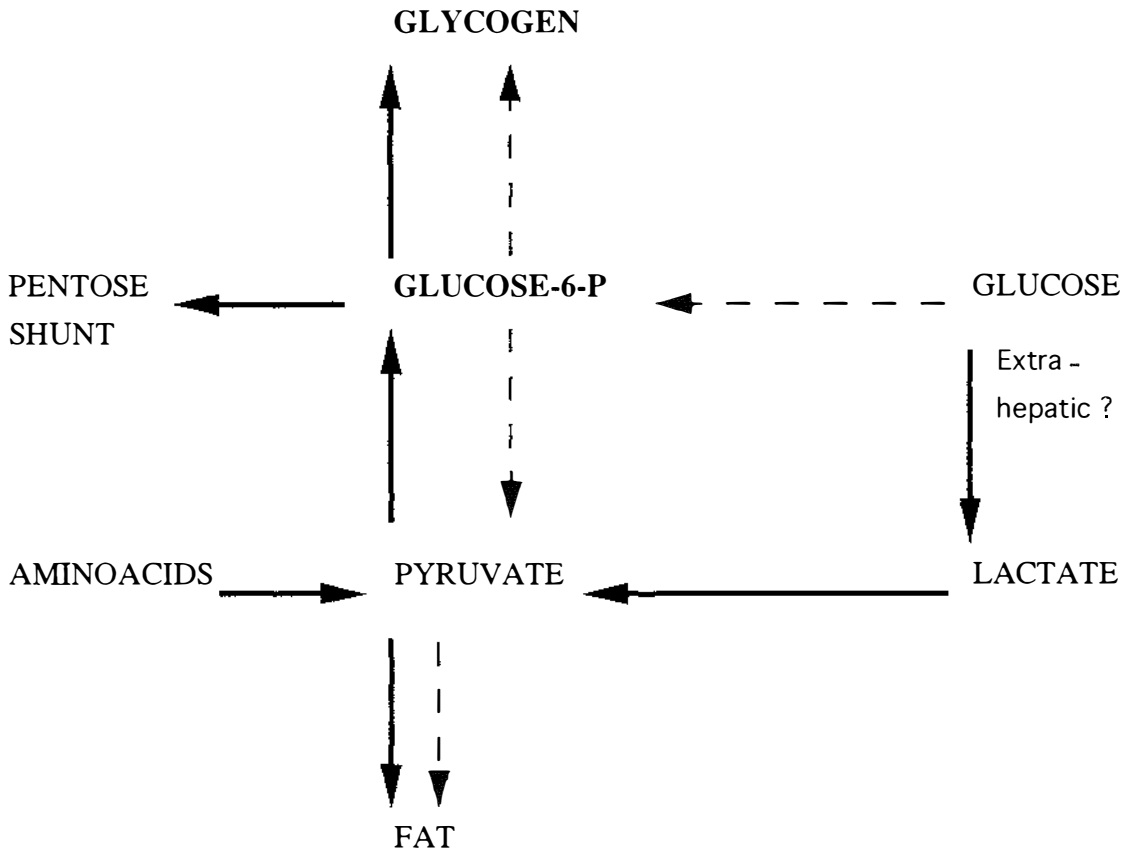

Figure 4.3 Hepatic Glucose Metabolism After Refeeding-Indirect pathway
(Redrawn from Foster, 1984; McGarry *et al.*, 1987).

Direct uptake of glucose by the liver is considered limited and glycogen is formed from glucose only indirectly after metabolism to lactate. Although hepatic glucose output falls abruptly, gluconeogenic carbon flow continues for a period of several hours . In the indirect pathway, dietary glucose is taken up by peripheral tissues and metabolized to lactate. Lactate is transported to liver and converted to glucose 6-phosphate by the gluconeogenic pathway, and glucose 6-phosphate is then incorporated into hepatic glycogen.

Studies on cultured adult (Spence and Koudelka, 1985; Salhanick *et al.*, 1989) and foetal (Bismut and Plas, 1989; 1991) rat hepatocytes have shown that the classical direct pathway of glucose incorporation into glycogen functions concomitantly with the indirect one mediated by triose phosphate (C3) formation, and that both direct and indirect pathways are stimulated by insulin.

Studies *in vivo* based on isotopic techniques have yielded conflicting estimates (10 - 88%) for the contribution of the indirect pathway. Attempts have been made to quantitate the extent of the indirect pathway of glycogen formation from three carbon precursors by:

(1) Randomization of the carbon label in glycogen (Shulman *et al.,* 1985; 1987; Newgard *et al.,* 1984b; Scofield *et al.*, 1985).
(2) Comparison of the $^{3}H/^{14}C$ ratio in liver glycogen with that of mixtures of administered (3-^{3}H) and (U-^{14}C) glucose (Huang and Veech, 1988; Newgard *et al.*, 1983; Lang *et al.*, 1986).
(3) Comparison of the specific activity of (^{14}C) glucose with that of glycogen (Newgard *et al.*, 1983; Lang *et.al,* 1986; Kalderon *et al.*, 1986).
(4) The incorporation of ^{3}H into glycogen from $^{3}H_2O$ (Postle and Bloxham, 1980; Kuwajima *et al.,* 1986).

The use of all of these isotopic methods to determine the rate of hepatic glycogen synthesis has been criticised because of uncertainty as to the extent by which dilution of label could have occurred via the Krebs cycle (Landau and Wahren, 1988). Discrepancies may also be related to differences in experimental procedures, particularly (a) the dietary state of the animal, (b) the size and form of the glucose load, (c) the route of the administration and (d) the analytical methods used.

The importance of gluconeogenesis for glycogen synthesis suggests that the liver must take up lactate and pyruvate after refeeding. However, this is apparently not the case since the liver releases net lactate and glycolysis appears to be enhanced (Sacca *et al.*, 1984; Williamson *et al.*, 1985; Holness *et al.,* 1986; Davis *et al.*, 1987). One possible explanation for the fact that liver produces and uses lactate at the same time is provided by metabolic zonation (see review by Jungermann and Katz, 1989). Hepatocytes from the periportal and perivenous regions of the liver have differing amounts of key enzymes, which has led to the proposal that the two regions perform different functions (Katz, 1976; Gumucio et al., 1981: see review by Jungermann and Katz, 1989). The lactate formed from glucose and released into the hepatic vein by perivenous hepatocytes is converted into glycogen in the periportal cells (Bartels *et al.,* 1987).

In contrast to the liver glycogenesis in muscle normally appears to occur via the classical direct pathway (Newgard *et al.*, 1984).

4.1.3.5 Hepatic Glycogen Synthesis and Fructose 2, 6-bisphosphate Concentrations After Refeeding

Fructose 2,6-bisphosphate is thought to be the primary regulator of glycolysis and gluconeogenesis in the liver (Hers and Hue, 1983), inhibiting gluconeogenesis and activating glycolysis. The concentration of fructose 2,6-bisphosphate in the liver is high during feeding and falls with fasting.

Fructose 2,6-bisphosphate is a potent stimulator of 6-phosphofructo-1-kinase, a key regulatory enzyme of glycolysis (see review Pilkis *et al.*, 1988; Pilkis and Granner, 1992). The concentration of fructose 2,6-bisphosphate (F 2,6-bis P) increased with refeeding only after the glycogen stores were largely repleted (Kuwajima *et al.*, 1984; Holness *et al.*, 1988a; 1988b). In the liver, glycolytic flux from glucose was negligible at a F 2,6-bis P concentration less than 5 nmol/g (Hue *et al.*, 1984). This concentration was only reached after 80% of the maximum glycogen concentration had been achieved (Kuwajima *et al.*, 1984; Holness and Sugden, 1988a). The maintenance of low F 2,6-bis P concentrations was observed for the first 4 hours of refeeding (Holness and Sugden, 1988a). After 6 hours of refeeding, a decline in the rate of glycogen synthesis, based on ^{3}H incorporation from $^{3}H_2O$, into glycogen, was observed in rats refed *ad libitum* after prolonged starvation (Holness and Sugden, 1988a). This may be the result of an increase in the utilization of glucose for glycogen synthesis via the direct pathway (Holness and Sugden, 1988a).

4.1.4 The Effects of Ethanol on Tissue Glycogen Content in the Fasted State, and After Fasting and Refeeding

There are few studies available on the effects of ethanol on tissue content of glycogen in the starved state. All of the studies published have been performed in fed or in fasted and refed animals. Cook *et al.* (1988) demonstrated that intragastric administration of ethanol (75 mmol/kg body wt, 3.45 g/kg) 1 hour before glucose refeeding of rats starved for 24 hours inhibited hepatic glycogen synthesis (by 70%) and deposition (by 69%), but was without significant effect on muscle glycogen deposition and synthesis. Furthermore, they found that administration of 4-methylpyrazole (an inhibitor of alcohol dehydrogenase) to ethanol-treated rats did not significantly diminish the inhibitory effect of ethanol on hepatic glycogen deposition after glucose refeeding,

indicating that the inhibition was not dependent on ethanol metabolism. They concluded that ethanol impairs hepatic glycogen repletion by inhibition at at least two distinct sites, namely (a) intestinal glucose absorption and (b) hepatic gluconeogenic flux.

Xu *et al.* (1992) examined the effects of ethanol on the pattern of glycogen deposition in individual cardiothoracic and skeletal muscles in response to oral and intraperitoneal glucose administration in rats starved for 40 hours. Rates of glycogen synthesis were consistently higher in oxidative muscles than in non-oxidative muscles. Intragastric ethanol administration was associated with an impaired glucose metabolism and the almost total abolition of glycogen deposition in oxidative muscles in response to oral or intraperitoneal glucose refeeding. This effect was dose-dependent and differential, in that ethanol produced no equivalent impairment in glycogen deposition in non-oxidative muscles. Ethanol treatment also selectively promoted glycogenolysis in oxidative muscles in the starved state. The selectivity of this impairment in glycogen metabolism was not observed in previous studies by the same research group (Cook *et al.*, 1988) since they examined effects of ethanol on glycogen deposition in mixed (thigh and calf muscles) as opposed to individual muscles. In the study by Xu *et al.* (1992) glycogen levels in oxidative muscles at 2 hours after intraperitoneal glucose administration were decreased in a dose-dependent manner by ethanol: in soleus muscle, levels were decreased by 20%, 28%, 39%, 74% and 79% at ethanol doses of 10, 20, 40, 60 and 75 mmol/kg body weight respectively, whereas in diaphragm glycogen levels were decreased by 12%, 31%, 63% and 66% at ethanol doses of 20, 40, 60 and 75 mmol/kg body weight respectively. Ethanol, irrespective of dose, produced no decrease in glycogen levels in plantaris or tibialis anterior, or the other non-oxidative muscles, or heart.

More recent research from Xu *et al.* (1993) examined the mechanism of the ethanol-induced impairment in glycogen synthesis in oxidative skeletal muscles. 4-Methylpyrazole, a potent inhibitor of alcohol dehydrogenase, potentiated the ethanol-mediated impairment in glycogen deposition in oxidative muscles and was associated with abnormalities in glycogen deposition in non-oxidative muscles. By contrast, disulfiram, a potent inhibitor of aldehyde dehydrogenase, had no effect on the ethanol-mediated impairment in glycogen deposition in both oxidative and non-oxidative muscles. They concluded that it is the ethanol molecule itself, and not one of its metabolites (acetaldehyde, acetate or NADH), that mediates the defect in glycogen metabolism.

4.1.5 **Aims of this Section**

Although there have been some studies of the effect of ethanol on glycogen metabolism in rats (see review by Palmer *et al.*, 1991), relatively little is known about the effect of ethanol on glycogen synthesis in skeletal muscle and liver following refeeding after starvation in the rat. A few studies have been performed recently in which ethanol was demonstrated to inhibit hepatic glycogen resynthesis (Cook *et al.*, 1988; Palmer *et al.*, 1991) and muscle glycogen resynthesis (Xu *et al.*, 1992) after oral glucose load given after 40 hours starvation. However, there have been no studies of the effects of ethanol on glycogen synthesis following chow refeeding after starvation. Chow refeeding is more analogous than oral glucose administration to the situation which would be encountered in human cases, where heavy alcohol intake occurs at the same time as food intake after a period without food. In addition, there are no data available about the effects of ethanol on the tissue content of glycogen in the starved state. All previous studies have been performed in fed or in fasted and refed animals.

The primary aim of this study was to investigate whether ethanol inhibits tissue glycogen deposition in response to chow refeeding after starvation taking into account low molecular weight (cytosolic) and high molecular weight (lysosomal) glycogen. In addition, experiments were designed to investigate whether ethanol affects tissue glycogen content in starved rats.

4.2 Methods

Animals were housed as described in Chapter 2, section 2.2.1, and ethanol was administered, animals were killed, and tissue samples were taken as described in section 2.2.3.

4.2.1 Effect of Starvation on Tissue Glycogen Content: Experimental Protocol.

In this experiment, rats were starved for 0, 24, 48 and 72 hours. Two rats were killed at each time, so that we could establish the level of glycogen depletion at various times during starvation.

4.2.2 Effect of Ethanol on Tissue Glycogen Content during Starvation: Experimental Protocols.

A single dose of ethanol, 4 g/kg in 4 ml of aqueous solution, was administered. After the ethanol dosing, two rats were housed in each grid-bottomed cage and were allowed access to water only.

Three different protocols were used in this part of the study.

(1) Fed rats were administered a dose of 4 g/kg ethanol, then starved and sacrificed after 24 and 48 hours. Two control rats and two treated rats were killed after each time interval.

(2) Fed rats were administered a dose of 4 g/kg ethanol, then starved and sacrificed after 3, 6, 12, 24 and 48 hours. Two control rats and two treated rats were killed after each time interval.

(3) Fed rats were administered a dose of 4 g/kg ethanol, then starved and sacrificed after 6, 12, and 24 hours. Two control rats and two treated rats were killed after each time interval.

4.2.3 Effect of Ethanol on Tissue Glycogen Content After Starvation and Refeeding: Experimental Protocols.

Two different protocols were used in this part of the study.

Protocol 1: Effect of ethanol on glycogen resynthesis after an oral glucose load.

In this experiment, 12 rats were selected and starved for 24 hours. At 1 hour after administration of intragastic ethanol (4 g/kg) rats were given an oral glucose load (2 mmol/100 g body weight) or water (1 ml/100 g body weight). Animals were killed for tissue sampling at 30, 60, 90, 120, 150 and 180 minutes after the glucose load. This protocol was the same as that used by Cook *et al.* (1988).

We decided to do this experiment to check the results of Cook *et al.* (1988) who used the above protocol for ethanol and glucose administration. However, Cook *et al.* (1988) killed the rats under light diethyl ether anaesthesia and took a considerable time for sample preparation, which, we believed, may have affected their results.

Protocol 2: Effect of ethanol on glycogen resynthesis after voluntary chow refeeding.

After 48 hours fasting the rats were given an intragastric ethanol dose of 4 g/kg or 6 g/kg and then free access to food. Animals were killed for tissue sampling at 5, 10, 24 and 48 hours after the ethanol dose.

We performed the whole experiment three times. For one experiment using a 6 g/kg ethanol dose, 20 rats were selected. However, the 6 g/kg ethanol dose, in combination with starvation, was detrimental to their health because four rats did not survive the initial ethanol dose, and those that did survive did not eat well. Therefore, the experiment was continued using 16 rats. Two control rats and two treated rats were killed after each time interval except at 5 hours, where three control and three treated rats were killed. In addition, two well-fed rats were killed at 0 hour.

Two experiments were carried out using an ethanol dose of 4 g/kg, with no adverse effects on the animals. 20 rats were used in each experiment, with two control and two treated animals being killed after each time interval except at 5 hours, when three rats were killed to purify sufficient glycogen for molecular weight analyses. In addition, two well-fed rats were killed at 0 time.

4.3 RESULTS AND DISCUSSION

4.3.1 Effect of Starvation Upon Body Weight and Liver Weight

Effects of starvation upon body weight and liver weight are shown in Table 4.2. Prolonged Starvation for 48 hours resulted in a 12.5% reduction in body weight and a 40% reduction in liver weight (Table 4.2). The liver accounted for 3.7% of body weight in the fed rat, in agreement with other studies (Lutkic *et al.*, 1974; Hebel and Stromberg, 1976; Fréminet *et al.*, 1984; Clark *et al.*, 1985; Geddes and Taylor, 1985 a,b; Calder and Geddes, 1992). After 48 hours starvation liver accounted for only 2.5% of body weight (Table 4.2).

Table 4.2 Effect of Starvation on Body Weight and Liver Weight

	Fed	**Starved (48 hours)**
Body weight	289 ± 6 (n=12)	256 ± 7 (n=12)
Liver weight	10.6 ± 0.2 (n=12)	6.4 ± 0.4 (n=12)

Data are means ± SEM for the number of animals shown in parentheses.

4.3.2 Effect of 48 hours Starvation on Tissue Glycogen Content

Table 4.3 shows the effect of 48 hours of starvation on tissue glycogen content. The data obtained from control animals in several experiments have been grouped to provide average values to compare with those in the literature. The hepatic glycogen levels in well-fed rats were 362 ± 17 μmol/g, in agreement with previous investigators (see Table 3.2).

Table 4.3 Effect of Starvation on Tissue Glycogen Content

	Glycogen content (μmol/g tissue wet weight)	
Tissues	**Fed**	**Starved (48 hours)**
Liver	362 ± 16 (n=12)	40.5 ± 09 (n=10)
Muscle	42 ± 5 (n=10)	16.9 ± 2.8 (n=10)

Glycogen content was determined as described in Section 2.3 Data are means ± S.E.M for the number of animals shown in parentheses.

After 48 hours starvation, the average remaining liver glycogen was 40 μmol/g wet weight, about 0.7% of the liver weight (Table 4.3). Other investigators have reported results in the range of 0.1-0.5% tissue weight (Lutkic and Fister, 1969, 1972; Cardell *et al.*, 1973; Boyd *et al.*, 1981; Fréminet *et al.*, 1984; Holness *et al.*, 1986; Geddes and Taylor, 1985b; Calder and Geddes, 1992).

Depletion of liver glycogen reserves during starvation would be expected to contribute to the reduction in the size of the organ, because glycogen makes up about 6-7% of liver wet weight in normal well-fed animals. However, the drop in liver weight was higher than 6-7%, probably due to water loss and protein breakdown.

Table 4.3 also shows that the skeletal muscle glycogen content was 42 μmol/g of tissue wet weight in the well-fed rat, 0.6% of the tissue weight. Starvation for 48 hours depleted the muscle glycogen content by 60% (Table 4.3), which is less than the 90-95% depletion observed in liver tissue. This result is in good agreement with those observed by other researchers (Table 4.4). Conlee *et al.* (1976) reported that 24 hours of starvation caused rat muscle glycogen to fall by 23-49%; Fréminet *et al.* (1984) demonstrated that 48 hours of starvation depleted muscle glycogen level by 45%; Calder and Geddes (1992) found that after 40 hours starvation the skeletal muscle concentrations were decreased by 55% (Table 4.4).

Table 4.4 Literature Values for Muscle Glycogen Content Following Starvation

Type of muscle	Fed (μmol/g)	Starved (μmol/g)	Reference
Hind limb	39.5	18.5 (40 h)	Calder and Geddes, 1992
Hind limb	35.0	14.8 (120 h)	Geddes and Chow, 1994b
Quadriceps	43.0	27.0 (48 h)	Winder *et al.*, 1995
Thigh muscle	37.0	18.5 (48 h)	Fréminet and Leclerc, 1980
4 types of muscle	52 - 40	40.7 - 20.3 (24 h)	Conlee *et al.*, 1976
4 types of muscle	47.8 - 38.2	25.3 - 38.2 (48 h)	Holness *et al.*, 1988c

A comparison of glycogen content in different muscle types after various starvation times. Rats were starved as indicated by the hours shown in parentheses.

4.3.3 Effects of Different Starvation Times on Liver and Muscle Glycogen Content.

Figure 4.4 and Table 4.5 and show the values of liver glycogen content of rats fasted for 0, 24, 48 and 72 hours. The liver glycogen content of rats starved for 24 hours was lower than the amount of glycogen present in the liver of rats fasted for 48 hours. Surprisingly, the livers of rats fasted for 72 hours contained even more glycogen than after 48 hours.

Although other researchers have done experiments starving rats for up to 96 hours, we did not wish to starve rats for longer than 72 hours for ethical reasons. Similar results to those above were recorded by Minassian *et al.* (1994) who reported that the liver of 72-hour and 96-hour fasted rats contained more glycogen (about 5 mg/g, 28 μmol/g, wet weight) than the livers of 24 and 48 hours fasted rats (about 2 mg/g, 11 μmol/g). This phenomenon has received little attention up to now, although it could be the result of a metabolic adaptation to fasting of physiological importance.

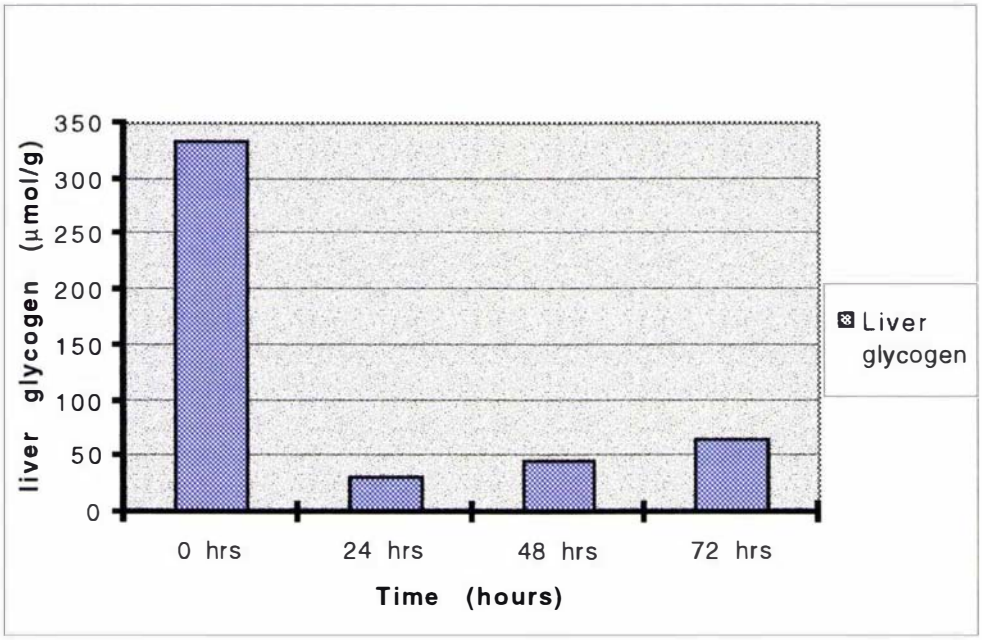

Figure 4.4 Effect of Starvation on the Liver Glycogen

Rats were starved for the times indicated. Two rats were killed at each time. Glycogen content was determined as described in section 2.3.

Table 4.5 Effect of Starvation on Liver Glycogen Content

Fasting time (hours)	Glycogen content (µmol/g wet weight)	Mean	Glycogen depletion (%)
0	337.0, 330	333.3	0
24	35.1, 27.0	31.0	91
48	47.2, 43.2	45.2	86
72	72.4, 58.5	65.4	80

Rats were starved for the times indicated. Two rats were killed at each time. Glycogen content was measured as described in section 2.3.

In our experiments, in starved rats, glycogen levels had decreased to 31 µmol/g after 24 hours starvation, increased to 45 µmol/g after 48 hours starvation and further increased to 65.4 µmol/g after 72 hour starvation whereas Minassian *et al.*, (1994) reported that glycogen levels had fallen to 11 µmol/g after 24 and 48 hours starvation and increased to 28 µmol/g after 72 and 96 hours. Their values for liver glycogen are considerably lower than our results, which may be due to different diet composition, mode of killing and analytical methods. In their experiments, fed and fasted rats were anaesthetized by a single injection of pentobarbital, 7 mg/100 g body weight. They waited for 15 minutes for the anaesthetic to act, then killed the rats.

Results for the effect of starvation for different times on muscle glycogen content are shown in Figure 4.5 and Table 4.6. The skeletal muscle (hindlimb) glycogen was 35 µmol/g tissue wet weight in the fed rats. 24 and 48 hours starvation resulted in a 60% and 54% fall in muscle glycogen content and there is not a noticeable difference between 24 and 48 hours starvation. Our results at 48 hours are almost identical to those reported by Calder and Geddes, (1992) at 40 hours (see Table 4.4). After 72 hours starvation the glycogen content was depleted by 49% (Table 4.6).

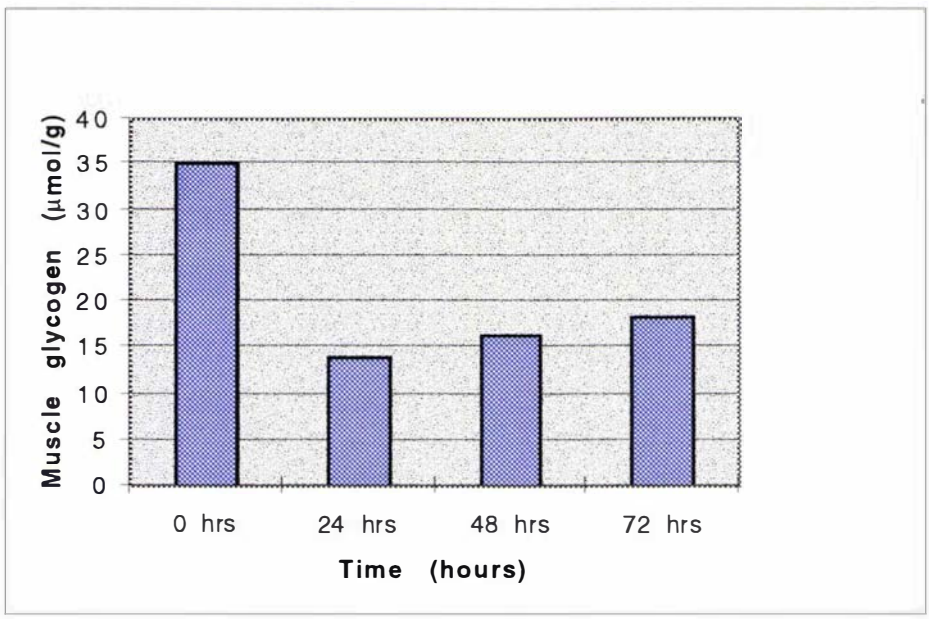

Figure 4.5 Effect of Starvation on the Muscle Glycogen

Rats were starved for the times indicated. Two rats were killed at each time. Glycogen content was measured as described in section 2.3.

Table 4.6 Effect of Starvation on Hind Limb Muscle Glycogen Content

Fasting time (hours)	Glycogen content (μmol/g wet weight)	Mean	Glycogen depletion (%)
0	37.4, 32.8	35.1	0
24	17.1, 10.8	13.9	60
48	15.8, 16.6	16.2	54
72	18.4, 18.0	18.2	49

Rats were starved for the times indicated. Two rats were killed at each time. Glycogen content was measured as described in section 2.3.

As we observed in the liver, after 72 hours starvation glycogen rebound (resynthesis) was observed in muscle. In contrast to the liver, where all hepatic glycogen reserves are mobilised to provide blood glucose during mild starvation and 48 hours is sufficient to deplete them almost totally, muscle glycogen stores are not as extensively depleted after this length of starvation. During this period of starvation it is likely that muscle glycogen reserves are spared to a certain extent by the use of fatty acids mobilised from adipose tissue, and by glucose from the blood (originating from liver glycogen and gluconeogenesis).

4.3.4 Effects of Ethanol on Tissue Glycogen Content in Starvation

4.3.4.1 Effects of Ethanol on Liver Glycogen Content in Starved Rats

Figure 4.6 and Table 4.7 show the effects of a single dose of ethanol on the liver glycogen content of rats fasted for 3, 6, 12, 24 and 48 hours. The results for 3 separate experiments, outlined as protocols 1, 2 and 3 in Section 4.2.2 have been combined.

Results for control rats showed that considerable glycogen depletion (approximately 100 μmol/g) had occurred between 3 and 6 hours of fasting. Holness and Sugden (1989) observed that the most rapid rates of depletion of hepatic glycogen occurred over a relatively short period of 4-6 hours duration, and depletion was almost complete after 24 hours of fasting. In our experiments, glycogen content fell most rapidly between 6 and 12 hours fasting, by about 255 μmol/g.

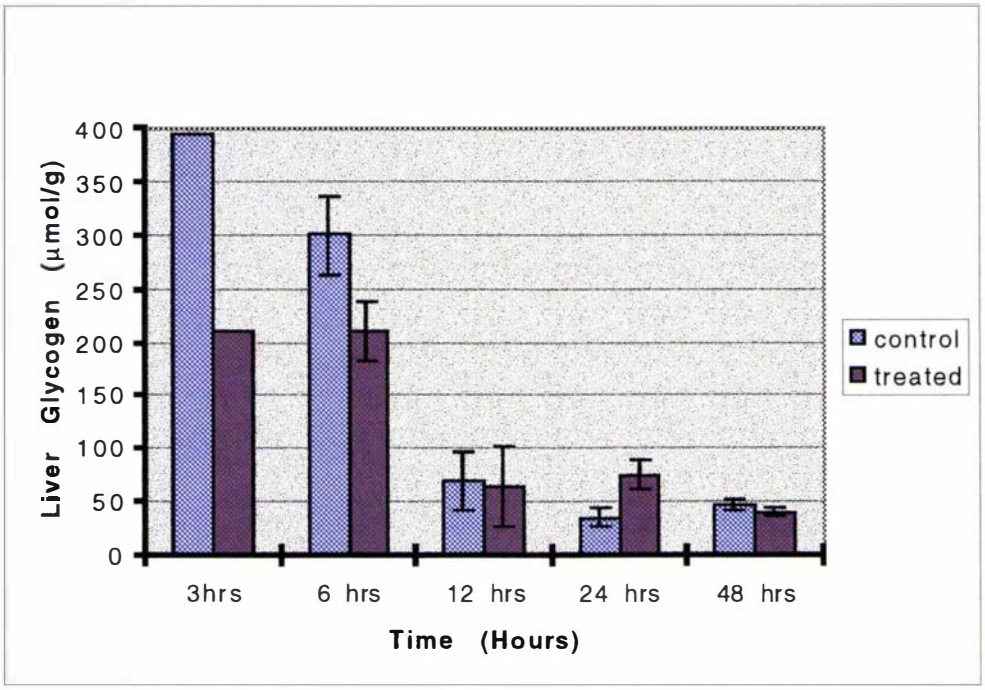

Figure 4.6 Effects of Ethanol on Liver Glycogen Content After Starvation

An ethanol dose of 4 g/kg or an equivalent volume of water was administered. Thereafter rats were allowed access to water only for up to 48 hours. The animals were sacrificed at the times indicated after treatment. Liver glycogen content was determined as described in section 2.3. Numbers of animals were as for Table 4.7.

Table 4.7 Effects of Ethanol on Liver Glycogen Content After Starvation

Fasting time (hrs)	Control (µmol/g wet weight)	Treated (µmol/g wet weight)
3	394.0 (n=2)	212.6 (n=2)
6	301.2 ± 36.4 (n=4)	211.6 ± 28.0 (n=4)
12	71.0 ± 27.2 (n=4)	65.2 ± 37.9 (n=4)
24	35.8 ± 8.4 (n=6)	76.5 ± 14.5 (n=6)
48	47.0 ± 4.9 (n=4)	41.4 ± 3.2 (n=4)

Rats were treated with ethanol 4g/kg or water (control) at time 0 as described in section 4.2.2. Glycogen content was measured as described in section 2.3. Data are mean ± SEM for the number of animals shown in parentheses.

In ethanol treated rats, after 3 hours starvation, glycogen content was depleted to almost half of the fed levels (about 235 μmol/g). A similar level, rather than a continuing decrease as in controls, was observed after 6 hours fasting. There was a sharp drop between 6 and 12 hours and at 12 hours the liver glycogen contents in control and ethanol treated rats were similar (Figure 4.6 and Table 4.7). At 24 hours, glycogen content increased slightly in the ethanol-treated rats, with the liver content being more than twice that in the control animals. The initial ethanol-induced depletion of liver glycogen content is similar to that observed for fed rats in Chapter 3. In fed rats, the depletion is followed by a large over-synthesis of glycogen at 24 hours. In starved rats, with no food intake during the 24 hours after the ethanol dose, an oversynthesis effect was not expected. However, the liver glycogen content did show an increase, although not large, between 12 and 24 hours in ethanol-treated, starved animals.

Ethanol-induced glycogen retention in starved rats has not been reported before. At 24 hours after ethanol administration, it is likely that the ethanol has been metabolized completely, so the increased liver glycogen content in treated animals at 24 hours must be due to effects of the presence of ethanol at earlier times. It is possible that lipolysis is stimulated by ethanol under the conditions of starvation. Increased lipolysis in adipose tissue generates glycerol, which could act as a gluconeogenic substrate. Other non-carbohydrate precursors could also contribute to net gluconeogenesis. During starvation, ethanol may accelerate the release of amino acids from skeletal muscles and uptake of glucogenic amino acids could occur in the liver, leading to an increase in gluconeogenesis and hence glycogen synthesis after 24 hours starvation. In addition, glycogen converted to glucose in treated rats at 3-6 hours may have been metabolized to lactate. While ethanol remained in the liver, gluconeogenesis from accumulated lactate would be inhibited, but once all the ethanol had been metabolized, the accumulated lactate may have allowed some glycogen resynthesis which would have decreased the rate of glycogen depletion due to starvation.

It is also possible that ethanol itself or one of its hepatic metabolites may have induced glycogen synthase activation or glycogen phosphorylase inhibition, or both. Which of these possible mechanisms is responsible for the resynthesis of glycogen in ethanol-treated starved rats is something that will have to be investigated in the future.

4.3.4.2 Effects of Ethanol on Muscle Glycogen Content in Starved Rats

The effects of ethanol on muscle glycogen contents in the starved rats are shown in Table 4.8 and 4.9. In control rats, starvation for 24 hours resulted in a 64% reduction in hindlimb muscle glycogen content. After 48 hours starvation there was no further decrease.

Table 4.8 Effects of Ethanol on Hindlimb Muscle Glycogen in Starved Rats

Fasting time (hours)	Muscle Glycogen content (μmol/g wet weight)		Percentage of difference
	Control	Treated	
0	42.0		0
24	13.7, 17.0 (15.3)	22.5, 17.1 (19.8)	29.4
48	18.2, 14.3 (16.2)	12.2, 15.3 (13.8)	14.8

Table 4.9 Effects of Ethanol on Gastrocnemius Muscle Glycogen in Starved Rats

Fasting time (hours)	Muscle Glycogen content (μmol/g wet weight)		Percentage of difference
	Control	Treated	
24	20.4, 17.6 (19.0)	28.8, 21.6 (25)	31.6
48	18.2, 17.0 (17.6)	13.5, 14.4 (14)	20.4

Effects of ethanol 4 g/kg on 2 types of muscle glycogen content in starved rats. Rats were treated with ethanol or water (control) as described in section (4.2.2). Glycogen concentrations were measured as described in section 2.3. The average is shown in parentheses. Two control rats and two treated rats were killed at each time interval. Protocol 4.2.2.1 was used in this experiment. Control fed data (hindlimb) obtained from Table 4.3.

Although this experiment was designed to address the hypothesis that a single dose of ethanol would cause muscle glycogen depletion in starved rats, an ethanol dose of

4 g/kg did not have this effect. In ethanol treated rats, there was actually an increase in glycogen content in both hindlimb (Table 4.8) and gastrocnemius (Table 4.9) muscles at 24 hours fasting. The experiments were conducted using only two rats, and since variation among the rats was significant, more experiments would need to be done to confirm this result, which was not persued further in this thesis. Our earlier studies (see section 3.2.1.2.b) confirmed that neither a 5 g/kg nor a 6 g/kg dose of ethanol caused any significant glycogen depletion in muscle in fed rats, so the occurrence of an apparent increase in muscle glycogen content at 24 hours in fasting, ethanol-treated animals is even more surprising, as it cannot be viewed as an oversynthesis response to earlier depletion, as seen in liver.

Although there have been some studies of the effects of ethanol on muscle glycogen metabolism, there is no previous study of the effect of ethanol on glycogen content in skeletal muscle in rats after starvation.

Our control values are in reasonable agreement with those of Calder and Geddes (1992) who reported 39.4 μmol/g (fed value) glycogen for hindlimb muscle and reported 50% depletion of glycogen after 40 hours starvation (Table 4.4). Fréminet and Leclerc (1980), reported a muscle glycogen level of 37 μmol/g in fed animals and found 50% drop after 48 hours starvation (Table 4.4). Conlee *et al.*, (1976) reported that four types of skeletal muscle had different glycogen contents, all in the range 20-50 μmol/g tissue wet weight and showed 23-43% glycogen depletion following 24 hours starvation in the rat (Table 4.4). Holness *et al.*, (1988c) reported glycogen concentrations of 38-48 μmol/g tissue wet weight for five muscle types in the fed rat. They observed 19-43% depletion of glycogen in five muscle types following 48 hours starvation (Tables 4.1 and 4.4).

4.3.5 **Effects of Ethanol on Molecular Weight Distributions of Glycogen in the Livers of Starved Animals**

The molecular weight distribution of glycogen purified from control and ethanol treated rats starved for various times is shown in Figure 4.7 and Table 4.10. In control animals, both high and low molecular weight glycogen are depleted to a similar extent by starvation.

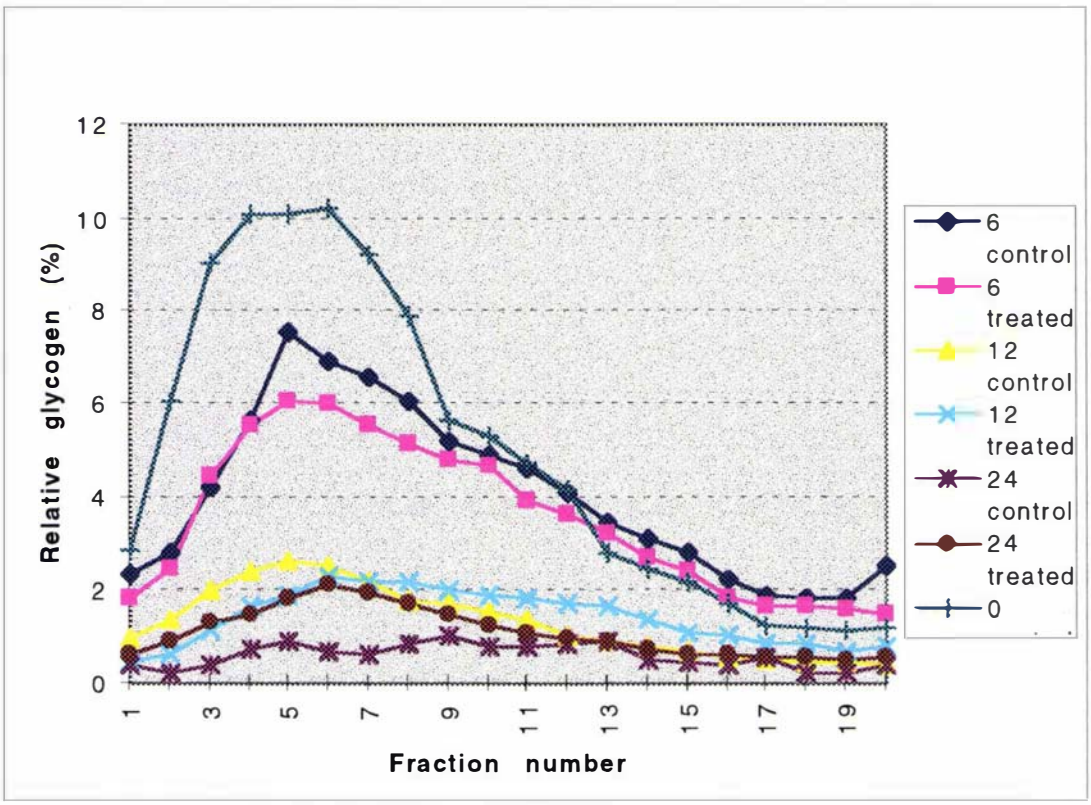

Figure 4.7 Effects of Ethanol on Molecular Weight Distribution of Glycogen in the Livers of Fed and Starved Animals

An ethanol dose of (4 g/kg) or an equivalent volume of water was administered. Thereafter rats were given access to water only for 24 hours. The animals were killed at the times indicated after treatment. The liver glycogen was purified as described in section 2.4.2.1. Glycogen contents were determined as described in section 2.4.2.5. The amount of glycogen in each fraction is expressed as a percentage of the total amount of tissue glycogen present in fed liver. Results are the average of three independent gradients from one experiment. Two control rats and two treated rats were killed at each time interval.

In control rats, 6 hours starvation depleted low and high molecular weight glycogen by 19% and 14% respectively (Table 4.10). Subsequently, there was a big glycogen drop between 6 and 12 hours starvation, when approximately 81% of the high molecular weight and 78% of the low molecular weight glycogen was mobilized. At 24 hours starvation, there was a further small depletion leaving the low molecular weight fraction 88% depleted and the high 83% depleted. Calder and Geddes (1992) reported that 40 hours starvation depleted both high and low molecular weight glycogen pools. In their study approximately 95% of the high molecular weight glycogen and approximately 98% of the low molecular weight glycogen were mobilized. Therefore, although their values are for 40 hours starvation, and slightly more depletion is seen, the pattern is the same as for the results of this study.

Table 4.10 Effects of Ethanol on Molecular Weight Distribution of Glycogen in the Livers of Fed and Starved Animals

	Amount of glycogen as % of total glycogen in fed control		Amount of glycogen in molecular weight range as % of that in fed control	
Starvation Time (h)	**Low MW $< 250 \times 10^6$**	**High MW $> 250 \times 10^6$**	**Low**	**high**
Fed	61.0	39.0	100	100
6 h control	49.5	34.0	81	86
6 h Treated	41.5	32.5	68	82
12 h control	13.0	7.5	22	19
12 h treated	11.5	13.0	19	34
24 h control	8.0	7.0	13	17
24 h treated	13.0	8.0	21	20

An ethanol dose of (4 g/kg) or an equivalent volume of water was administered. Thereafter rats were given access to water only for 24 hours. The molecular size range has been divided into two classes, corresponding to low ($< 250 \times 10^6$ Da) and high ($> 250 \times 10^6$ Da) molecular weight. The amount of glycogen in each size range is expressed as a percentage of the total amount of tissue glycogen present in fed liver. The animals were killed at the times indicated after treatment. The liver glycogen was purified as described in section 2.4.2.1. Glycogen contents were determined as described in section 2.4.2.5. The control data were obtained from Table 3.8.

It can be seen from Table 4.10 that in ethanol treated animals, the rate and extent of depletion was greater in low molecular glycogen than high molecular weight glycogen at 6 hours and 12 hours. After 6 hours starvation, it was noted that about one third of the low molecular weight glycogen was mobilized while only 20% of high molecular weight glycogen was depleted. It is apparent that both low molecular weight and high molecular weight glycogen showed rapid rates of depletion between 6 and 12 hours starvation. However, a significant observation was that after 12 hours starvation, high molecular weight glycogen was elevated in treated animals compared to their respective controls, while low molecular weight glycogen was not. It seems that although in ethanol treated rats, total glycogen content was about two fold higher than in control animals, all of this additional glycogen was of the high molecular weight form. These findings have prompted us to believe that there is a feedback mechanism that might

have controlled the metabolism of both pools of glycogen. However, it is not clear from this study whether ethanol did have an effect on a feedback mechanism. In contrast to the results seen in this section, in our dose response experiments on fed animals (section 3.2.1.3b) glycogen levels were evenly depleted in both low and high molecular weight ranges. It is possible that ethanol may impair glycogen synthesis differently in the rough endoplasmic reticulum (high molecular weight) and smooth endoplamic reticulum (low molecular weight).

4.3.6 Effects of Ethanol on Tissue Glycogen Content During the Starved-to-Refed Transition.

4.3.6.1 Effects of Ethanol on Food Consumption During Refeeding Following Starvation

Protocol 2, section 4.2.3 was used for this part of the study. Food intake was measured 10, 24 and 48 hours after ethanol administration. Initially 250 g pellets (standard laboratory) were added to each cage and two rats were housed in each cage. After 10, 24 and 48 hours, the weight of left-over food was subtracted from 250 g. A problem with measuring food consumption correctly was encountered, because the cages used were not well designed for this purpose. Some spillage of food occurred, but this was weighed and accounted for in the calculation. The final results provide a reasonably accurate estimate of the relative food consumption by control and treated animals.

The effects of ethanol on chow intake on refeeding after 48 hours starvation are presented in Table 4.11. The results indicate that after 10 hours chow refeeding, ethanol treated rats had consumed slightly less food than the control animals. The lesser intake in ethanol treated animals may be due to the sedative effects of ethanol. There was no significant difference observed between control and treated rats after 24 hours and 48 hours refeeding respectively.

Table 4.11 Effects of Ethanol on Food Consumption Refeeding Following Starvation

	Chow consumption (g)	
Refeeding Time (hours)	**Control rats**	**Treated rats**
0-10	46.6 ± 6.0 (6)	39.6 ± 3.5 (6)
0-24	83, 85 (4)	69, 95 (4)
0-48	162 (2)	170 (2)

Data indicate food consumption in grams per two rats. Two rats were housed in each cage. The results shown are from a single experiment and the total number of rats used in the experiment is indicated in parenthesis. Rats starved for 48 hours were administered ethanol (4 g/kg) or an equivalent volume of water before refeeding with chow. Rats were sacrificed 10, 24 or 48 hours after ethanol treatment.

4.3.6.2 Effects of Ethanol on Liver Glycogen Content in the Starved-to-Refed Transition.

The results obtained using Protocol 2, section 4.2.3, with a 4 g/kg ethanol dose are presented in Fig. 4.8 and Table 4.12. The results show that the content of glycogen within the livers of the 2 well-fed rats used in this experiment was approximately 400 μmol/g of tissue wet weight which agrees with other results for fed control animals used in Chapter 3 (Section 3.2.1.2 and 3.2.1.3). On refeeding after 48 hours starvation, there was an rapid increase in liver glycogen concentration. As shown in Table 4.3, after 48 hours starvation the liver glycogen content had fallen to about 40 μmol/g wet weight. By 5 hours after refeeding started, in control animals the glycogen content had already increased to 312 μmol/g. The liver glycogen content would have returned to the fed level after about 6-7 hours, but resynthesis continued. The peak in liver glycogen content was 2.10 times the normal fed level. After 48 hours of refeeding the liver glycogen level was still approximately 1.9 times the normal fed level.

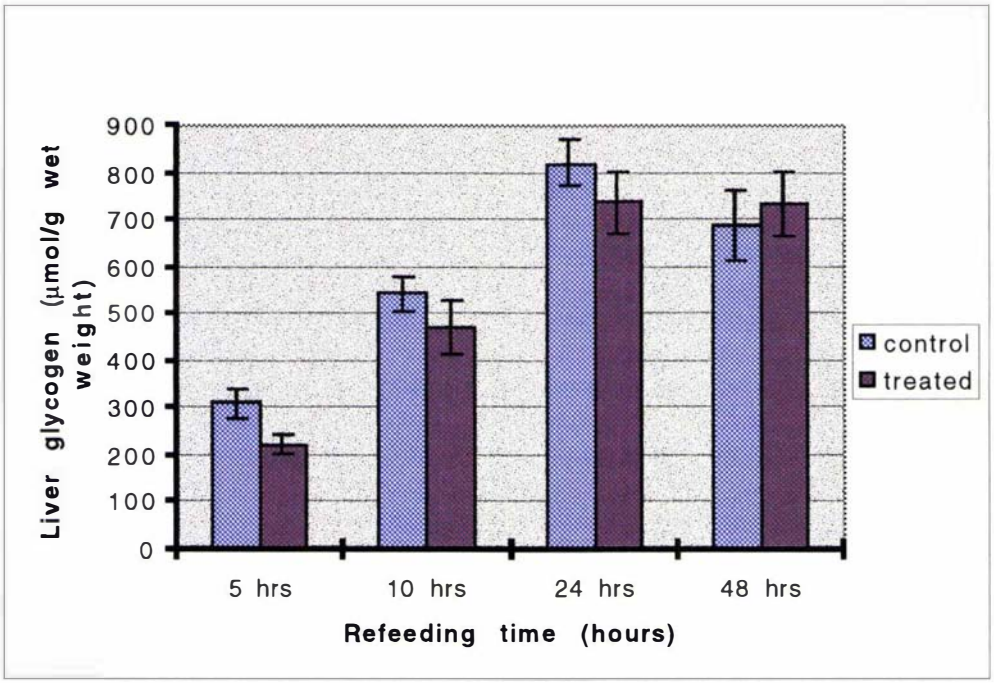

Figure 4.8 Effects of Ethanol (4 g/kg) on Liver Glycogen Content During Refeeding Following 48 hours Starvation

Rats starved for 48 hours were administered ethanol (4 g/kg) or an equivalent volume of water before refeeding with chow. Thereafter rats were sacrificed after 5, 10, 24 and 48 hours. Liver glycogen content was determined as described in section 2.3. Data presented are the mean ± S.E.M of 4 rats

Table 4.12 Effects of Ethanol (4 g/kg) on Liver Glycogen Content During Refeeding Following 48 hours Starvation

Refeeding time (h)	Liver glycogen content (control) (µmol/g)	Liver glycogen content (treated) (µmol/g)
5	312 ± 31	224 ± 21
10	546 ± 37	473 ± 58
24	823 ± 49	739 ± 67
48	691 ± 75	733 ± 68
fed level	387	

For conditions see Figure 4.8. The animals were sacrificed at the times indicated after treatment. Two well-fed rats were killed at 0 time, and the result shown is an average value for the two animals.

Over-production of liver glycogen similar to that shown in Table 4.12 and Figure 4.8 has been reported after a period of starvation in rats (Lutkic and Fister, 1969; Konishi and Fuwa 1983; Holness *et al.*, 1988; Calder and Geddes, 1992), rabbits (Geddes and Stratton, 1977) and humans (Nilsson and Hultman, 1973). Calder and Geddes (1992) reported that the liver glycogen concentration had returned to normal by 48 hours of refeeding, which contrasts with the observation in this study that at 48 hours the liver glycogen content was still 1.9 times the normal fed level.

Fig 4.9 shows a comparison of different studies on liver glycogen synthesis after various refeeding times following starvation. The hepatic glycogen level at various refeeding times is also shown for this study (H). It is significant to note that in all these studies, including this one, over-synthesis of glycogen was observed except in study B. It is also evident that the results of this study showed the one of the highest peaks of glycogen content (approximately 12.5% of wet weight) among those shown in Figure 4.9. The levels of peak after refeeding increased proportionally to the length of previous starvation except in study B. Given the fact that the normal fed level of liver glycogen is about 6% tissue wet weight, all the studies showed a peak content following refeeding of 1.5 - 2 times this value, except study B. The variation in the rate and extent of glycogen resynthesis between studies can be explained by the different lengths of starvation used, the different strains, sizes and ages of rats used, and the type of food available at refeeding. However, there is general agreement between the results of our study and a number of other studies.

Effects of ethanol on liver glycogen deposition during refeeding after 48 hours starvation are also presented in Table 4.12 and Figure 4.8. Intragastric administration of ethanol (4 g/kg) before chow refeeding inhibited hepatic glycogen deposition by 28.5% at 5 hours. Ethanol treated rats ate slightly less than respective control rats during the first 10 hours of the refeeding phase (Table 4.11). It appears that liver glycogen repletion in response to chow refeeding at both 10 hours and 24 hours was not significantly inhibited by administration of 4 g/kg ethanol. There was no difference observed in food intake between control and treated rats after 24 hours and 48 hours refeeding respectively. Therefore, while a dose of 4 g/kg ethanol caused a slight decrease in the content of liver glycogen reaccumulated at 5 hours, this decrease could be explained at least in part by decreased food intake, and there was not a significant inhibition by ethanol of glycogen resynthesis at later time intervals.

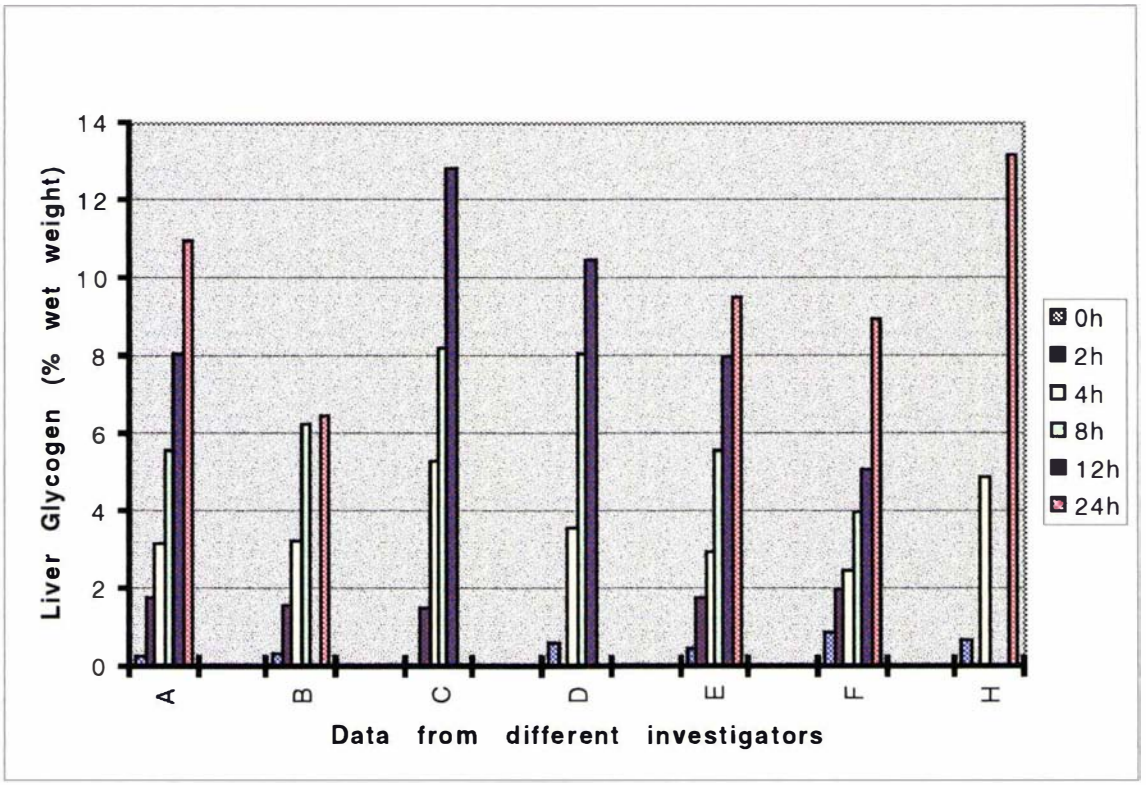

Figure 4.9 Comparison of Hepatic Glycogen Levels at Various Refeeding Times Following Starvation

Animals were starved for various times and then allowed to refeed *ad libitum*. The hepatic glycogen level at various refeeding times is shown for this study (H; rat, S 48) and those of (A- Calder and Geddes, 1992; rat, S 40H); Holness *et al.*, 1986; rat S 48) (C- Geddes and Stratton, 1977b; rabbit S 4 days); (D- Konishi and Fuwa, 1983; rat, S 48); (E- Lutkic and Fister, 1972; rat S 14) and (F- Lutkic and Fister, 1969). The normal fed level of liver glycogen is approximately 6% tissue wet weight.

Fig. 4.10 and Table 4.13 illustrate results obtained using protocol 2, section 4.2.3, with a 6 g/kg ethanol dose. The content of glycogen within the livers of the fed rats was 360 µmol/g of tissue wet weight. The results for control animals were similar to those for the 4 g/kg experiments except that the liver glycogen content had returned to the fed level after about 7-8 hours of refeeding. After 24 hours, the peak in the glycogen content observed was slightly higher than in the 4 g /kg experiment (Table 4.12).

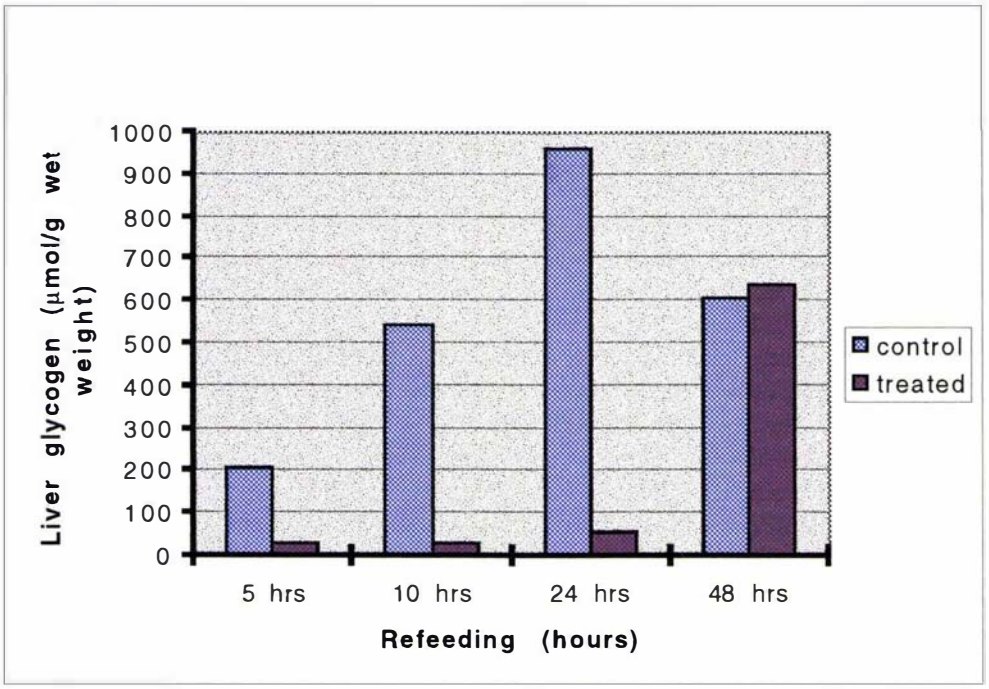

Figure 4.10 Effects of Ethanol (6 g/kg) on Liver Glycogen Content During Refeeding Following 48 hours Starvation

Rats starved for 48 hours were administered ethanol (6 g/kg) or an equivalent volume of water before refeeding with chow. Thereafter rats were sacrificed after 5, 10, 24, and 48 hours. Liver glycogen content was determined as described in section 2.3. Two controls rats and one treated rat were killed after each time interval, except that at 5 hours, 3 control and two treated rats were killed.

Table 4.13 Effects of Ethanol (6 g/kg) on Liver Glycogen Content During Refeeding Following 48 hours Starvation

Refeeding time (hours)	Liver glycogen content (control) (µmol/g)	Liver glycogen content (treated) (µmol/g)
5	208	33.5
10	545	34.6
24	962	54.0
48	609	641
fed level	360	

For conditions see Figure 4.13. The animals were sacrificed at the times indicated after treatment. Two control rats and one treated rat were killed at each time interval. At 5 hours, 3 control and two treated rats were killed.

The 6 g/kg ethanol dose inhibited glycogen resynthesis almost completely until 24 hours after refeeding. (Figure 4.10 and Table 4.13). Ethanol reduced the liver glycogen content by 84% compared with controls at 5 hours refeeding, by 93% at 10 hours and by 94% at 24 hours. Rats did not eat until 10 hours refeeding and but had eaten some food after 24 hours refeeding. Thus the inhibtion of glycogen resynthesis is due to both starvation and ethanol. The dramatic effect of the ethanol dose of 6 g/kg on starved rats, where some did not survive the dose and all were severely affected, contrasted with the relatively mild effects of the same dose in fed animals, which were sleepy for 1-2 hours but showed no other adverse effects.

Our results show that hepatic glycogen deposition was inhibited by ethanol doses of 4 g/kg or 6 g/kg. The effect of the 4 g/kg ethanol dose was relatively small with a significant decrease in liver glycogen content only being apparent at 5 hours after refeeding started. The inhibition by the 6 g/kg ethanol dose was severe for up to 24 hours. There are several possible ways in which ethanol might inhibit glycogen resynthesis following starvation. Firstly, ethanol decreased the food intake in rats treated with a 4 g/kg ethanol dose at the 10 hours sampling time and this factor could account for lower liver glycogen content seen at 5 hours. In rats treated with a 6 g/kg dose, food intake was dramatically inhibited and this probably accounts for much of the decrease in glycogen synthesis. Other factors that may contribute to an effect of ethanol on glycogen resynthesis are inhibition of gluconeogenesis and of glucose absorption by ethanol. Ethanol is a known inhibitor of gluconeogenesis (Hawkins and Kalant, 1972; Badawy, 1977). Since hepatic glycogen repletion on refeeding is predominantly (up to 70%) from C3 metabolites of glucose (indirect pathway; section 4.1.3.4), ethanol may impair hepatic glycogen repletion via the specific inhibition of gluconeogenesis. It has been shown that inhibition of hepatic glycogen repletion by ethanol after glucose refeeding of rats starved for 24 hours can be overcome by provision of glycerol, indicating that the site of action of ethanol is before the formation of triose phosphate in the indirect pathway of glycogenesis (Cox *et al.*, 1988). Ethanol is also known to interfere with the intestinal absorption of nutrients, including glucose and amino acids (Cox *et el.*, 1988; Cook *et al.*, 1988). At doses higher than 20 mmol/kg (1.09 g/kg) body wt., ethanol produced substantial malabsorption of glucose in the rat, higher than 60% of administered glucose remaining unabsorbed at 1 hour after administration (Cook *et al.*, 1988). It is clear from the results of the present experiments that the effects of ethanol on absorption of glucose or glucose precursors after chow refeeding cannot be as dramatic as this, since a much larger dose of ethanol (4 g/kg compared with 1.09 g/kg) has produced little effect on the reaccumulation of liver glycogen.

4.3.6.3 Effects of Ethanol on Muscle Glycogen Content in the Starved-to-Refed Transition.

Effects of ethanol on muscle glycogen deposition on refeeding after 48 hours starvation are presented in Figures 4.11 and 4.12 and Tables 4.14 and 4.15. Protocol 2 was used in this part of the study. Muscle glycogen was rapidly resynthesised upon refeeding in both control and treated animals. In control rats, the muscle glycogen content returned to the fed level much faster than liver glycogen had returned to the fed level. At 5 hours refeeding, the control level was approximately 1.5 times the fed level. From Table 4.14, we can see that the peak in muscle glycogen concentration was reached after 24 hours refeeding and was 2.3 times the normal fed level. By 48 hours of refeeding the control muscle glycogen concentration had returned to the normal level.

Intragastic administration of ethanol (4 g/kg) before chow refeeding following 48 hours starvation decreased muscle glycogen content by 53% at 5 hours, 11% at 10 hours, and 20% at 24 hours, relative to respective control animals. The glycogen content returned to the fed level between 5 hours and 10 hours refeeding. The peak in ethanol treated muscle glycogen content was 1.8 times the normal fed level at 24 hours. After 48 hours, the glycogen content fell to 61% of the muscle glycogen content in comparison with control animals.

A 6 g/kg ethanol dose decreased the muscle glycogen level much more than a 4 g/kg ethanol dose (Figure 4.12 and Table 4.15). The glycogen content returned to close to the normal fed level by 24 hours, but no over-synthesis was seen, and at 48 hours, further depletion was apparent. Ethanol decreased the glycogen content by 70% at 5 hours refeeding, by 67% at 10 hours, by 55% at 24 hours, and by 35% at 48 hours. These values are again calculated relative to respective control animals.

Cook *et al.* (1988) have reported that ethanol treatment (75 mmol/kg) does not inhibit muscle glycogen synthesis and deposition on glucose refeeding (2 mmol/100 g body wt.) after 24 hours starvation. From their dose-response experiments, they observed that there was no effect of ethanol on muscle glycogen repletion even though glucose absorption was impaired. However, in our study with a similar ethanol dose (4 g/kg), muscle glycogen deposition was inhibited after chow refeeding. This discrepancy could be due to differences in the methods used in our study and that of Cook *et al.*

(1988). Differences between our study and Cook *et al.* are the different length of starvation times used, the different mode of ethanol and food administration and different strains and sizes of rats used. Cook *et al.* in their experiment starved the animals for 24 hours. Ethanol was administered thereafter. After one hour the rats were given an oral glucose load (2 mmol/100 g body weight) and after another one hour under light anaesthesia the animals were killed for the first tissue sampling. In our study the rats were starved for 48 hours prior the administration of ethanol. The rats had free access to food and after five hours animals were killed, without use of anaesthesia, for the first tissue sampling.

As indicated in section 4.1.4, Xu *et al.* (1992) confirmed that ethanol decreased glycogen resynthesis in oxidative muscles in response to oral and intraperitoneal glucose refeeding after 40 hours starvation, but not in non-oxidative muscles. Xu *et al.* (1993) were unable to demonstrate how ethanol administration differentially impaired glycogen deposition in oxidative muscles .

There are a number of possible ways in which ethanol may impair muscle glycogen metabolism. Xu *et al.*, (1993) suggested that the defect did not arise secondary to intramuscular ethanol oxidation, since muscle had little ADH and ALDH indicating that any metabolic impairment must be mediated by ethanol itself, or its hepatic metabolites. There is a possibility that the impairment may arise from ethanol-induced endocrine disturbances, alterations in plasma corticosterone (Ellis, 1966) and catecholamine levels (Perman, 1961). Additionally, ethanol may perturb membrane transport processes either via in membrane fluidity, or via a pathway of non-oxidative ethanol metabolism, by which ethanol forms ethyl esters with long chain of fatty acids (Laposata and Lange, 1986). Organs lacking oxidative alcohol metabolism which are frequently damaged by ethanol abuse have high fatty acid ethyl ester synthetic activities (Laposata and Lange, 1986). The ethanol-induced inhibition of glycogen deposition is unlikely to be mediated via changes in the activites of the enzymes of glycogen phosphorylase and glycogen synthase, since the levels of these enzymes are not altered in response to ethanol treatment (Cook *et al.*, 1992). Perhaps, effects of ethanol metabolites generated in the liver might impair muscle glycogen levels and metabolism (acetate, acetaldehyde, redox imbalance).

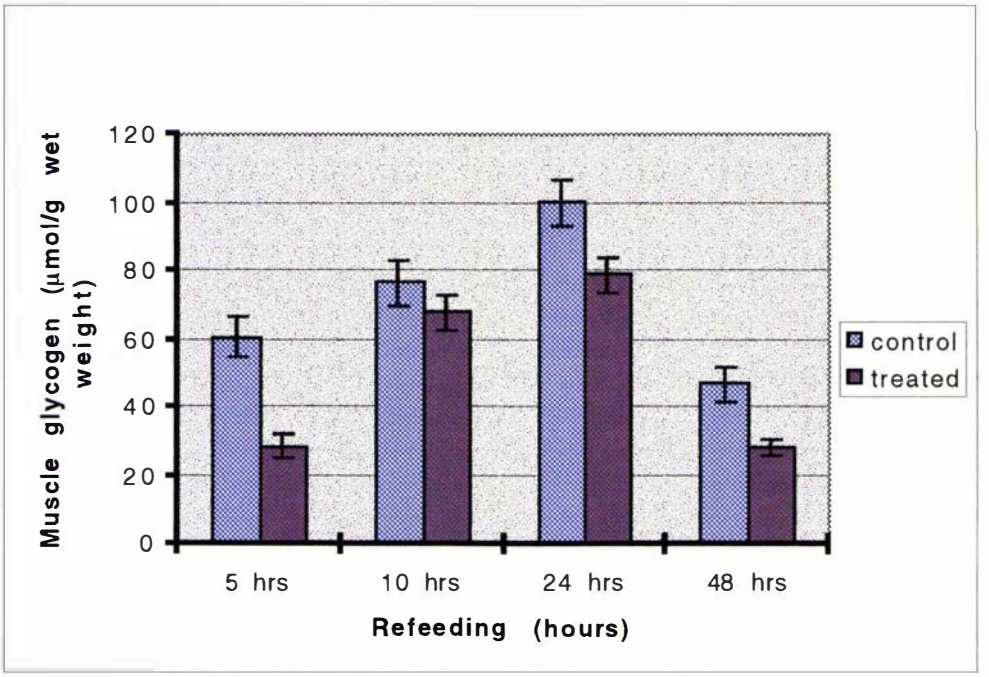

Figure 4.11 Effects of Ethanol (4 g/kg) on Muscle Glycogen Content During Refeeding Following 48 hours Starvation

Rats starved for 48 hours were administered ethanol (4 g/kg) or an equivalent volume of water before refeeding with chow. Thereafter rats were sacrificed after 5, 10, 24 and 48 hours respectively. Muscle glycogen content was determined as described in section 2.3. Data presented are the mean ± SEM of 4 rats, two rats from each of two independent experiments.

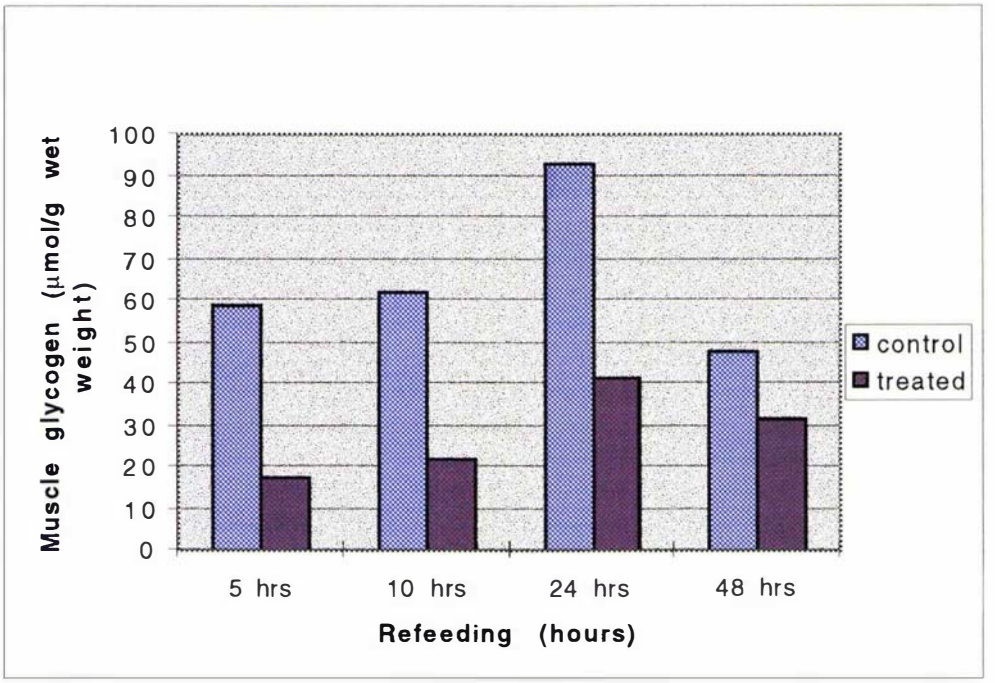

Figure 4.12 Effects of Ethanol (6 g/kg) on Muscle Glycogen Content During Refeeding Following 48 hours Starvation

Rats starved for 48 hours were administered ethanol (6 g/kg) or an equivalent volume of water before refeeding with chow. Thereafter rats were sacrificed after 5, 10, 24 and 48 hours respectively. Muscle glycogen content was determined as described in section 2.3. Two control and one treated rats were killed at each time interval.

Table 4.14 Effects of Ethanol (4 g/kg) on Muscle Glycogen Content During Refeeding Following 48 hours Starvation

Refeeding time (hours)	Muscle glycogen content (control) (µmol/g)	Muscle glycogen content (treated) (µmol/g)
5	60.5 ± 5.8	28.4 ± 3.5
10	76.6 ± 6.9	68.2 ± 5.9
24	100.2 ± 6.7	79.1 ± 5.1
48	46.6 ± 4.9	28.4 ± 2.4
fed level	45	

For conditions see Figure 4.11. Data are mean ± S.E.M of 4 rats, two rats from each of two independent experiments. The animals were sacrificed at the times indicated after treatment. Two well fed rats were killed at 0 time.

Table 4.15 Effects of Ethanol (6 g/kg) on Muscle Glycogen Content During Refeeding Following 48 hours Starvation

Refeeding time (hr)	Muscle glycogen content (control) (μmol/g)	Muscle glycogen content (treated) (μmol/g)
5	59.1	17.7
10	62.2	21.7
24	93.1	41.0
48	47.9	31.5
fed level	42	

For conditions see Figure 4.12. Two control and one treated rats were killed at each time interval. Two well fed rats were killed at 0 time.

Table 4.16 illustrates a comparison of results found by different investigators for hindlimb muscle glycogen resynthesis after various refeeding times following starvation. There is variation between studies in terms of the rate and extent of muscle glycogen resynthesis. This variation is probably due to the different lengths of starvation used, the different strains, sizes and ages of rats used, the amount of exercise and the amount of carbohydrate available at refeeding.

Our control data show general agreement with Geddes and Chow (1994b) in terms of rate and extent of muscle glycogen resynthesis. In both studies, a peak was observed at 24 hours refeeding. In contrast to these results, Calder and Geddes, (1992) found the peak in glycogen content at 13 rather than 24 hours. Rapid resynthesis of skeletal muscle glycogen has also been reported following exercise-induced glycogen depletion in the rat (Terjung *et al.*, 1974; Conlee *et al.*, 1978) and man (Maehlum *et al.*, 1977; Hermansen and Vaage, 1977). In the study by Calder and Geddes (1992), skeletal muscle glycogen concentration had returned to the fed level after about 30 minutes of refeeding. These researchers also reported the peak was approximately 50 μmol/g tissue wet weight or 1.3 times the normal fed level, reached after 13 hours of refeeding. Holness *et al.* (1988c) showed a peak in muscle glycogen content of 1.3-1.8 times the fed level at 8 hours of refeeding.

Table 4.16 Comparison of Hindlimb Muscle Glycogen Synthesis Upon Various Refeeding Time Following Starvation

Length of Refeeding (hour)	Our data (μmol/g wet weight)	Calder and Geddes (1992) (μmol/g wet weight)	Geddes and Chow (1994b) (μmol/g wet weight)
0	16.9 (48 hours)	18.5 (40 hours)	14.8 (120 hours)
0.5		38.3	
4		51.2	
5	59.8 (n=4)		
6			51.8
10	70.6 (n=4)		
13		51.9	
24	81.4 (n=4)	46.3	84.0
48	37.8 (n=4)	36.4	
fed	42.3 (n=10)	39.5	35

4.3.6.4 Effects of Ethanol on Molecular Weight Distribution of Liver Glycogen on Refeeding Following Starvation.

The effect of ethanol on molecular weight distribution of glycogen in the livers of starved and refed rats presented in Figure 4.13 and Table 4.17. Starvation severely affected the content of both high and low molecular weight glycogen. In the liver, prolonged starvation (48 hours) resulted in the mobilisation of approximately 95% of the high molecular weight glycogen and 95% of the low molecular weight glycogen. On refeeding, there was rapid resynthesis of glycogen of all size ranges but there was early over-synthesis of high molecular weight material. After 5 hours of refeeding rat chow *ad libitum*, the overall liver glycogen level reached 79% of the normal level but the high molecular weight component had returned to the fed level, while the low molecular weight glycogen was present at only 70% of the normal level. The over-synthesis of high molecular weight glycogen continued with further refeeding and

after 10 hours and 24 hours the levels were 1.8 times and 2.42 times the fed level, respectively. Re-synthesis led to levels of low molecular weight glycogen after 10 hours and 24 hours of 1.08 times and 1.86 times the fed level respectively.

In ethanol treated rats, after 5 hours, high molecular weight material was present at only 72% of the fed level while low molecular weight glycogen was only 47%. In treated rats, the total glycogen content, after 10 hours and 24 hours was 1.20 times and 1.90 times the normal level respectively. Although the oversynthesis of high molecular weight glycogen continued with further refeeding after 10 hours and 24 hours, the rate and extent are slower than the corresponding data for control animals indicating that ethanol might preferentially inhibit the resynthesis of high molecular weight glycogen. By 48 hours of refeeding, the high molecular weight glycogen content in treated animals was 2.4 times the fed level, which was identical to that in control animals. However, there was also increased over-synthesis of low molecular weight material. It appears that, apart from at 5 hours, ethanol does not significantly inhibit the resynthesis of low molecular weight glycogen.

Liver glycogen is built upon a protein backbone (Krisman and Barengo, 1975) and the backbones are joined to one another by disulphide bonds (see reviews: Geddes, 1985; Calder, 1991). Due to the presence of these disulphide bonds, glycogen molecules are aggregates in which smaller subunits combine to give a vast molecular size (see section, Fig 1.3; Geddes *et al.*, 1977a,b). Although we do not have direct evidence for disulphide bond reduction, it appears from Table 4.17 that, in liver, ethanol preferentially decreases the resynthesis of high molecular weight, rather than low molecular weight, glycogen. The increase in NADH concentration which occurs in liver during ethanol metabolism could lead to increased disulphide bond reduction, which might then cause loss, or decreased assembly, of high molecular weight glycogen.

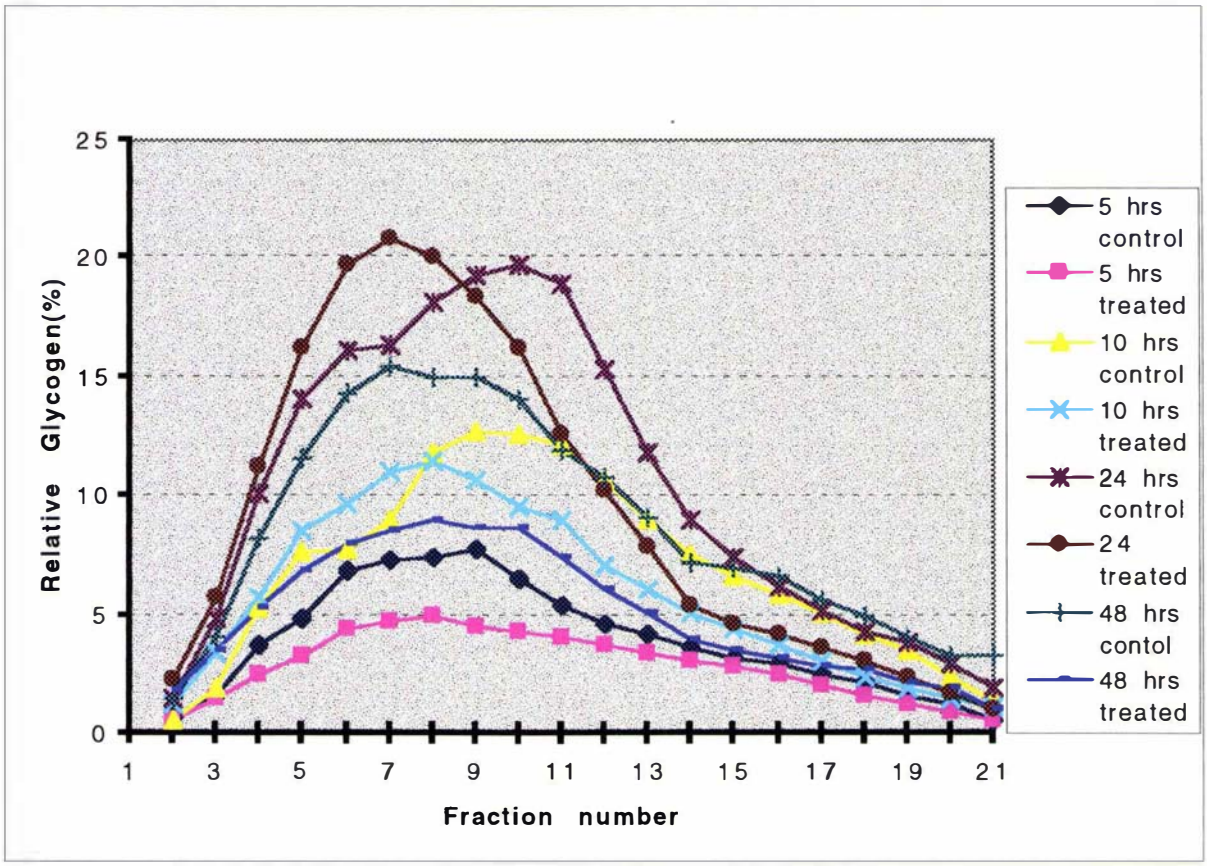

Figure 4.13 Effects of Ethanol on Molecular Weight Distribution of Glycogen in the Livers of Fed, Starved and Starved/Refed Rats

Rats starved for 48 hours were administered ethanol (4 g/kg) or an equivalent volume of water before refeeding with chow. Rats were killed after 5, 10, 24 and 48 hours. Liver glycogen purification and molecular weight distributions were described in section 2.4.1.1 and 2.4.1.3 respectively. The amount of glucogen in each fraction expressed as a percentage of the total amount of tissue glycogen present in fed liver. Glycogen contents were determined as described in section 2.4.2.4. All values were averaged over three sucrose density gradients.

Table 4.17 Effects of Ethanol on Molecular Weight Distribution of Glycogen in the Livers of Fed, Starved and Starved/Refed Rats

		Amount of glycogen as % of total glycogen in fed control		Amount of glycogen in molecular weight range as % of that in fed control	
Refeeding time	Liver glycogen content (μmol/g)	Low MW $< 250 \times 10^6$	High MW $> 250 \times 10^6$	Low	high
0	40.0	2.7	2.0	4.22	5.55
5 h con	312	44.3	36.4	69.0	101.0
5 h treated	224	30.5	25.8	47.5	72.0
10 h con	546	69.5	66.9	108.2	186.0
10 h treated	473	71.1	46.0	110.8	128.0
24 h con	823	120.0	86.6	187.0	242.0
24h treated	739	130.6	55.8	203.4	155.0
48 h con	691	85.1	88.0	132.5	245.0
48 h treated	733	94.0	87.5	146.0	244.0
Fed	396	64.0	36.0	100.0	100.0

Rats starved for 48 hours were administered ethanol (4 g/kg) or equivalent volume of water before refeeding with chow. The animals were killed at the times indicated after treatment. Two control rats and two treated rats were killed at each time interval except at 5 hours, where three control and three treated rats were killed. The livers were pooled to allow purification of sufficent glycogen for the gradients which were carried out as described in Figure 4.13. The liver glycogen was purified as described in section 2.4.1.1. Glycogen contents were determined as in section 2.4.2.4. The molecular size range has been divided into two classes, corresponding to low ($< 250 \times 10^6$ Da) and high ($> 250 \times 10^6$ Da) molecular weight. The amount of glycogen each size range is expressed as a percentage of the total amount of tissue glycogen present in the fed liver .

Calder and Geddes (1990a) proposed that the initial step in lysosomal glycogen degradation may be disulphide bond reduction in *post mortem* glycogen degradation. This reduction would produce a larger pool of low molecular weight glycogen. High molecular weight glycogen has a higher protein content than the low molecular weight glycogen and it has been proposed that this is the result of two different sites of synthesis (Geddes, 1986). High molecular weight glycogen is proposed to be synthesised in the rough endoplasmic reticulum (RER). The small ß-particles are known to be built upon a single protein backbone (Krisman and Barenco, 1975) in groups of 2-3 which may then aggregate by covalent bonding through disulphide bridges to form the large α-particles (see section 1.1.4). The results of this study suggest that ethanol may preferentially inhibit resynthesis of high molecular weight glycogen in the RER for up to 24 hours after refeeding.

4.3.6.5 **Effects of Ethanol on Molecular Weight Distribution of Muscle Glycogen on Refeeding Following Starvation.**

The effects of ethanol on molecular weight distribution of glycogen in the muscles of starved and starved and refed rats are shown in Figure 4.14 and Table 4.18. Starvation markedly reduced the content of both high and low molecular weight glycogen. In the muscles, prolonged starvation (48 hours) resulted in the mobilisation of approximately 32% of the high molecular weight glycogen and 81% of the low molecular weight glycogen.

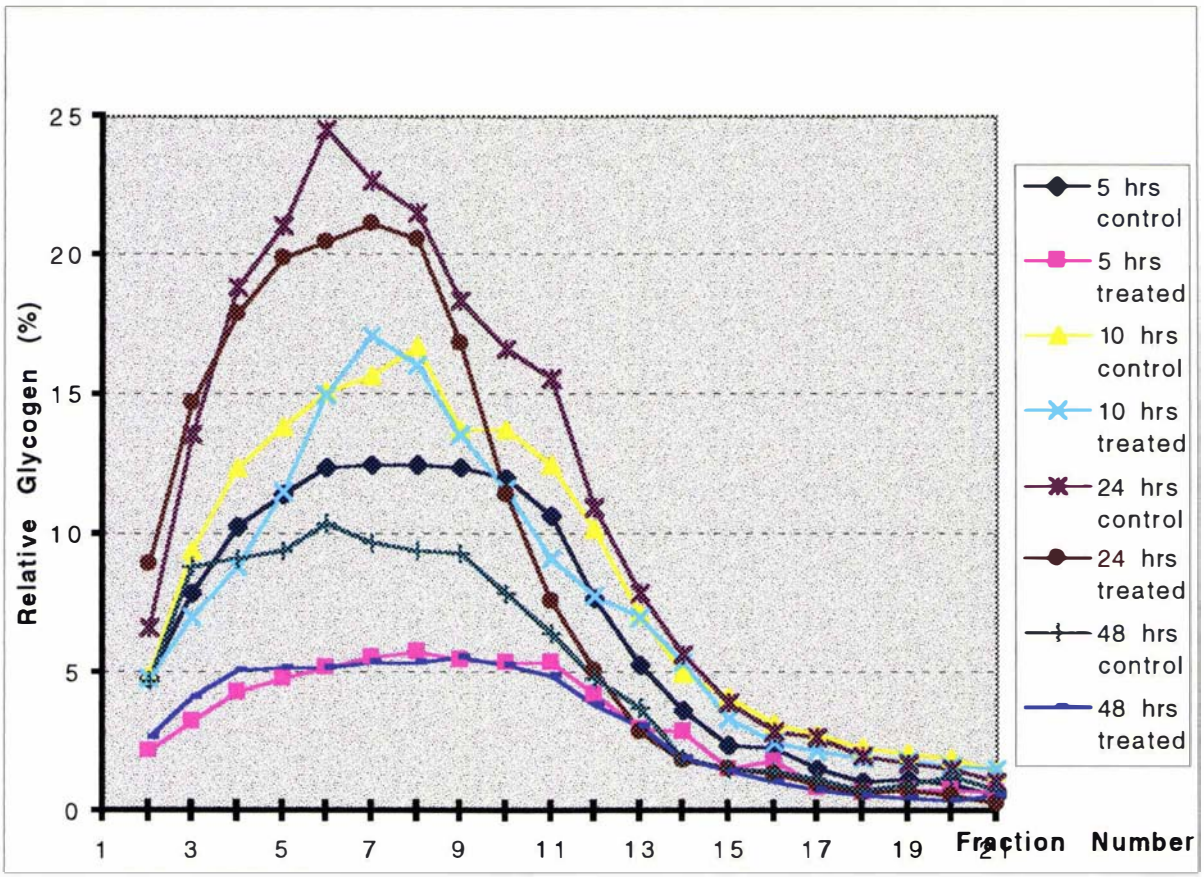

Figure 4.14 Effects of Ethanol on Molecular Weights Distribution of Glycogen in the Muscles of Fed, Starved and Starved/Refed Rats

Rats starved for 48 hours were administered ethanol (4 g/kg) or equivalent volume of water before refeeding with chow. Rats were killed after 5, 10, 24 and 48 hours. Muscle glycogen purification and molecular weight distributions were described in section 2.4.1.2 and 2.4.1.3 respectively. The amount of glycogen in each fraction is expressed as a percentage of the total amount of tissue glycogen present in fed muscle. Glycogen contents were determined as described in section 2.4.2.4. All values were averaged over three sucrose density gradients.

Table 4.18 Effects of Ethanol on Molecular Weights Distribution of Glycogen in the Muscles of Fed, Starved and Starved/Refed Rats

		Amount of glycogen as % of total glycogen in fed control		Amount of glycogen in molecular weight range as % of that in fed control	
Refeeding time	**Muscle glycogen content (μmol/g)**	**Low MW < 250 x 10^6**	**High MW > 250 X 10^6**	**Low**	**High**
0	16.9	11.8	26.5	19.3	68.0
5 h con	60.5	84.4	48.7	138.2	122.7
5 h treated	28.4	36.7	28.5	60.2	71.0
10 h con	76.6	101.9	66.3	167.0	170.0
10 h treated	68.2	93.9	55.9	153.0	143.0
24 h con	100.2	147.3	72.6	241.3	186.0
24 h treated	79.1	140.5	35.7	230.0	91.5
48 h con	46.6	71.1	32.5	116.5	83.3
48 h treated	28.4	38.5	24.4	63.1	62.5
Fed	45 (100)	61.0	39.9	100.0	100.0

For conditions see Figure 4.14. The molecular size range has been divided into two classes, corresponding to low (< 250 x 10^6 Da) and high (> 250 x 10^6 Da) molecular weight. The amount of glycogen in each size range is expressed as a percentage of the total amount of tissue glycogen present, taking the present in the fed muscle. Glycogen contents were determined as described in section 2.4.2.4. All values were averaged over three sucrose density gradients.

Upon refeeding, there was rapid resynthesis of glycogen of all size ranges. In control animals, muscle glycogen levels returned to the fed level soon after resumption of feeding. At 5 hours of refeeding rat chow *ad libitum*, total muscle glycogen levels of approximately 1.34 times of the normal level were seen. The high molecular weight

glycogen content was 1.23 times the control, and low molecular weight glycogen was present at 1.38 times the control. The oversynthesis of both low and high molecular weight glycogen continued with further refeeding. After 10 hours the levels were 1.7 times the normal fed level, and after 24 hours the levels were even higher, 2.4 times the fed level for low molecular weight glycogen and 1.9 times the fed level for high molecular weight. By 48 hours after refeeding, both low and high molecular weight glycogen content had returned to very close to the normal fed level (1.2 and 0.83 times, respectively). These results support previous findings (Calder and Geddes, 1992) that both low and high molecular weight glycogen are rapidly resynthesised in muscle on refeeding after starvation. However, Calder and Geddes (1992) reported a higher rate of high than of low molecular weight glycogen synthesis which is not seen in this study.

In ethanol treated rats, after 5 hours refeeding, the levels of both low and high molecular weight glycogen and total muscle glycogen content were considerably lower than water-treated control rats. It is expected that sedative effects of ethanol could have contributed to the lower consumption of food intake during early stages of refeeding. If we consider muscle glycogen resynthesis during the refeeding period from 0-5 hours, it was marked by accumulation of predominantly low molecular weight glycogen in the treated rats. However, in subsequent refeeding up to 10 hours, both low and high molecular weight glycogen accumulated to a similar extent. High molecular weight glycogen resynthesis peaked at 143% after 10 hours refeeding whereas low molecular weight glycogen accumulation continued until 24 hours after refeeding. By 48 hours of refeeding, the content of both low and high molecular weight glycogen had dropped again to about 60% of the normal fed content.

In ethanol treated rats, there was little high molecular weight glycogen synthesis between 0 and 5 hours, and between 10 and 24 hours there was a decrease in high molecular weight glycogen content. It is difficult to attribute the decreased synthesis of high molecular weight glycogen in muscle to an effect of ethanol on the redox state in this tissue leading to reduction of disulphide bonds, as was suggested for liver. Ethanol is not rapidly metabolised in muscle, and therefore does not cause a direct increase in the $NADH/NAD^+$ ratio. It is possible, however, that hepatic metabolites entering circulation as a consequence of ethanol metabolism might have impaired high molecular weight glycogen accumulation in muscle.

Using labelled glucose precursors, Geddes and Chow (1994b) showed that there is a control mechanism in muscle that enables the synthesis of high molecular weight glycogen to be switched off during the initial part of the refeeding period. This is confirmed in the present study, where it is seen that low molecular weight glycogen

content increases by about 7-fold in the first 5 hours of refeeding, while high molecular weight glycogen content increases by only about 2-fold. In the presence of ethanol, this effect is accentuated, as high molecular weight glycogen content shows almost no change in the first 5 hours of refeeding, while low molecular weight content still increases by about 3-fold. An increase in the tissue content of lactate in the muscle could result from ethanol metabolism in the liver. It is therefore interesting to speculate that either lactate concentration or a change in $NADH/NAD^+$ ratio as a result of the presence of lactate, or both, may be controlling factors for synthesis of high molecular weight glycogen in muscle.

However, studies in humans have suggested that neither lactate nor acetate mediates changes in glucose utilisation in muscle (Lundquist, 1962; Lundquist *et al.* 1962; Lundquist *et al,.* 1973). Measurement of arterial and venous lactate/pyruvate ratio across the forearm indicates that the cytoplasmic redox state in muscle is unchanged by ethanol administration (Lundquist *et al.,* 1973). It has also been shown that acetate infusion has no effects on rates of glucose disposal in muscle (Yki-Järvinen *et al.,* 1988).

The decrease in high molecular weight glycogen content seen between 10 and 24 hours is even more difficult to explain in terms of the effects of ethanol or its metabolites as ethanol should have been completely cleared by this time, and the metabolic changes due to its presence reversed. The current observation is a single result, and needs repeating to confirm that the effect is reproducible. The results of this study do suggest, however, that ethanol inhibition may provide a useful tool in the study of regulation of the synthesis of low and high molecular weight glycogen in muscle.

4.3.7 **Effect of Ethanol on Glycogen Resynthesis After an Oral Glucose Load**

This experiment was performed using Protocol 1, Section 4.2.3, the same as that used by Cook *et al.* (1988). Figure 4.15 and Table 4.19 illustrate the effect of ethanol on hepatic glycogen resynthesis after an oral glucose load (2 mmol/100 g body weight).

In the control rats, liver glycogen deposition increased steadily up to 120 minutes and thereafter decreased because of the limited amount of glucose administered. The intragastric administration of ethanol (3.45 g/kg) 1 hour before glucose refeeding decreased liver glycogen deposition by 21% at 60 min, by 38% at 90 min, and by 31%

at 120 min. However, at 150 min, the treated glycogen level was slightly higher than the respective control and at 180 min, the glycogen content was 43% higher than in control rats.

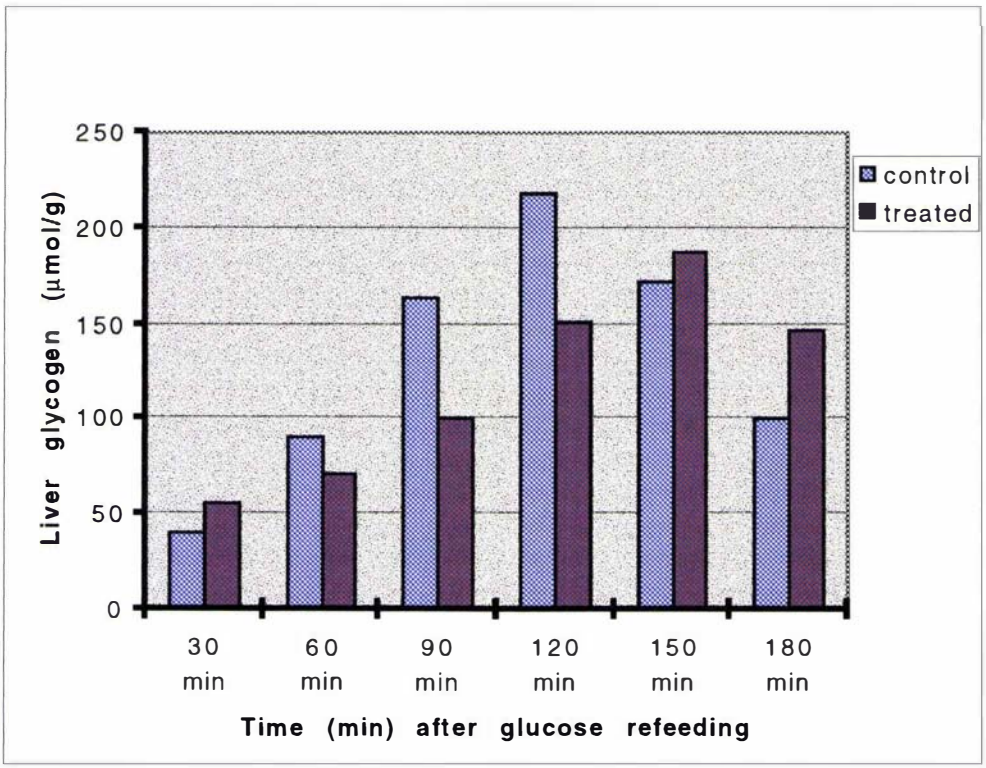

Figure 4.15 Effects of Ethanol (75 mmol/kg body wt.) on the Time Course of Glycogen Repletion after an Oral Glucose Refeeding (2 mmol/100 g body wt.)

Rats were starved for 24 hours. Ethanol or an equivalent volume of water was administered 1 hour before glucose refeeding. Animals were killed for tissue sampling at after 30, 60, 90, 120, 150 and 180 min. Data are the average of two rats.

Table 4.19 Effects of Ethanol (75 mmol/kg body wt.) on the Time Course of Glycogen Repletion After Oral Glucose Refeeding (2 mmol/100 g body wt.)

Time (min)	Liver glycogen (μmol/g wet weight)		Percentage of difference
	Control	Treated	
30 min	39, 41 (40.0)	60, 51 (55.5)	+ 40%
60 min	88, 93 (90.5)	74, 69 (71.5)	- 21%
90 min	161, 165 (163)	103, 100 (101)	- 38%
120 min	217, 220 (218.5)	151, 151 (151)	- 31%
150 min	175, 169 (172)	183, 190 (187)	+ 8%
180 min	104, 98 (101)	149, 143 (146)	+ 43%

Rats were starved for 24 hours. Ethanol or an equivalent volume of water was administered 1 hour before glucose refeeding. Animals were killed at time intervals as indicated. The average is shown in parentheses.

The glycogen content variation between rats was minimal in glucose refed animals compared to chow-refed animals. The variation in chow-refed animals is partly due to inter-individual differences in ethanol metabolism and the amount of food consumed at refeeding.

From Figure 4.15, it is evident that that ethanol has delayed the peak reached in liver glycogen content, by either decreasing glucose absorbtion, by inhibiting gluconeogenesis or glycogen synthesis, or a combination of all these factors. There are two important phases in this refeeding. In the first phase, glycogen deposition occurred, until 120 min for control animals and 150 min for ethanol-treated rats. In the second phase glycogen mobilization occurred and the liver glycogen content declined again. This is due to the limited amount of glucose supplied.

Figure 4.16 illustrates a comparison of our study and that of Cook *et al.* (1988) Although we used the same protocol as Cook *et al.* (1988), the results we obtained are significantly different from theirs. Both the rate and extent of glycogen repletion are significantly different between the two studies. Cook *et al.* (1988) concluded from their dose-response experiments that high concentrations of ethanol could affect the intestinal absorption of glucose. Ethanol at a dose of 10 mmol/kg body weight

inhibited hepatic glycogen repletion (by 43%) without affecting intestinal glucose absorption and gastric fluid volume. However, ethanol at a dose of 75 mmol/kg body weight impaired the intestinal absorption of glucose. Whereas control rats absorbed > 80% of an oral glucose load within 90 min, ethanol pretreatment caused a marked delay in absorption. Less than 1% of the glucose load was absorbed in the first 1 hour after its administration, and about 40% remained unabsorbed even after 3 hours. Contrary to the findings of Cook *et al.* (1988), our data from Fig. 4.16 shows that although the high dose of ethanol (75 mmol/kg; the same as that used by Cook *et al.* 1988) appears to have delayed the peak of glycogen content by about 30 minutes, the maximal content reached is not much lower in treated animals (207 μmol/g) than in controls (242 μmol/g). Both these glycogen contents are far higher than the maximal levels reached in the study by Cook *et al.* (1988) and our results suggest that even at the high ethanol dose, glucose absorption was not greatly inhibited.

The foregoing observations can also be analysed theoretically. The calculation of liver glycogen content from 2 mmol/kg glucose refeeding shows that liver glycogen repletion could have reached 400 μmol/g on the assumption that 100% oral glucose would convert into liver glycogen (assuming liver weight is about 10 g for a 200 g rat). If it is considered that 50% of the oral glucose load might be converted into liver glycogen, our results are in reasonable agreement with this theoretical calculation. Measurements of glycogen in muscle and liver after an intragastric glucose load given to rats (Niewöhner *et al.* 1984) or mice (Cox and Palmer, 1987) starved for 24 hours have suggested that during the first hour, total glycogen deposition in both tissues is about equal.

There are two significant differences in methodology that could have contributed to the different results, the size of the rats used and the use of anaesthesia by Cook *et al.* (1988). In another study from our laboratory (Cheng, 1997) it was shown that liver glycogen content is lower in 150-180 g rats than in 220-250 g rats. Cook *et al.* (1988) used much smaller rats (100-120 g). The use of anaesthesia by Cook *et al.* (1988) may also have led to a decrease in glycogen synthesis, if blood flow to the liver and hence oxygenation were decreased. Their rats were kept under light anaesthesia from the time of ethanol and glucose administration until tissue sampling.

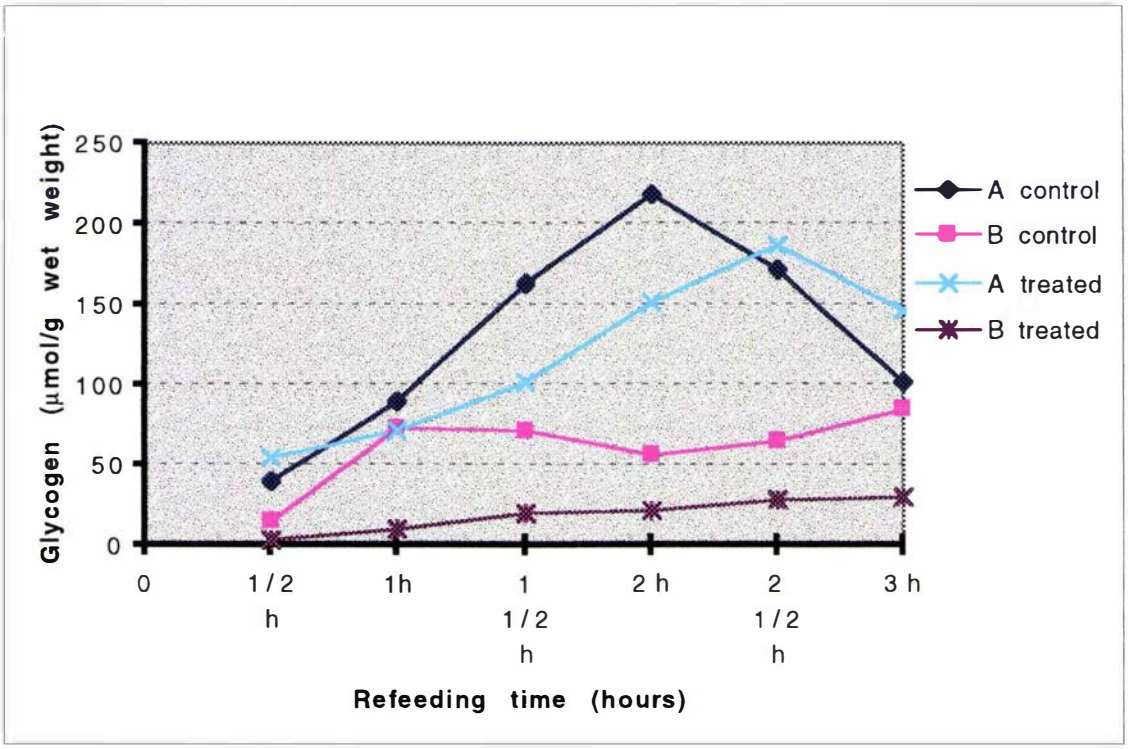

Figure 4.16 Comparative Study of Effects of Ethanol on the Time Course of Glycogen Repletion After Glucose Refeeding of Cook *et al.* and Ours.

Ethanol (75 mmol/kg body wt. or 3.45 g/kg) or an equivalent volume of water was administered 1 hour before glucose refeeding. The rats were starved for 24 hours before the experiments. Our results were determined as described in section 2.9. A-our data are the average values from Table 4.19 and B-Data from Cook *et al.*, (1988).

The results of both our studies on the effects of ethanol on hepatic glycogen resynthesis following starvation (using either chow refeeding or glucose administration) show far less inhibition than that observed by Cook *et al.* (1988). The results of our replication of their experiment with glucose refeeding indicates that the differences observed are not simply due to chow refeeding compared with glucose refeeding. It appears that the effects of ethanol on hepatic glycogen repletion either by direct inhibion of gluconeogenesis or by inhibition of glucose uptake are not as great as indicated by Cook *et al.* (1988). Their results probably reflect the combined effects of ethanol and diethyl ether anaesthetic on hepatic glycogen synthesis in small rats, where liver glycogen content may be lower anyway. In our larger rats, without the additional effect of anaesthetic, ethanol at a dose of 4 g/kg causes little inhibition of glycogen re-synthesis. A larger dose of ethanol of 6 g/kg causes much greater inhibition in chow fed animals, probably due primarily to lack of food intake.

4.4 Conclusions

This chapter was designed to investigate the affects of ethanol on starved and starved-refed animals. This chapter has several objectives.

Basically, the starvation study was divided into two parts, (1) the effect of starvation on tissue glycogen content and (2) the effect of ethanol on tissue glycogen content during starvation. In the first part of the study, the rats starved for 72 hours contained more liver glycogen than those starved for 24 hours and 48 hours rats. In the second part of the study, fed rats were administered a dose of 4 g/kg ethanol, then starved and sacrificed at various times. After 3 hours starvation, liver glycogen content had depleted to almost half of the fed levels in ethanol treated rats. Interestingly, at 24 hours glycogen content increased in the ethanol treated rats, to as much as twice than in the control animals. Nevertheless, an ethanol dose of 4 g/kg did not have any significant effect on muscle glycogen. Since we had used only two rats for this study, further experiments need to be done. The rate and extent of depletion was greater in LMW glycogen than HMW glycogen at 6 hours and 12 hours. An important observation was that after 12 hours starvation, HMW glycogen had elevated in treated animals compared to their respective controls.

Effects of ethanol on tissue glycogen content after starvation and refeeding studies were undertaken using two different protocols. The first part of the study was the effect of ethanol on glycogen resynthesis after voluntary chow refeeding. After 48 hours starvation, rats were given an intragastric ethanol dose of 4 g/kg or 6 g/kg and then permitted free access to food. Animals were killed at 5, 10, 24 and 48 hours after the ethanol dose. It appears that liver glycogen repletion in response to chow refeeding at 10 hours and 24 hours was not significantly inhibited by administration of 4 g/kg ethanol except at 5 hours in which inhibition was around 30%. However, a 6 g/kg ethanol dose inhibited glycogen resynthesis almost completely until 24 hours after refeeding. It is important to note that the inhibiton of glycogen resynthesis is due both to starvation and ethanol. The rate and extent of resynthesis of HMW glycogen was slower in treated rats than in control animals indicating that ethanol might preferentially inhibit the synthesis of HMW glycogen, possibly through disruption or prevention of formation of disulphide bonds in the protein component of high molecular weight glycogen.

Unlike liver, intragastric administration of ethanol (4 g/kg) before chow refeeding following 48 hours starvation decreased muscle glycogen repletion by 53% at 5 hours, by 11% at 10 hours, and by 20% at 24 hours, relative to the respective control rats. Like wise, 6 g/kg ethanol decreased the muscle glycogen level much more than a 4 g/kg ethanol dose.

Second part of the study was the effect of ethanol on hepatic glycogen resynthesis after an oral glucose load (2 mmol/100 g body wt.). The intragastric administration of ethanol 1 hour before glucose refeeding decreased liver glycogen content by 21% at 60 min, by 38% at 90 min, and by 31% at 120 min. Undoubtedly ethanol has delayed the peak reached in liver glycogen content by either decreasing glucose absorbtion, by inhibiting gluconeogenesis or glycogen synthesis, or a combination of all these factors. Although we used the same protocol as Cook *et al.* (1988), the results we obtained are significantly different from theirs.

CHAPTER 5

Effects of Repeated Ethanol Doses on Tissue Glycogen Content in Fed Rats.

Introduction:

5.1 The Effects of Chronic Administration of Ethanol on Glycogen Metabolism.

5.1.1 The Effects of Chronic Administration of Ethanol on Liver Glycogen Content.

Experimental studies in animals have shown that after chronic administration of ethanol glycogen stores are decreased in liver (Walker and Gordon, 1970; Lefèvre *et al.*, 1970; Winston and Reitz, 1980, 1981) and muscle (Palmer*et al.*, 1991, Cook *et al.*, 1992). Winston and Reitz (1980) reported that liver glycogen levels were decreased about 61% in both male and female rats after chronic ethanol ingestion. In contrast, the blood glucose concentration was decreased only in males. This suggests that there may be differences between males and females in the way ethanol affects glycogen metabolism. In an attempt to explain these data, these investigators measured six enzymes involved with the metabolism of glucose and glycogen. Sex differences were found in the effects of ethanol on the activities of glucose 6-phosphate dehydrogenase, UDPG-pyrophosphorylase and phosphoglucomutase. Glucose 6-phosphate dehydrogenase and UDPG-pyrophosphorylase activities were decreased by ethanol in males by 42 and 62%, respectively, while in females the activities were not changed. Phosphoglucomutase was decreased 29% in females, and in males was not changed. Glucose 6-phosphatase was increased and hexokinase and phosphorylase a were decreased by about the same extent in both sexes by ethanol. Although the decrease in liver glycogen may be explained by the increased utilization of glucose by peripheral tissues, the lack of a decrease in blood glucose in females argues against this. Winston and Reitz (1981) studied the chronic effects of ethanol on glycogen phosphorylase activity in rats and found that in the absence of AMP, a significant decrease in specific activity was observed in both males (19%) and females (30%). AMP addition

stimulated phosphorylase activity and completely obliterated the ethanol-induced decreases in both male and female animals. They performed kinetic studies in the absence of AMP, and found that only V_{max} had been changed by ethanol. They concluded that decreases in liver glycogen after chronic ethanol ingestion may not be related to the specific activity of glycogen phosphorylase.

5.1.2 **The Effects of Chronic Administration of Ethanol on Muscle Glycogen.**

It has long been recognised that chronic alcohol abusers have defects in skeletal muscle physiology and function, the important manifestation of which is muscle weakness. Detailed studies by various research groups have shown that the chronic form of alcoholic muscle disease occurs very much more frequently than the acute type (Martin *et al.*, 1985; Urbano-Marquez *et al.*, 1989; Peters and Preedy, 1991). Chronic abuse causes an alcoholic skeletal myopathy in up to two-thirds of alcoholic patients (Urbano-Marquez *et al.*, 1989; Peters and Preedy, 1991), which in its most severe form may involve the loss of 25% of the body skeletal muscle mass.

Although muscle has little capacity to oxidize ethanol, ethanol consumption has profound effects on muscle metabolism and function. In man, ethanol inhibits glucose uptake by leg muscles (Jorfeldt and Juhlin-Dannfelt, 1978), and decreases the stimulatory effect of prolonged exercise on glucose uptake by the exercising leg (Juhlin-Dannfelt *et al.*, 1977). In addition, ethanol inhibits conversion of glucose into lactate (Kreisberg *et al.*, 1971) and may cause acute peripheral insulin resistance and impaired glucose utilization (Shelmet *et al.*, 1988; Yki-Jarvinen *et al.*, 1988; Boden *et al.*, 1993). Ethanol also impairs glucose uptake by the isolated rat diaphragm (Clarke and Evans, 1960). These findings may imply that ethanol consumption may diminish glucose uptake and oxidation primarily via effects on skeletal muscle.

Although muscle glycogen plays a vital role in exercise, the effects of chronic ethanol intake on muscle glycogen metabolism are poorly understood. Indeed, few studies had been carried out until the 1990's. Recently, one study has focused on the chronic effects of ethanol on muscle glycogen metabolism (Cook *et al.*, 1992, see review by Palmer *et al.*, 1991). Cook *et al.* (1992) investigated whether the cause of myopathy may be related to abnormalities in carbohydrate and lipid metabolism in different muscles. Glucose metabolism was examined in two muscles with different fibre compositions, the extensor digitorum longus (EDL) muscle, which contains predominantly type II muscle fibres, and the soleus muscle, which is composed primarily of type I muscle fibres (see Table 4.1). Feeding on the ethanol-supplemented

Lieber-De Carli liquid diet for 2 or 6 weeks was associated with disturbances in glucose metabolism in both EDL and soleus muscles, particularly in relation to rates of glycogen and alanine formation.

5.1.3 **Effects of Ethanol on Energy Balance**

The effects of ethanol on energy balance are complex. Ethanol not only represents a source of calories, but also affects energy expenditure through its action on activity, as well as its effects on utilization of other nutrients (such as carbohydrates and lipids) in terms of digestion, absorption and subsequent metabolic steps.

Several studies have shown that a negative relationship between ethanol consumption and body weight exists (Jones *et al.*, 1982; Fisher and Gordon, 1985; Gruchow *et al.*, 1985; Colditz *et al.*, 1991). Colditz *et al.* (1991) have studied the relationship between alcohol intake, body mass index, and diet in 89,538 women and 48,493 men in two cohort studies and they concluded that total energy intake increased with alcohol consumption but without an associated increase in body mass. Lieber (1988, 1991) reported that equal energy replacement of dietary carbohydrate with ethanol resulted in weight loss in men. However, more recently, Rumpler *et al.* (1996) have proposed that on an energy basis ethanol and carbohydrate are utilized in the diet with the same efficiency.

There are two possible explanations for the apparent low energy value of dietary ethanol: ethanol would have to either increase the metabolic rate of the individual (Suter *et al.*, 1994) or decrease the digestibility of another component in the diet by an amount equivalent to the metabolizable energy content of the ethanol consumed. Pirola and Lieber (1972) reported weight losses in humans given ethanol as up to 25% of total energy intake as a substitute for dietary carbohydrate. They also demonstrated that when chocolate was substituted on an equal caloric basis for ethanol the subjects gained weight. Subsequently, Lieber and co-workers (1988) suggested that inefficiency of utilization of ethanol could be explained, at least in part, by the induction of the microsomal ethanol oxidizing system (MEOS). (The 16 ATP per mol of ethanol oxidized to carbon dioxide via the ADH pathway is significantly greater than the 10 ATP produced via the MEOS pathway. Thus oxidation of ethanol via the MEOS pathway would yield only 67% of the useful energy from the ADH pathway.)

Lands and Zakhari (1991) proposed an alternative mechanism for the apparent low yields of useful energy from ethanol. They hypothesized that virtually all of the energy in the ethanol molecule might be transformed to heat by a futile cycle which linked ethanol and acetaldehyde, dissipating 6 ATP equivalents at each turn. This unregulated futile cycle employs an irreversible oxidation of alcohol to acetaldehyde (using $NADPH/O_2$) (-3 ATP) and a reduction of the acetaldehyde to alcohol (-3 ATP). Such a cycle would dissipate 6 ATP equivalents for each turn of the cycle. Two or three futile cycles for each net mole of alcohol metabolism would eliminate any net calorie gain from ingested alcohol. More recently, Murgatroyd *et al.* (1996) have demonstrated that alcohol-induced thermogenesis dissipated only 15% of the alcohol energy. Alcohol addition had no significant effect on protein or carbohydrate oxidation but fat oxidation was suppressed to an extent equivalent to storing 74% of the alcohol energy. Shelmet *et al.* (1988) have also shown that infused ethanol inhibits the oxidation of fat and, to a lesser extent, of protein and carbohydrate. Both Carpenter (1940) and Weststrate *et al.* (1990) had concluded that 10% of the energy of alcohol is dissipated as post-ingestive thermogenesis.

5.1.4 **Aims of this section**

Experimental studies described earlier in this thesis had shown that ethanol affected both high (lysosomal) and low (cytosolic) molecular weight glycogen in fed animals (Chapter 3), and in the fasted and refed animals (Chapter 4). All these studies were performed with a single dose of ethanol. In Chapter 3, ethanol was shown to reduce the content of liver glycogen by about two-thirds at a 6 g/kg dose. The effect seems to be maximal at about 90 min after the ethanol dose. Although there have been some studies which investigated the effect of chronic ethanol administration via a liquid diet on tissue glycogen content and metabolism, there have been no previous studies on the effects of ethanol on glycogen content which included investigation of the effects on molecular weight distribution. The aims of the work described in this chapter were to investigate the effects of repeated doses of ethanol on tissue glycogen content and molecular weight distribution. Furthermore, we wanted to investigate the effects of ethanol on weight gain in the treated animals.

5.2 METHODS

5.2.1 Alcohol Administration for Repeated Dose-Response Experiments.

Repeated ethanol dosing experiments were performed on male Sprague-Dawley rats weighing 230-260 g. These rats were maintained at 25 ^{0}C on a 14-hour light 10-hour dark cycle. Food (standard rat chow) and water were supplied *ad libitum.* When necessary ethanol or water were administered via a stomach tube. The animals were killed by cervical dislocation.

20 male rats (8 control and 12 ethanol treated) were selected for this study. Two rats were kept in each grid bottomed cage. Initially, rats were administered a 4 g/kg dose of ethanol (in 4 ml of aqueous solution) every 12 hours for 3 days. Rats were checked every 6 hours for any signs of discomfort. We found that after 3 days the ethanol-treated rats were not gaining weight (see Table 5.1). Therefore, we subsequently reduced the ethanol dose to 3 g/kg for another 4 days.

Ethanol dosing was always carried out between 8 and 9 am and 8 and 9 pm in order to minimise diurnal variation. Control rats were given an equivalent volume of water and were killed at the same time after dosing. Rats were sacrificed 3, 12, 24 and 48 hours after the last ethanol dose. Rats were handled quietly and gently to avoid muscle contraction as much as possible and care was taken to avoid stressing the rats before killing.

5.3 RESULTS AND DISCUSSION

5.3.1 Effects of Repeated Ethanol Doses on Body Weight

Figure 5.1 and Table 5.1 show the effects of repeated ethanol doses on body weight. Despite the doses of ethanol given (4 g/kg for 3 days and 3 g/kg for 4 days), the ethanol-treated rats increased 18% in body weight during this period, compared to a 22% gain in the control rats. Table 5.1 indicates that ethanol-treated rats did not gain any weight during the first three days, when they were receiving 4 g/kg ethanol twice daily. Indeed, during this period, treated rats lost an average of about 2 grams or 1% of body weight (n=12) and control rats (n=8) gained an average of 22 g or 9% of body

weight. Subsequently, ethanol-treated rats gained 8-10 g daily (except the 8th day) and control rats gained 4.5-8 g only.

These data show disagreement with previous investigators Winston and Reitz (1981) who reported that the daily weight gain was 3.8 ± 0.8 g/day for males and 3.0 ± 0.7 g/day for females. It is not a surprise that their rats did not gain much weight compared to our rats because they administered a larger amount of ethanol (15 g of ethanol per kg body weight per day) for a longer period of time (40 days). It would appear that Winston and Reitz (1981) primarily designed the experiment to measure weight gain in male and female rats rather than for their control and treated rats. In contrast in our experiment, we administered 8 g/kg/day for the first 3 days and 6 g/kg/day for another 4 days, and all rats were male rats.

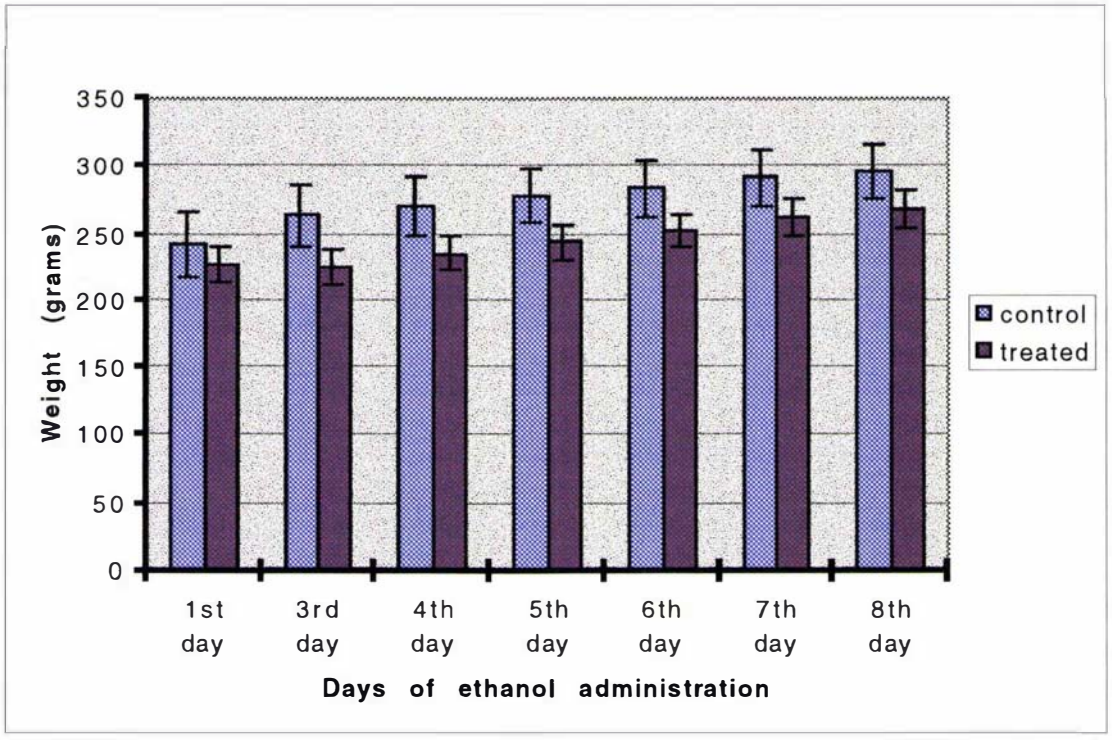

Figure 5.1 Effects of Repeated Ethanol Doses on Weight Profile

The blue and red bars represent data from controls and ethanol treated rats, respectively. Ethanol administration was described in Section 5.2.1. Data were measured from 1 to 8 days of treatment. Data presented are the mean ± S.E.M of 8 rats for control and 12 rats for treated.

Table 5.1 Effects of Repeated Ethanol Doses on Body Weight

Days of ethanol adminis-tration	Body weight (g) Control rats (n=8)	Average gain per day (g)	Body weight (g) Ethanol treated rats (n=12)	Average gain per day (g)
1st day	242.6 ± 24.7 (start of day)		227.1 ± 13.5 (start of day)	
3rd day	264.5 ± 23.0	21.9	225.5 ± 13.5	-1.6
4th day	271.6 ± 21.8	7.1	236.0 ± 13.0	10.5
5th day	279.5 ± 19.9	7.9	244.5 ± 13.4	8.5
6th day	284.2 ± 20.6	4.7	253.0 ± 12.6	8.5
7th day	292.0 ± 20.9	7.8	263.1 ± 13.5	10.1
8th day	296.5 ± 19.4	4.5	269.0 ± 13.0	5.9

An ethanol dose of 4 g/kg (every 12 hours) was administered for the first 3 days and then 3 g/kg was administered every 12 hours for 4 days and weights were measured from 1-8 days. Data are presented as the means ± S.E.M of 8 rats for control and 12 rats for treated.

Table 5.2 illustrates the effects of repeated ethanol doses on body weight and liver weight change. Body weight has increased after 8 days, by 22% in control rats and by 18.5% in ethanol treated rats. There is no significant difference in the liver weight/body weight ratio in control and treated rats.

Table 5.2 Effects of Chronic Ethanol on Body Weight and Liver Weight Change

Weight (g)	Control rats (n=8)	Ethanol treated rats (n=12)
Initial weight	242.6 ± 24.7	227.1 ± 13.5
Final weight (after 8 days)	296.5 ± 19.4	269 ± 13.0
Change (%)	+ 22.3	+ 18.5
Final liver weight (g)	12.8 ± 1.0	12.0 ± 1.1

An ethanol dose of 4 g/kg (every 12 hours) was administered for first 3 days and then 3 g/kg was administered every 12 hours, for 4 days and weights were measured from 1-8 days. Data are presented the means ± S.E.M of 8 rats for control and 12 rats for treated.

5.3.2 Effects of Repeated Ethanol Doses on Food Intake

The effects of ethanol on food intake are recorded in Figure 5.2 and Table 5.3. There was some error in measurement of food intake, as the cages were not well designed for this purpose and there was some food spillage. However, this was collected and weighted, and the final figures give a reasonable estimate of the relative food intake of two groups of animals. As the Table and Figure indicate, there was little difference in food intake between the control and treated animals, except in the measurement at 5 days where the treated rats had eaten slightly less.

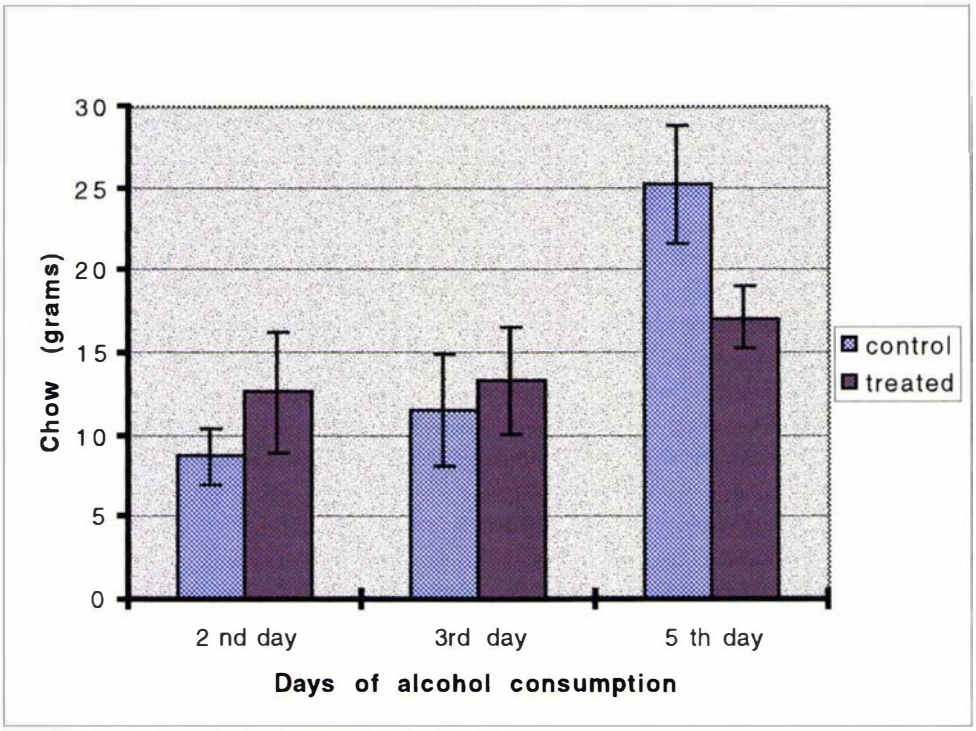

Figure 5.2 Effects of Ethanol (Repeated dose) on Food Consumption

The blue and red bars represent data from controls given water and ethanol treated rats, respectively. Ethanol administration was described under section 5.2.2. Data were measured after 2, 3 and 5 days of treatment. Data presented are the means ± S.E.M of 4 measurements on 8 rats for control and 6 measurements on 12 rats for treated animals, which were kept two per cage. An ethanol dose of 4 g/kg (every 12 hours) was administered for the first 3 days and then 3 g/kg was administered every 12 hours for 4 days.

Table 5.3 Effects of Ethanol (Repeated dose) on Food Consumption

State of the animal	2 nd day of Ethanol Consumption (g/day/rat)	3rd day of Ethanol Consumption (g/day/rat)	5th day of Ethanol Consumption (g/day/rat)
Control	8.75 ± 1.7	11.5 ± 3.4	25.3 ± 3.7
Treated	12.71 ± 3.7	13.4 ± 3.2	17.2 ± 1.9

For conditions see Figure 5.2. Data indicate food consumption in grams per rat per day and two rats were housed in each cage.

5.3.3 Effects of Repeated Doses of Ethanol on Glycogen Content in Liver

The effects of repeated doses of ethanol on liver glycogen content are presented in Figure 5.3 and Table 5.4. At 3 hours, both control and treated rats had the same amount of liver glycogen (approximately 540 μmol/g/wet weight). This batch of fed rats showed a higher than normal fed glycogen level. By 12 hours after the last ethanol dose, livers from treated rats exhibited a slightly higher glycogen content than their respective controls. Indeed, liver glycogen content in the control rats had decreased slightly, which is expected as the animals eat less during the day, whereas in the treated rats the glycogen content remained unchanged. We designed the repeated dose experiments on the assumption that repeated doses of ethanol would deplete more glycogen content than single dose experiments, but contrary to our expectation after 24 hours and 48 hours the liver glycogen content in the treated rats had increased further, to about 25% higher than in the control rats (Table 5.4).

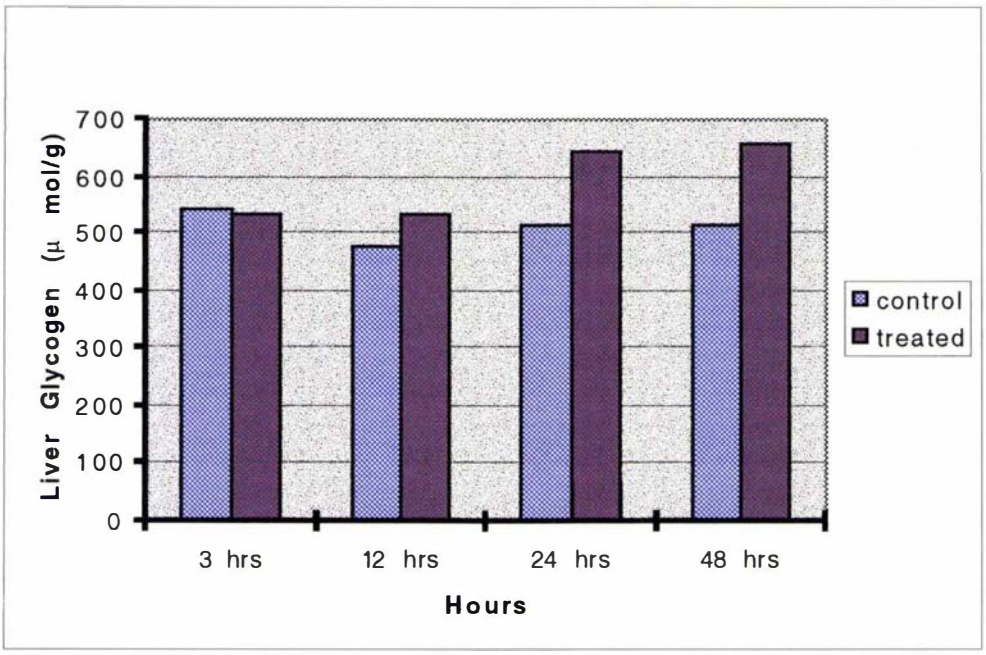

Figure 5.3 Effects of Repeated Ethanol Doses on Liver Glycogen

An ethanol dose of 4 g/kg(every 12 hours) was administered for first 3 days and thereafter 3 g/kg was administered every 12 hours, for 4 days. Animals were sacrificed after 3, 12, 24 and 48 hours respectively. Liver glycogen content was determined as described in section 2.3. Data are the average of two animals.

Table 5.4 Effects of Repeated Ethanol Doses on Liver Glycogen

Time (hours)	Glycogen (μmol/g wet weight)		Percentage of difference
	Control	Treated	
3	542, 544 (543)	519, 546 (532)	- 1.8%
12	449, 509 (479)	533, 534 (533)	+ 11.3%
24	521, 510 (515)	661, 625 (643)	+ 24.8%
48	516, 511 (514)	737, 578 (657)	+ 28.5%

An ethanol dose of 4 g/kg (every 12 hours) was administered for first 3 days and then 3 g/kg was administered every 12 hours, for 4 days and animals were sacrificed after 3, 12, 24 and 48 hours respectively. Liver glycogen content was determined as described in section 2.3. Data are individual results for two animals, with the average shown in parentheses.

The experiments described in Chapter 3 were designed to address the hypothesis that a single dose of ethanol would cause depletion of liver glycogen in fed rats. In that part of the research, rats were administered an ethanol dose of 2, 4 or 6 g/kg. Depletion of liver glycogen compared to the respective control animals was observed at each ethanol dose, with the 2 g/kg giving 16-27% depletion, 4 g/kg giving 44-64% depletion and 6 g/kg giving 61-78% depletion. The depletion of liver glycogen was maximal at 90 min after ethanol dosing (section 3.3.2.1). Overproduction of glycogen with resynthesis following ethanol-induced depletion peaked at 24 hours. After 12 hours there was no overproduction of glycogen in ethanol-treated rats given a single ethanol dose. Glycogen depletion followed by overproduction was also seen in starved-refed rats, in the experiments described in Chapter 4.

As indicated above, the surprising observation in the repeated dose experiments described here is that although no depletion of liver glycogen content was observed at 3 or 12 hours, a marked oversynthesis was observed at 24 and 48 hours. Generally, glycogen oversynthesis follows depletion, as seen in the earlier experiments in this Thesis.

One possible explanation for the failure to observe glycogen depletion is that glycogen content might have depleted between 0 and 3 hours and another reason could be that elevation of the glycogen level might have followed earlier depletion, after the ethanol dose given at 8 - 9 pm the previous evening. The results certainly indicate that with the

ethanol dosing regime used here, and the tissue sampling times used, there was no evidence that 7 days of continual ethanol intake caused depletion of liver glycogen content. These results contrast with others that have been published.

In another published study (Lefèvre *et al.* 1970) using rats pretreated with alcohol (3 g/kg) for 3 days, hepatic glycogen was decreased. Glycogen content from control animals was 37 mg/g (205 μmol/g) whereas the ethanol treated animals showed 20 mg/g (111 μmol/g). Walker and Gorden, (1970) reported that isocaloric replacement of either the fat or carbohydrate content of the diet by ethanol (36% of the total caloric intake) decreased the liver glycogen content. Winston and Reitz (1980) found that liver glycogen levels were decreased about 61% in both male and female rats after chronic ethanol ingestion. In contrast, blood glucose concentration was decreased only in males, indicating different mechanisms between the sexes. Winston and Reitz (1980, 1981) used a specifically formulated low fat liquid diet containing 36% of calories as ethanol.

From the foregoing, it is apparent that all the three investigators found the same results, namely that chronic treatment with alcohol depleted liver glycogen content. This contrasts with our finding that liver glycogen content was higher in the ethanol treated rats. We attribute the difference between the results of this study and those of others to the methodology adopted in supplying the food and ethanol together. In the other studies, liquid diet was used as opposed to our standard laboratory pellets. Additionally, the amount of ethanol, and the duration of ethanol administration were also different in their studies.

The increase in liver glycogen content at 48 hours in ethanol-treated rats is apparently due to an increase in the content of both low and high molecular weight glycogen, as shown in Figure 5.4.

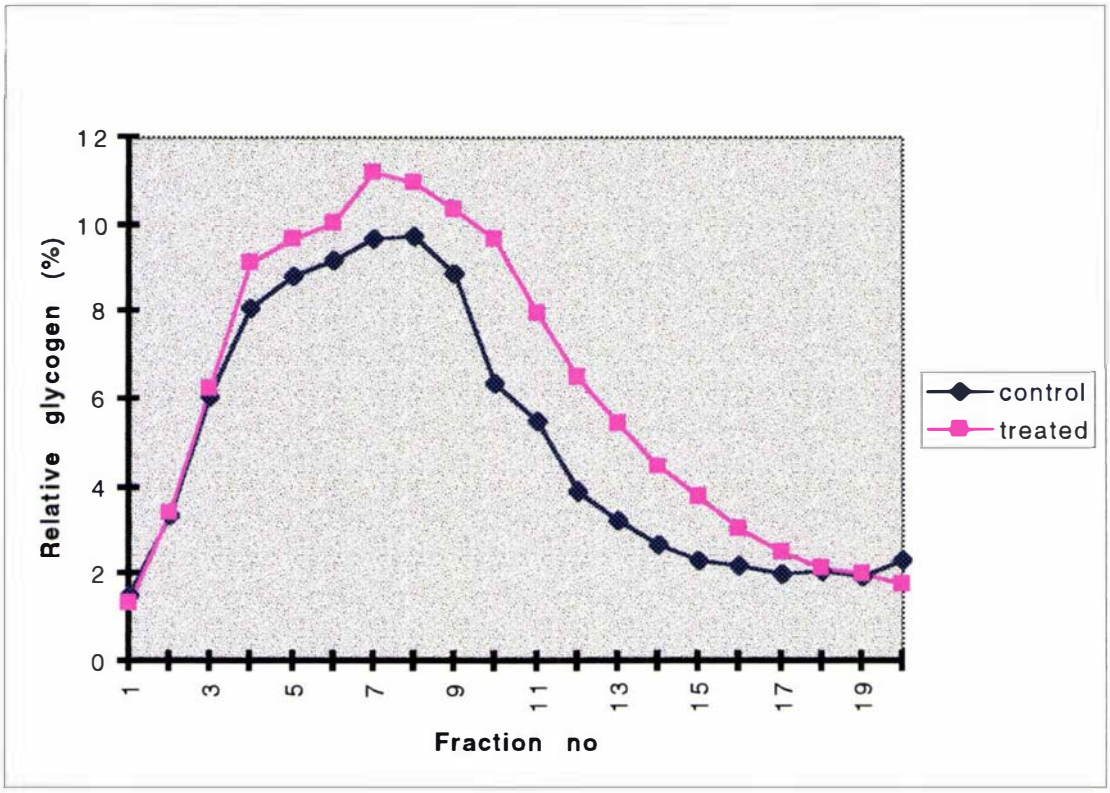

Figure 5.4 Molecular Weight Distribution of Liver Glycogen with Repeated Doses

An ethanol dose of 4 g/kg (every 12 hours) was administered for first 3 days and thereafter 3 g/kg was administered every 12 hours, for 4 days. Rats were sacrificed after 48 hours. Molecular weight distributions were determined as described in section 2.7. Each point represents the mean of 3 determinations from individual gradients.

There has been no previous report of glycogen rebound in the liver of rats receiving repeated ethanol doses. This study does not provide any indication of the reason for this increase, except that it appears to occur almost uniformly over all molecular weight ranges. However, it is unlikely that the increase in glycogen content in the treated rats is an artefact caused by changes in liver weight. As indicated earlier in this section, the liver weight/body weight ratio was the same in treated and control animals. Recently the mechanism of the glycogen content increase in the liver of 72 hours fasted rats was examined by Minassian *et al.* (1994) (see section 4.3.3). They found that the increase in liver glycogen does not involve glycogen synthase activation or glycogen phosphorylase inhibition. It may be dependent on the concentration of the precursor substrate of glycogenesis, i.e glucose 6-phosphate.

5.3.4 **Effects of repeated Ethanol Doses on Kidney Glycogen**

The effects of repeated doses of ethanol on kidney glycogen content are presented in Fig. 5.5 and Table 5.5. Kidney glycogen content decreased by 6-26% in the treated animals compared with the controls and the depletion of kidney glycogen was maximal at 24 hours after ethanol dosing. Experiments were carried out only on two rats, but the depletion in kidney glycogen content is consistent between the two rats at each time interval from 3-24 hours. The decrease at 48 hours is very small. It is interesting that the effect of repeated ethanol doses on kidney glycogen is opposite to that seen on liver glycogen content, where an increase followed 7 days of repeated ethanol dosing. There have been no previous studies on the effect of ethanol on kidney glycogen content.

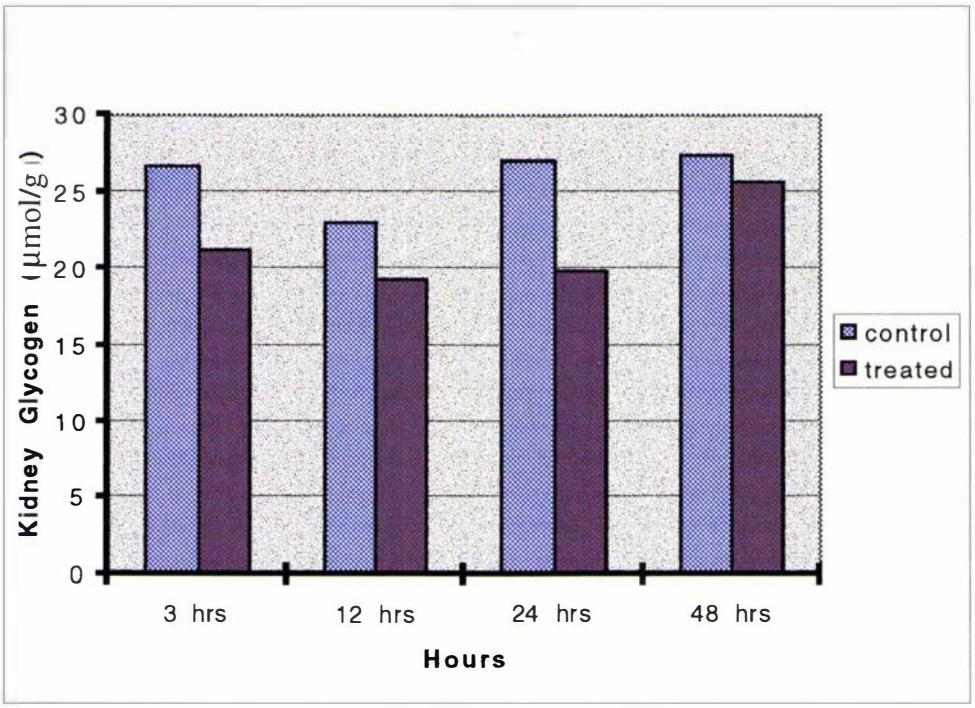

Figure 5.5 Effects of Repeated Ethanol Doses on Kidney Glycogen

An ethanol dose of 4 g/kg (every 12 hours) was administered for first 3 days and thereafter 3 g/kg was administered every 12 hours, for 4 days. Animals were sacrificed after 3 hours, 12 hours, 24 hours and 48 hours respectively. Kidney glycogen content was determined as described in section 2.3. Data are the average of two animals.

Table 5.5 Effects of Repeated Ethanol Doses on Kidney Glycogen

Time (hours)	Glycogen (μmol/g wet weight)		Percentage of difference
	Control	Treated	
3	25.8, 27.5 (26.6)	20.7, 21.9 (21.3)	- 18.6
12	22.5, 23.4 (23.0)	18.9, 19.8 (19.4)	- 15.7
24	27.0, 27.0 (27.0)	18.9, 20.7 (19.8)	- 26.7
48	27.9, 27.0 (27.4)	25.6, 25.6 (25.6)	- 6.55

An ethanol dose of 4 g/kg (every 12 hours) was administered for first 3 days and then 3 g/kg was administered every 12 hours, for 4 days and animals were sacrificed after 3, 12, 24 and 48 hours respectively. Kidney glycogen content was determined as described in section 2.3. The average is shown in parentheses.

5.3.5 Effects of Repeated Ethanol Doses on Muscle (Hind limb) Glycogen and Heart Glycogen

Table 5.6 and 5.7 illustrate the effects of repeated ethanol doses on muscle and heart glycogen. Despite two doses of ethanol per day being given to the rats for seven days, there were no effects on muscle and heart glycogen content. In another study of the effects of chronic ethanol intake on muscle glycogen content, Cook *et al.* (1992) reported that the pattern of changes in glycogen contents of individual skeletal muscles in response to chronic ethanol feeding for 2 weeks was somewhat conflicting. Animals were anaesthetised under halothane anaesthesia and the individual muscles and liver were rapidly dissected out and freeze-clamped in aluminium clamps precooled in liquid nitrogen. They found that there was no correlation between muscle fibre composition and glycogen storage. They observed that the glycogen contents of the EDL and quadratus lumborum muscles (both type II muscle fibres) were significantly lower in ethanol-fed rats, by 14% and by 29% respectively. In contrast, the glycogen contents of other type II fibre rich muscles were not significantly altered after 2 weeks of ethanol feeding. Their findings imply that muscle fibre composition is not the only factor which determines the effects of chronic ethanol feeding on muscle glycogen content.

In order to identify the site of the biochemical lesions involved with chronic ethanol treatment, Cook *et al* (1992) also examined muscle metabolism with isolated muscle preparations *in vitro*. They selected two skeletal muscles with different fibre compositions, the EDL (type II) and soleus (type I) fibres. At the end of 2 weeks or 6

weeks, soleus and EDL muscles were dissected out of the hind-legs and incubated in Krebs-Ringer bicarbonate buffer, pH 7.4, supplemented with 1% bovine serum albumin and 5 mM glucose. The incubations were terminated by the addition of perchloric acid into the medium. They found the glycogen content of EDL muscle alone was reduced by 35% after 2 weeks of ethanol feeding, although rates of glycogen synthesis were significantly impaired in both the soleus (by 29%) and EDL (by 33%) muscles. In contrast to 2 weeks, chronic ethanol feeding for 6 weeks had no significant effects on muscle glycogen content, although there were some abnormalities in glucose metabolism particularly in the soleus muscle. The reason why 2 weeks ethanol feeding selectively decreased the EDL muscle glycogen content remains to be established.

Table 5.6 Effects of Repeated Ethanol Doses on Muscle (Hind limb) Glycogen

Time (hours)	Glycogen (μmol/g wet weight)	
	Control	Treated
3	27.7, 33.3 (30.3)	29.7, 33.3 (31.5)
12	33.8, 27.9 (30.8)	33.3, 34.6 (34.0)
24	42.8, 47.7 (45.2)	47.2, 44.1 (45.6)
48	47.7, 46.8 (47.2)	46.8, 45.0 (45.9)

An ethanol dose of 4 g/kg (every 12 hours) was administered for first 3 days and then 3 g/kg was administered every 12 hours, for 4 days and animals were sacrificed after 3, 12, 24 and 48 hours respectively. Muscle (hindlimb) glycogen content was determined as described in section 2.3. Data are for two animals with the average shown in parentheses.

Table 5.7 Effects of Repeated Ethanol Doses on Heart Glycogen

Time (hours)	Glycogen (μmol/g wet weight)	
	Control	Treated
3	18.9, 20.0 (19.4)	20.4, 21.2 (20.8)
12	21.2, 19.8 (20.5)	18.9, 20.2 (19.6)
24	21.3, 19.8 (20.5)	20.2, 20.9 (20.6)
48	22.5, 20.7 (21.6)	20.2, 18.9 (20.0)

An ethanol dose of 4 g/kg (every 12 hours) was administered for first 3 days and then 3 g/kg was administered every 12 hours, for 4 days and animals were sacrificed after 3, 12, 24 and 48 hours respectively. Heart glycogen content was determined as described in section 2.3. Data are for two animals with the average shown in parentheses.

In our study, hindlimb samples which contain both type I (soleus) and type II (EDL) muscles were used. It is perhaps not surprising that the small decrease in glycogen content seen by Cook et al. (1992) in EDL muscles in freeze-clamping experiments was not observed in this study, as the ethanol treatment was carried out for only 7 days, and mixed muscle samples were taken. The larger decrease seen in incubated EDL muscle samples by Cook *et al.* (1992) after two weeks treatment is difficult to explain considering that at six weeks, no decrease was observed. Overall it seems safe to conclude from the results of both this study and that of Cook et al (1992) that short-term repeated ethanol dosing has very little effect on muscle glycogen content.

5.3.6 **Conclusions**

This chapter was designed to address the effects of repeated doses of ethanol on tissue glycogen content and molecular weight distribution. These studies were initiated with the expectation that repeated ethanol doses might deplete liver glycogen content more than single doses had done. Contrary to this expectation, in animals killed at 12, 24 and 48 hours after the 7 days of administration of ethanol finished, the liver glycogen content in the ethanol treated rats had increased relative to that in the control rats. Indeed in the treated rats after 24 hours and 48 hours, liver glycogen content was as much as 25% higher than that in the control rats. It was also evident that both low and high molecular weight glycogen had increased almost uniformly at 48 hours in the ethanol treated rats. It appears that while there is a depletion of liver glycogen content with a single dose of ethanol, with repeated dosing there may be an adaptation to counteract this effect which leads, on cessation of alcohol intake, to oversynthesis of liver glycogen. Ethanol, however, had decreased kidney glycogen content by 6-26% in the treated rats compared with the control rats. Further it was observed that the depletion of kidney glycogen was maximal at 24 hours after ethanol dosing. Significantly there were also no effects on muscle and heart glycogen, although two doses of ethanol were given daily for seven days.

CONCLUSIONS AND FUTURE WORK

The results reported in this thesis have expanded current knowledge about effects of ethanol on glycogen structure and metabolism in liver and muscle.

Ethanol affected liver glycogen levels in a dose-dependent and time-dependent manner in fed animals. A single dose of ethanol was shown to cause glycogen depletion in liver of fed and fed-to-starved animals. Ethanol was found to inhibit glycogen resynthesis in starved-refed animals, in both chow refeeding and glucose intubation experiments. The inhibition observed was not as great, however, as that seen in other studies.

Carefully controlled methodology should be adopted in future studies to differentiate starvation-induced and ethanol-induced glycogen depletion in different physiological conditions. Food intake should also be calculated accurately to meet this objective.

The results of this thesis confirm that ethanol-induced overproduction of glycogen occurs in fed, fed-starved and starved-refed animals in both single and repeated dose experiments. This finding is potentially of great importance to exercise physiology and sports science, in helping to develop recommendations for alcohol intake during training regimes. However, further experiments are needed to confirm the results of this study on the effects of repeated ethanol doses on liver and muscle glycogen content as only a single experiment was performed.

Glucose disposal on carbohydrate refeeding after starvation is a multistep process. How ethanol affects the two largest stores of glycogen within the body, liver and muscle, requires further research. This research should take into account ethanol metabolism, intestinal glucose absorption, extrahepatic glycogen deposition, extrahepatic glucose uptake, glycogen cycling, conversion of glucose to lactate and the Cori cycle.

In the present study, the effects of ethanol on tissue glycogen content after chow refeeding were monitored for a set starvation time (48 hours) and at intervals of 5, 10, 24 and 48 hours after refeeding. In future work, to extend those experiments, animals could be starved for various times (e.g, 3, 6, 12, 24 and 48 hours). After administration of ethanol, animals could be killed for tissue sampling at shorter time intervals (e.g. 30 min, 1, 2, 3, 4, and 5 hours). At the same time, it would be important to monitor the

food intake closely. Such experiments would help to clarify the role of ethanol in inhibiting glycogen resynthesis following ethanol intake.

The effect of ethanol on lysosomal glycogen metabolism also requires further investigation. In particular, the effects of ethanol on the uptake of glycogen into lysosomes could be studied. The breakdown of lysosomal glycogen is important since this has a role in controlling the liberation of free glucose.

It has recently been proposed that the amount of glycogenin will influence how much glycogen the cell can store. The rise and fall in glycogen content that occurs in response to glucose intake can be accounted for by oscillation between the low (proglycogen) and high (macroglycogen) molecular weight forms of glycogen (Alonso *et al.,* 1995). How ethanol interacts or regulates the balance of glycogenin, proglycogen and macroglycogen may have important implications in the understanding and management of ethanol-induced hypoglycemia, non-insulin dependent diabetes and for exercise physiology.

Since chronic alcohol abuse is one of the most important human health problems, repeated dose experiments in both male and female animals need further investigation. In light of the results reported from this thesis, future experiments should take into account the following factors: length of the ethanol dosing period, diurnal variation of glycogen content; food intake; and weight gain. Repeated dose experiments could also be carried out in different physiological situations, such as resting and exercised animals.

It would be worthwhile to investigate the combined effects of ethanol and exercise on muscle glycogen content. Different muscles have different amounts of glycogen and some muscles function oxidatively while others do not. Glycogenesis increases during exercise in exercising muscles and glycogen sparing during exercise is promoted by fatty acids and probably glucose. It is possible that ethanol would have a similar effect.

In summary, there is tremendous scope for further investigation of both the acute and chronic effects of ethanol on structure and metabolism of glycogen in liver and skeletal muscle and also in other tissues.

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