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THE EFFECT OF ETHANOL ON LIVER GLYCOGEN OF FED ANIMALS

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ABSTRACT

A hyperglycemic effect of ethanol has been reported in fed animals, but is poorly documented and in general, little work has been done on the effects of ethanol on carbohydrate metabolism in the fed state. This study is a further extension of research investigating the effect of ethanol on liver carbohydrate metabolism, as liver is a major source of glucose output in fed animals. It has been suggested that the hyperglycemic condition might be caused by an ethanol-stimulated liver glycogenolysis.

Because it was possible that the actions of ethanol were a direct effect on carbohydrate stores rather than an effect mediated by hormones, ethanol was tested on a simple unicellular organism *Ochromonas danica* where hormonal mechanisms are absent. It appears that ethanol does cause a reduction of *Ochromonas* carbohydrate stores in the absence of hormones. The major difficulty in using this organism was that its carbohydrate content could change according to the osmotic pressure in the external environment which therefore had to be strictly controlled.

Following the initial work using *Ochromonas danica*, further experiments were carried out using fed rats to assess the effects of ethanol on tissue glycogen stores. Sprague-Dawley rats were administered with an acute dose of ethanol (6g/kg). The effect of ethanol on the liver glycogen content of the animals was examined at 45, 90 and 180 minutes after the dose given. It appears that ethanol would lead to a significant decrease in liver glycogen content in both male and female rats at any given time. However, the decrease was not as much as that reported in the literature. Presumably this is due to the differences in ethanol administration, assays of liver glycogen and the strains of animals used in the experiment. The glycogen content in other tissues such as heart, kidney and muscle was also investigated but little difference was observed with ethanol treatment except in muscle, which showed some increase in glycogen content especially in the males. It is interesting that the free glucose concentrations in these tissues were not elevated as might have been expected if liver glycogen breakdown had occurred. Moreover, ATP levels were also observed to be unchanged.

The female rats were found to metabolise ethanol at a slower rate than males. The ethanol concentration in their extrahepatic tissues was similar to the calculated theoretical value for initial ethanol absorption. However in male rats, it is lower than the theoretical value. This indicates that the ethanol clearance curve for these tissues was not linear, and this implies that other factors such as delayed absorption or a first-pass effect (Lieber et al., 1994) might occur.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

GENERAL

ATP	adenosine-5'-triphosphate
BSA	bovine serum albumin
cAMP	cyclic adenosine 3',5'-monophosphate
EDTA diNa	sodium ethylenediamine tetra acetic acid
FRS	free reducing sugar
H^+	hydrogen ion
H_2O	water
HCl	hydrochloric acid
$KHCO_3$	potassium hydrogen carbonate
$KClO_4$	potassium perchlorate
KOH	potassium hydroxide
$MgCL_2$	magnesium chloride
NAD^+	nicotinamide adenine dinucleotide (oxidised form)
NADP	nicotinamide adenine dinucleotide phosphate (reduced form)
OAA	oxaloacetate
PCA	perchloric acid
PEG	polyethylene glycol
RS	reducing sugar
αKG	α-ketoglutarate

ENZYMES

ADH	alcohol dehydrogenase
AGS	amyloglucosidase
G6PD	glucose 6-phosphate dehydrogenase
GP	glycogen phosphorylase
GS	glycogen synthase
HK	hexokinase
MDH	malate dehydrogenase
PhK	phosphorylase kinase
PKA	protein kinase A
PP1G	protein phosphatase 1G

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Chapter 1

INTRODUCTION

1.1 GENERAL

Ethanol is a primary alcohol which is completely miscible with water. Because of its small molecular size and weak charge, it passes freely and easily through all membranes of the body by passive diffusion. The most common route of ethanol entering the blood is absorption through the gastrointestinal tract, mostly from the small intestine, and to a lesser extent, from the stomach and large intestine. Absorption continues as long as a concentration gradient exists between the gastrointestinal tract and the blood in the capillary network. The rate of absorption varies considerably among individuals and depends on factors such as: concentration and type of alcohols, rate of consumption, presence of food in the stomach, physical exercise, disease, and drugs (Wilkinson, 1980). Ethanol is mainly oxidised in liver which accounts for 70-95 % of the oxidation (Lundquist, 1971; von Wartburg, 1971), although some other tissues (eg., kidney, muscle, lung, intestine, and possibly even brain) may metabolise ethanol in smaller quantities (Kirka and Clark, 1979; Pawan, 1972).

Ethanol has been known to affect carbohydrate metabolism for many decades. It is generally accepted that ethanol affects carbohydrate metabolism via its metabolites acetate and acetaldehyde, and via the effect of a change in NAD^+/NADH ratio, on the intermediary metabolism. When ethanol is added to the perfusion of livers from fed rats, it stimulates hepatic glucose output (Topping et al., 1979; Topping et al., 1982). In addition, blood glucose levels were reported elevated in fed animals administered ethanol orally (Potter and Morris, 1979). Hyperglycemia was also observed in fed subjects after heavy drinking of alcohol (Shelmet et al., 1988; Sneyd, 1989). As liver is the major source of glucose output, it is likely that ethanol affects carbohydrate metabolism in liver and hence contributes to the high blood glucose in fed subjects. One recent experiment has suggested that ethanol stimulates glycogenolysis in livers from fed rats (Kubota et al., 1992).

The present study is an extension of research to investigate further the effects of ethanol on carbohydrate metabolism in liver. Since the hyperglycemic effect of ethanol has been ascribed to effects on the secretion of catecholamines and glucagon (Erwin and Towell, 1983), one interesting experimental approach is to use a model carbohydrate

system where no hormones are involved, to see whether ethanol has any direct effect on carbohydrate stores. In the present study, a single cell organism Ochromonas danica is used, and the direct effects of ethanol on its carbohydrate reserve are observed by growing the cells in ethanol-treated medium. In addition, further experiments are carried out to investigate the effects of ethanol on glycogen content in liver as well as other tissues (muscle, heart, kidney) to clarify the effects of ethanol on glycogen stores as reported in literature. Little work has been done with isolated human tissues for obvious reasons, and in the current experiments rats are used as experimental animals.

1.2 ETHANOL METABOLISM

Ethanol is primarily oxidised in the liver (Li, 1977). In mammals, ethanol elimination is simply a two-step system in which ethanol is oxidised to acetaldehyde and then to acetate. The metabolism is considered finished at the stage of acetate since acetate is a naturally-occurring metabolite in the body. The main enzyme catalysing the initial conversion of ethanol to acetaldehyde is alcohol dehydrogenase (ADH) (EC 1.1.1.1) located in the cytosol (the soluble fraction of the cell). The catalysed reaction is readily reversible and is subject to product inhibition by acetaldehyde, and NADH.

$$C_2H_5OH + NAD^+ \overset{ADH}{\rightleftharpoons} CH_3CHO + NADH + H^+$$

The enzyme has been extensively studied in many respects including, for many of its isozymes, their location, structure and substrate specificities.

1.2.1 Alcohol dehydrogenase system

Alcohol dehydrogenase (ADH) is an enzyme capable of catalysing the oxidation of a large variety of alcohols including ethanol. Human and rodent ADH exists as a heterogenous group of isozymes that can be placed in three classes based on structural and functional distinctions (Strydom and Vallee, 1982; Algar et al., 1983). Class I ADH isozymes include homodimers and heterodimers of the closely related α, β and γ protein chains. Class II ADH is composed of homodimers of the π protein chain, and class III is composed of homodimers of the χ protein chain (Duester, 1991). Most isozymes are dimers with polypeptides of about 40,000 in molecular weight (Jörnvall et al., 1989). The subunits

consist of two domains; one catalytic domain and one coenzyme-binding domain. The active site is situated between the two domains.

Under physiological conditions (pH 7.5), class I isozymes have a wide substrate specificity and generally have low K_m values (1.4 mM) for ethanol as a substrate (Boleda et al., 1989). Class I ADH accounts for 96% of total ADH activity in mammals (Boleda et al., 1989). Liver is the major organ for ethanol oxidation because it contains more Class I isozyme (90% hepatic) than any other organs. Class I is also found in other tissues like lung, intestine and kidney but in very small amounts (6% extrahepatic) (Boleda et al., 1989). Class II is especially concentrated in the external organs: buccal and nasal mucoses, esophagus, stomach, rectum and sexual organs (Boleda et al., 1989). It also oxidises ethanol but with a k_{cat}/K_m value 100 times less than that of the class I (Burnell and Bosron, 1989). This suggests that class II ADH is less efficient in oxidising ethanol. Moreover, it has intermediate K_m values (5 mM) and a high k_{cat} (1000 min^{-1}) for all substrates. These constants imply that the class II ADH will be active and participate in ethanol disposal when ethanol concentrations are high. This was supported by the finding that stomach and small intestine activities represented 10% of the liver ADH activity at 33 mM ethanol concentration (Boleda et al., 1989). However, one recent report has indicated that the stomach has significant low K_m ADH activity (Mirmirian-Yazdy et al., 1993). Class III isozyme is found present in all organs but has poor activity with ethanol. The k_{cat}/K_m value is 1000 times lower than the values at pH 7.5 for the class I (Vallee and Bazzone, 1983). Thus it oxidises long-chain alcohols more efficiently than it oxidises ethanol. Recently, a new isozyme of ADH has been purified from human stomach (Moreno and Pares, 1991). It exhibits a high K_m for ethanol (41 mM) and it has an extraordinarily high activity (Moreno and Pares, 1991). This enzyme is responsible for 30-50 % of the overall ADH activities in the stomach and is mainly responsible for the gastric first pass metabolism of ethanol (Seitz et al., 1994).

Although class I ADH is undoubtedly the dominant enzyme in catalysing the conversion of ethanol to acetaldehyde in mammalian liver, there is evidence to suggest that other systems may make some contribution (Shigeta et al., 1983). These alternate or minor systems still remain controversial but research in the past ten years has greatly increased our understanding of the individual systems. They are the microsomal ethanol oxidising systems (MEOS), located in the endoplasmic reticulum; and the catalase system, located in the peroxisomes.

1.2.2 Microsomal ethanol oxidising systems (MEOS)

MEOS are the most studied non-ADH systems of ethanol metabolism (Lieber, 1983). It is recognised that MEOS includes both enzymic ethanol oxidation catalysed by a group of cytochrome P-450s and nonenzymic oxidation by an iron-catalysed reaction. The cytochrome P-450 portion oxidises ethanol by the following reaction which depends upon NADPH and oxygen.

$$NADPH + H^+ + O_2 + C_2H_5OH \longrightarrow CH_3CHO + NADP^+ + 2H_2O$$

It has been reported that P-450 was responsible for 30% of microsomal activity in untreated rabbits, but after ethanol treatment, the enzyme was responsible for 70-80% of the microsomal activity (Koop et al., 1984). Similar results were observed with microsomes from ethanol-treated rats (Koop, 1989) and deermice (Handler et al., 1988). The system appears to have a relatively high K_m for ethanol (10 mM). This indicates that it accounts for an increasing percentage of ethanol oxidation at high ethanol concentrations (Lieber, 1983). The nonenzymic oxidation of ethanol involves an oxygen species which has the characteristics of the hydroxyl radical ($^{\cdot}OH$). The uncoupling of the microsomal electron transport system results in the one- and two-electron reduction of oxygen to superoxide and hydrogen peroxide, respectively (White and Coon, 1980). The H_2O_2 formed participates in ethanol oxidation when a reduced metal chelate is present (most likely iron-EDTA). The latter facilitates a classical metal-dependent reduction of H_2O_2 (Fenton reaction) as follows (Halliwell and Gutteridge, 1986).

$$M^{n+} + H_2O_2 \longrightarrow M^{(n+1)+} + OH^- + {}^{\cdot}OH$$

Physiologically reduced iron chelates can catalyse an H_2O_2-dependent oxidation of ethanol, but this reaction may be limited by the availability of H_2O_2 and reduced iron-chelate.

1.2.3 Catalase system

It is well established that catalase compound I can oxidise ethanol to acetaldehyde. The enzyme is found in all mammalian tissues and is located in the peroxisomal fraction of the liver (deDuve, 1974). The peroxidatic reaction of catalase is well characterised and has been

reviewed extensively (Chance et al., 1979). The mechanism of action is shown below.

$$\text{catalase} + H_2O_2 \rightleftharpoons \text{catalase } H_2O_2 \text{ (compound I)}$$

$$\text{catalase } H_2O_2 + C_2H_5OH \longrightarrow \text{catalase} + CH_3CHO + 2H_2O$$

Generally, it is accepted that the rate of the peroxidatic reaction of catalase is dependent on the rate of H_2O_2 production, but it has been reported that ethanol concentration and catalase heme concentration could also affect the rate of action (Oshino, et. al., 1973). Apparently, the question of participation of catalase in ethanol metabolism is a question of the rate of hydrogen peroxide formation. This rate has been reported to be 0.05-0.1 μ mole/min/g wet weight for rat liver (Sies, 1974). Although the physiological rates of H_2O_2 production are too low to make a significant contribution in ethanol elimination, addition of fatty acids can raise the rate of hydrogen peroxide formation to 0.17 μmol/g/min (Thurman et al., 1975), which may be sufficient to account for the non-ADH system, at least at low ethanol concentrations. Some experiments suggest that the nutritional state is an important factor regarding the rate of ethanol oxidation (Handler and Thurman, 1985).

1.2.4 Acetaldehyde dehydrogenase

Unlike ADH, which is located primarily in the liver, acetaldehyde dehydrogenase (AlDH) is located in virtually every organ in rats with the majority of activity situated in liver (Deitrich, 1966). Essentially 90-95% of the acetaldehyde produced from ethanol oxidation is metabolised to acetate by AlDH in liver (Eriksson, 1977). The small amount of acetaldehyde that is not metabolised in liver could be oxidised by aldehyde dehydrogenase found in various tissues throughout the body (Weiner, 1979). In addition, some acetaldehyde can bind to proteins, both in hepatic and non-hepatic tissues (Lin et. al., 1990; Sillanaukee et. al., 1992).

Also, unlike ADH, which is found exclusively in the cytosol, AlDH is found in microsomes and mitochondria as well as in the cytosol. The molecular weight of highly purified liver AlDH is reported to be over 200,000 and the subunit is about 50,000-60,000 (Duncan, 1977). The enzyme is typically a tetramer (Pietruszko, 1983; Weiner and Wang, 1994), though some forms are dimeric. Two minor forms have been noted on gel electrophoretic analysis (Harada et al., 1980), but they have

been identified as different enzymes of other substrate specificities: glyceraldehyde-3-phosphate dehydrogenase (Rizlak and Pietruszko, 1985) and glutamyl-γ-semialdehyde dehydrogenase (Forte-McRobbie and Pietruszko, 1986).

Acetaldehyde is primarily formed and metabolised in liver after ethanol ingestion. This is an irreversible reaction and is shown below.

$$CH_3CHO + NAD^+ + H_2O \xrightarrow{AlDH} CH_3COO^- + NADH + 2H^+$$

Because acetaldehyde is toxic and not a natural metabolite, it is necessary for the body to effectively remove it. In rat liver, it has been shown that AlDH exists in two forms. One form has high affinity for acetaldehyde (K_m = 1-25 μM), while the other form has a poor affinity (K_m >1 mM) (Greenfield, 1976). Most researchers appear to agree that in rats, the low K_m enzyme exists in mitochondria whereas the high K_m form is associated with the cytosolic or microsomal fractions (Marjanen, 1972; Horton and Barrett, 1975; Siew et. al., 1976). The microsomal enzyme appears to be specific for long chain fatty aldehyde substrates (Nakaysu et al., 1978). Kidney, brain and skeletal muscle were investigated with respect to their ability to oxidise acetaldehyde (Cederbaum and Rubin, 1977). The results supported the above conclusion that the bulk of oxidation was related to a mitochondrial activity. In a more recent investigation, studies performed with liver slices obtained from different animals have shown that acetaldehyde is primarily oxidised by the mitochondrial matrix space enzyme (Cao et al., 1988). During moderate drinking, when the blood ethanol concentration is below 5 mM and the liver acetaldehyde level is below 100 μM, ethanol oxidation occurs primarily in mitochondria. During heavy drinking, when ethanol levels reach as high as 25-40 mM and the liver acetaldehyde level is as high as 200 μM, some may be oxidised in the cytosolic fraction (Weiner, 1979).

The end product of ethanol metabolism, acetate, is a common metabolite of carbohydrate, fat and some amino acids. It is readily converted to CO_2 via the tricarboxylic acid cycle (TCA cycle), or can undergo reactions to form fatty acids, ketone bodies, amino acids, and steroids via its activated form acetyl CoA (Lieber, 1984; Skutches et al., 1983).

1.2.5 NADH

1.2.5.1 Reoxidation of endogenous NADH

Under normal conditions when ethanol is absent, NADH is formed by glycolysis in the cytoplasm and the TCA cycle in the mitochondrial matrix. NADH produced by these intracellular processes must be reoxidised back to NAD^+ for them to continue. The reoxidation is mainly fulfilled by the electron transport chain situated in the inner mitochondrial membrane.

NADH generated by the TCA cycle can be readily reoxidised in the electron transport system. However, NADH produced in the cytoplasm by glycolysis cannot be transferred and reoxidised in mitochondria since the inner mitochondrial membrane is impermeable to NADH. Instead the reducing equivalents produced by oxidation of NADH in the cytosol are transferred to the mitochondria by means of specific shuttle mechanisms, primarily the malate-aspartate shuttle (Nordmann et al., 1975) mediated by two trans-membrane carriers and four enzymes. This is illustrated in Fig.1. Electrons from cytoplasmic NADH are transferred to oxaloacetate (OAA) in the cytosol, forming malate. This reaction is catalysed by malate dehydrogenase (MDH). Malate is then transported into mitochondria, via a specific carrier catalysing a one-for-one exchange of malate for α-ketoglutarate (αKG), where it is oxidised to OAA with the concomitant production of NADH. NADH can now be oxidised in the electron transport system. Because OAA transport through the mitochondrial membrane is very low at physiological OAA concentrations, transaminations on each side of the inner membrane are required to complete the cycle. Aspartate, generated in the mitochondria from OAA via aspartate aminotransferase is transported to the cytosolic side. This is achieved by the glutamate-aspartate cotransport system which exchanges glutamate for aspartate. Aspartate is subsequently transaminated with αKG by a similar enzyme in the cytosol to produce OAA. The transport of aspartate and αKG to the cytosol for the latter enzyme reaction occurs in exchange with glutamate and malate, respectively (Williamson and Tischler, 1979).

1.2.5.2 NADH from ethanol oxidation

As discussed in the previous sections, ethanol oxidation requires NAD^+ but generates NADH via the alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (AlDH) reactions. This results in a decrease in NAD^+/NADH ratio (Christensen and Higgins, 1979) because the liver

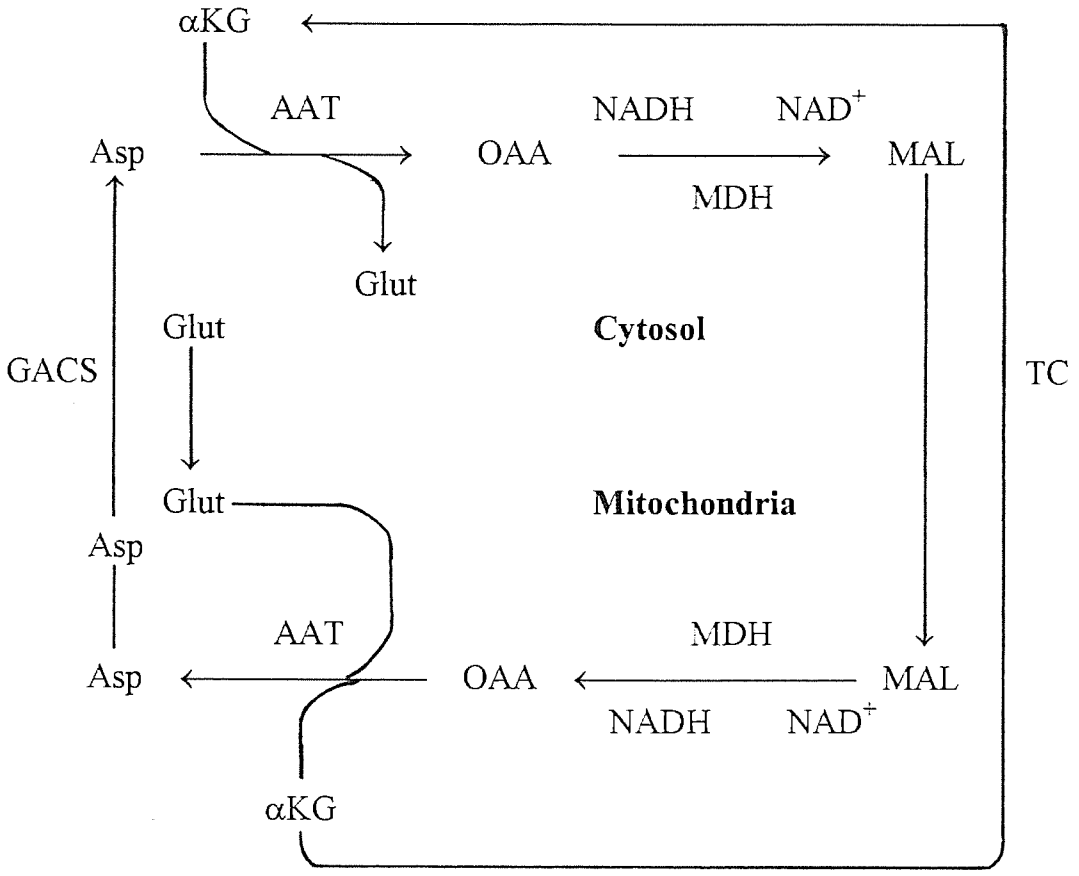


Figure 1 Scheme showing the malate-aspartate shuttle.

Abbreviations: OAA, oxaloacetate; MAL, malate; Asp, aspartate; αKG, α-ketoglutarate; Glut, glutamate; MDH, malate dehydrogenase; AAT, aspartate aminotransferase; GACS, glutamate-aspartate cotransport system; TC, transmembrane carrier.

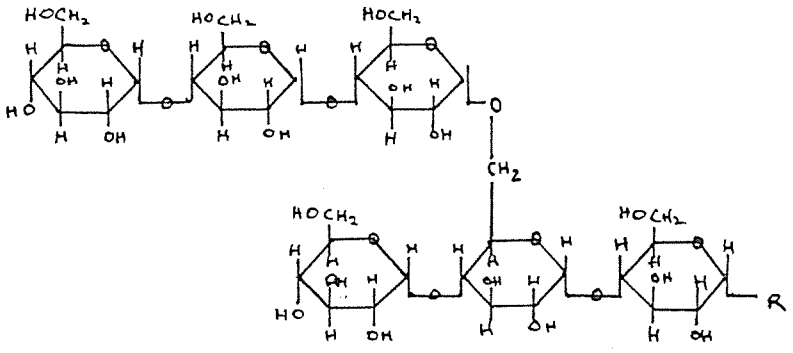


Figure 2 The structure of glycogen.

Glucose residues are linked by α-1,4 glycosidic bonds and branching achieved by α-1,6 linkages which accounts for the compactness of the structure. (Stryer, 1989)

does not oxidise NADH as fast as it is being produced. Since the ADH reaction is reversible and is inhibited by NADH, accumulation of this product would lead to a reduced rate of ethanol metabolism (Crow, 1985).

However, the rate-determining factor for ethanol oxidation is not the rate at which NADH can be reoxidised to NAD^+ in the malate-aspartate shuttle. Although some experiments have shown that rates of ethanol metabolism are decreased when the malate-aspartate shuttle is inhibited (Nordmann et al., 1975; Williamson et al., 1974b), under physiological conditions, NADH reoxidation is not rate-limiting. One recent approach using inhibitors of enzymes has demonstrated that the rate of ethanol oxidation is primarily limited by the amount of liver alcohol dehydrogenase although a number of other steps such as NADH reoxidation and acetaldehyde oxidation may contribute to rate limitation *in vivo* (Page et al., 1991).

The decreased NAD^+/NADH redox state in the liver has the potential to affect flux through other metabolic pathways in which NAD^+ and NADH are involved (Lieber et al., 1994), for instance, glycolysis and the TCA cycle are reported inhibited (Reitz, 1979). It was proposed that they were limited by the low availability of NAD^+ (Thurman and Scholz, 1977) but it is now recognised that they are inhibited by the increased NADH (Crow et al., 1982; Kitson et al., 1996). This is because, under physiological conditions, free cytosolic [NAD^+] is about 0.5 mM and the free [NADH] is about 0.5 μM. Therefore the change in NAD^+ concentration will be small relative to the change in NADH concentration. One analysis has shown that free cytosolic [NADH] increases to 1.0 or 2.5 μM, with no significant drop in free cytosolic [NAD^+] (Crow et al., 1983). This change in NADH is sufficient to alter the activity of some enzymes and possibly the overall direction of some enzyme reactions in carbohydrate metabolism (Crow and Greenway, 1989). Futhermore, the conversion of pyruvate to lactate by the increased NADH removes pyruvate from the system, and contributes to the decrease in TCA cycle activity (Reitz, 1979).

Mitochondria will use the hydrogen equivalents originating from ethanol rather than those from the oxidation of fatty acids that normally serve as the main energy source of the liver (Shelmet et al., 1988). Thus ethanol appears to act as a preferred fuel, almost totally replacing fat as a substrate. In addition, there is evidence that free radicals are generated due to NADH oxidation during ethanol metabolism (Mira et al., 1995). One study has shown that the production of superoxide and hydroxyl radical by microsomes are NADH-dependent after chronic ethanol treatment (Rashba-Step et al., 1993). Therefore the large amounts of

reducing equivalents available can result in many potent effects which will upset the normal functioning of the body.

1.3 GLYCOGEN METABOLISM

Glycogen is a promptly mobilised storage form of glucose present in animals and in some microorganisms. It is stored mainly in liver and muscle tissues in animals. It consists of large and branched polymers of glucose residues which are linked by either α-1,4- or α-1,6- glycosidic bonds (Fig.2). Because branching occurs frequently, it has a dense and compact structure which allows glucose molecules to be packed closely together in a given space. The presence of glycogen stores provides glucose that is readily available between meals and during muscular activities.

For decades, glycogen synthesis was thought to require only one UDP-glucose transglucosylase (glycogen synthase) and branching enzyme, but in the last few years, new evidence has required revision of the classical view of glycogen synthesis (Alonso et al., 1995). Glycogen synthesis has now been shown to involve two forms of glycogen namely proglycogen (intermediate glycogen), having M_r 400,000 and macroglycogen (mature glycogen), having M_r 10^7. Moreover, three UDP-glucose transglucosylases instead of one, have been recognised. They are glycogenin, activated by Mn^{2+}, and proglycogen synthase and macroglycogen synthase. The latter two are activated by glucose 6-phosphate. The new process of glycogen synthesis is elucidated by Lomako (1993). Glycogenin is the starting enzyme for glycogen formation. It has the ability of self-glucosylation in which glucose from UDP-glucose is added to its Tyr-194, followed by a further seven residues to form a protein-bound maltosaccharide that serves as the primer for synthesis of proglycogen by glycogen synthase. The proglycogen, a stable intermediate, can have its existing polymer chains extended through further elongation and branching by macroglycogen synthase. Whether there are distinct forms of phosphorylase, and branching and debranching enzymes for the separate stages, is not known. In muscle, there is no free glycogenin (Lomako et. al., 1990), therefore the supply of glycogenin limits the number of molecules of glycogen present. Muscle has a significant proportion of proglycogen which means that a very significant increase in the mass of glycogen could occur if the proglycogen were to be converted into macroglycogen.

Glycogen metabolism is affected by several hormones. Insulin (Maruyama et al., 1984) induces the synthesis of glycogen whereas glucagon and epinephrine trigger the breakdown of glycogen (Bazotte, 1989). Epinephrine is a catecholamine released from adrenal medulla and it markedly stimulates glycogen breakdown in muscle and, to a lesser extent, in liver (Bazotte, 1989; Dumm and Ralli, 1968). A review has given an overview of the regulation of liver glycogen metabolism (van de Werve and Jeanrenaud, 1987).

The coordinate control of glycogen metabolism is achieved by the hormone-triggered cyclic AMP cascades. The enzyme catalysing the formation of cAMP from ATP in cytosol is adenylate cyclase, a transmembrane enzyme linked to surface receptors in hepatocytes. When glucagon and epinephrine bind to the surface receptors, adenylate cyclase is activated and produces cAMP. The effects of the hormones are then amplified by a reaction cascade which involves reversible phosphorylation (Fig.3). The increased intracellular level of cAMP activates Protein kinase A (PKA) which is inactive in the absence of cAMP. Active PKA now phosphorylates both phosphorylase kinase (PhK) and glycogen synthase (GS). The two enzymes exist in two forms (a and b), only the a form is active. Therefore PhK now becomes active PhK a, while GS becomes inactive GS b. PhK a also inactivates GS to ensure glycogen is not synthesized. PhK a then activates glycogen phosphorylase (GP a) which catalyses glycogen breakdown (van de Werve and Jeanrenaud, 1987).

Insulin however triggers glycogen synthesis. It binds to its receptors and leads to activation of an insulin-sensitive protein kinase (ISPK) which phosphorylates and activates Protein phosphatase 1G (PP1G). The latter enzyme dephophorylates GS b, PhK a and GP a. The consequent dephosphorylation promotes glycogen synthesis and blocks its degradation.

There is evidence that ethanol leads to glycogenolysis via an increased secretion of catecholamines (Erwin and Towell, 1983) and glucagon (Thurman et al., 1989; Potter and Morris, 1980). However, ethanol has also been reported to induce alterations in cAMP metabolism by stimulating glucagon-responsive adenylate cyclase (Gorman and Bitensky, 1970). Therefore it is possible that ethanol may act in an absence of hormones.

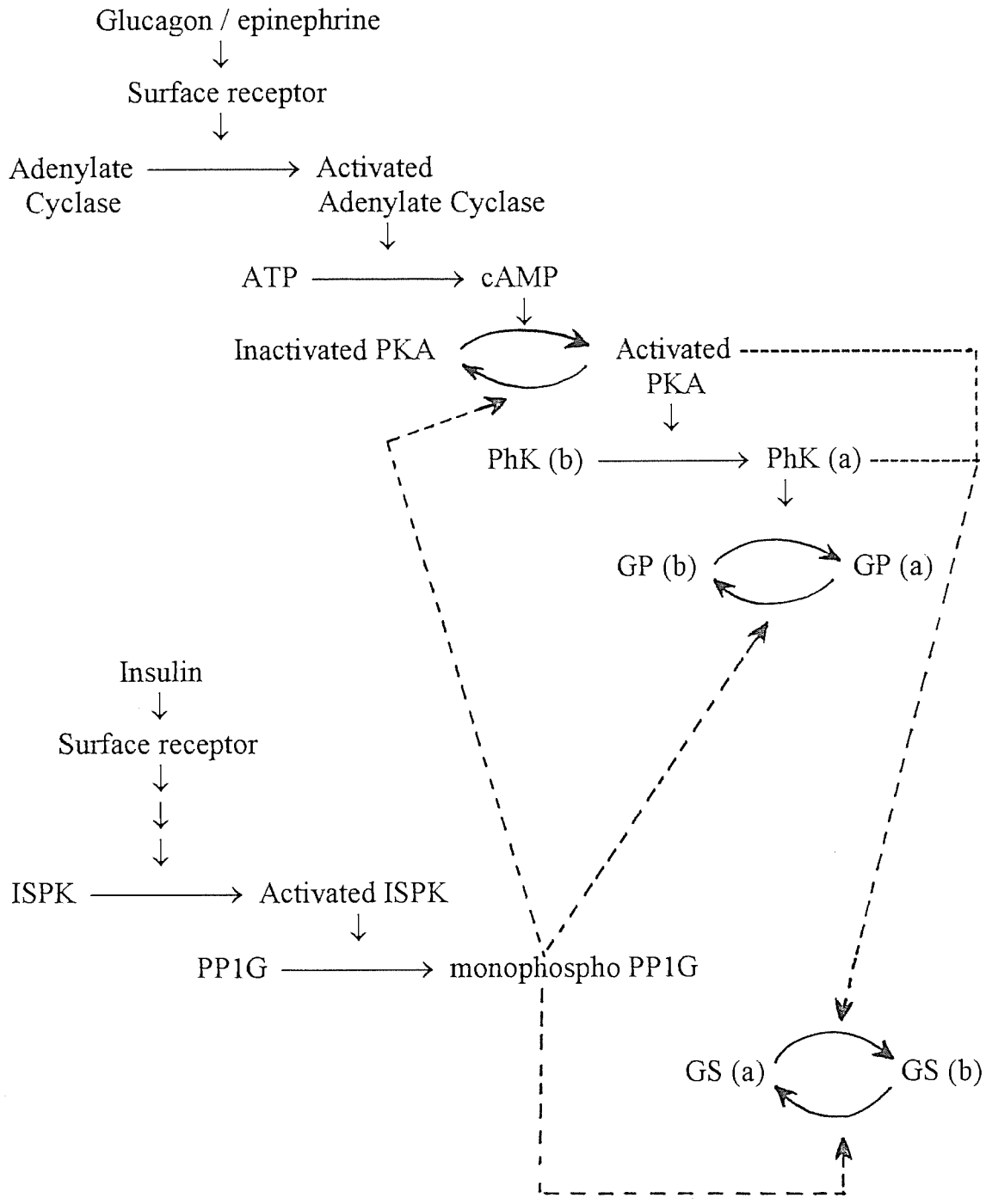


Figure 3 Reaction cascades for the hormonal control of glycogen metabolism.

Abbreviations: PKA, protein kinase A; PhK, phosphorylase kinase; GP, glycogen phosphrylase; GS, glycogen synthase; ISPK, insulin-sensitive protein kinase; PP1G, protein phosphatase 1G.

1.4 INTERACTIONS OF ETHANOL AND CARBOHYDRATE METABOLISM

The effects of ethanol on carbohydrate metabolism are complex and various factors including physical, nutritional, and hormonal may influence the biochemical sequelae. Generally ethanol affects carbohydrate metabolism through its metabolites such as acetaldehyde and acetate because these metabolites influence the normal flux of metabolites in metabolic pathways. The change in NAD^+/NADH ratio as a result of ethanol oxidation also contributes to the effect.

Acetate is a naturally occuring product of carbohydrate metabolism. It is derived from acetyl CoA by acetyl CoA deacylase. However, ethanol oxidation produces an increase in acetate concentration in blood (Korsten et al., 1975; Lundquist et al., 1962) since acetaldehyde is quickly metabolised to acetate in an irreversible reaction. Acetate is then converted to acetyl CoA via acetate thiokinase (Ballard, 1972). It has been pointed out by Topping (1982) that ethanol increases the concentration of acetyl CoA in the liver and increases the mitochondrial ratio of acetyl CoA to CoA. An increase in this ratio would inactivate pyruvate dehydrogenase and thus, direct carbon flux through pyruvate carboxylase into gluconeogenesis (Sneyd, 1989). Acetate has also been shown to stimulate gluconeogenesis in isolated liver cells (Whitton et al., 1979). It is possible that acetate-stimulated hepatic gluconeogenesis may contribute to the increase of plasma glucose levels in fed animals. Acetaldehyde is also found to stimulate glucose production from pyruvate (Cederbaum and Dicker, 1982). It was suggested that acetaldehyde may be involved in stimulating pyruvate carboxylase via acetyl CoA produced from acetaldehyde oxidation, or provision of reducing power for conversion of oxaloacetate to malate within the mitochondria, or provision of NADH for the glyceraldehyde 3-phosphate dehydrogenase reaction (Cederbaum and Dicker, 1982). Their mechanisms of action are presumably different since acetaldehyde stimulated gluconeogenesis from pyruvate, while acetate stimulated from lactate.

However, there is controversy in the contribution of gluconeogenesis to hyperglycemia as it has been reported that slices of rat liver showed direct impairment of glucose formation from lactate when ethanol was added (Freinkel et al., 1965). Inhibition of gluconeogenesis from lactate has also been observed in isolated rat hepatocytes (Phillips et al., 1985). It was suggested that the increase in [NADH] in the cytosol as a result of ethanol oxidation inhibits the conversion of lactate to pyruvate (Sneyd, 1989), a necessary first step in gluconeogenesis from lactate.

Thurman and coworkers have shown that ethanol inhibits glycolysis in perfused livers from fed rats (Thurman et al.,1989). This is supported by a report that pretreatment of rats with a large dose of ethanol leads to subsequent observation of low rates of glycolysis during liver perfusion (Yuki and Thurman, 1980). Apparently the inhibition of glycolysis is caused by the decrease in NAD^+/NADH ratio as a result of ethanol oxidation. Literature has also indicated that the tricarboxylic acid cycle (TCA) was inhibited after ethanol ingestion suggesting that NADH decreased the oxidation of various TCA cycle intermediates (Reitz, 1979). Therefore, ethanol can result in a number of metabolic disturbances in carbohydrate degradation and synthesis presumably by slowing the reactions that require NAD^+ or which are inhibited by NADH.

Apart from the effect of ethanol metabolites, ethanol may act on carbohydrate metabolism via hormones such as catecholamines and glucagon which can activate the enzymes involved in glycogen hydrolysis (Erwin and Towell, 1983). In a more recent report, it has been shown that ethanol when added to cultured hepatocytes, results in a substantial reduction in the levels of GLUT-2 glucose transporter mRNA (Nagamatsu et al., 1995). Therefore, it is likely that ethanol may act as a repressor and bind directly to the GLUT-2 gene interfering with transcription. This is consistent with the observation that GLUT-2 activity is reduced in diabetic rats (Unger, 1991). As a result, glucose cannot be transported into liver efficiently, hindering glycogen synthesis. This is supported by an early report which showed that ^{14}C-glucose incorporation into glycogen under insulin stimulation was massively reduced in ethanol-treated rats (Rifkin et al., 1983).

Therefore the effects of ethanol on carbohydrate metabolism are complicated, since ethanol may act directly to affect carbohydrate metabolism, may act through its metabolites acetaldehyde or acetate or through the change in NAD^+/NADH ratio, or may act indirectly via hormones.

Chapter 2

THE EFFECT OF ETHANOL ON THE RESERVE POLYSACCHARIDE OF OCHROMONAS DANICA

2.1 INTRODUCTION

Ochromonas danica (Chrysophyceae) is a freshwater single cell phytoflagellate. The organism possesses chloroplasts with chlorophyll and is capable of photoautotrophic growth (Brown, 1968) but the photosynthetic apparatus was found to be less efficient than in other algae (Myers and Graham, 1956). Mixotrophic nutrition habit seems to be an alternative strategy for survival (Andersson et al., 1989). The organism will grow heterotrophically in the dark when provided with a suitable culture medium composed of all of the required nutrients plus vitamin B_{12} and biotin (Ford, 1953). The organism is also capable of phagotrophy. During starvation in the dark, when both external and storage sources of nutrient are scarce, the organism may engulf or predate its own species for survival. The extraordinary nutritional versatility of *Ochromonas* is considered to be a primitive characteristic.

The major polysaccharide reserve material of *Ochromonas* is chrysolaminarin. It is composed of β-(1→3)-D-glucose residues with occasional β-(1→6)-branched glucose residues and has a degree of polymerisation of 30-40 residues (Archibald, et al., 1963). The polysaccharide is used as a carbon reserve and for osmoregulation (Hellebust, 1980).

Previous studies (Kauss, 1962) with the related organism *Ochromonas malhamensis* (syn. *Poterioochromonas malhamensis*) showed that chrysolaminarin became strongly labelled by ^{14}C during photosynthesis when the organism was supplied with $^{14}CO_2$. It was also found that when the organism was transferred from growth on a complete medium to a medium lacking carbon substrates and was maintained in darkness, the organism respired by using chrysolaminarin that had been synthesized and stored during heterotrophic growth (Chick, unpublished data).

Earlier it had been demonstrated using [$1^{14}C$]- and [$6^{14}C$]- glucose, that the major, if not sole, pathway of respiration when glucose was substrate was via the glycolytic pathway to pyruvate (Reazin, 1956). Thus in these respects, metabolism of chrysolaminarin by *Ochromonas* is analogous to

the metabolism of glycogen in rat liver. In addition, it was found using [1^{14}C]- and [6^{14}C]- glucose that *Ochromonas* fermented glucose via the glycolytic pathway to CO_2, ethanol and lactate (Reazin, 1956).

Because it is unicellular, Ochromonas affords a simple system for investigating the effect of ethanol on the catabolism of polysaccharide reserve material, without the confounding effects of hormones, which are possible in animals. However, a possible difficulty in using *Ochromonas* is that it is a wall-less organism and has been found to respond to changes in the osmolarity of its environment by the synthesis and degradation of an osmolyte, isofloridoside (O-α-D-galactopyranosyl-1,1-glycerol) (Schobert et al., 1972). Carbon for isofloridoside synthesis was derived from several sources but in the absence of exogenous carbon sources it was mainly provided by chrysolaminarin. Carbon from degradation of isofloridoside was converted to chrysolaminarin (Kauss, 1979). Thus it would be necessary when studying the effects of ethanol on chrysolaminarin catabolism to ensure that the organism was in osmotic balance to avoid changes in the amount of chrysolaminarin content due to osmolyte synthesis or degradation.

2.2 MATERIALS

Ochromonas danica (ATCC 3004) was obtained from the American Type Culture Collection (Rockville, Maryland, USA). Seaweed laminarin (from Laminaria digitata), laminarinase (EC 3.2.1.6 from Penicillium) and polyethylene glycol (average molecular weight 2000) were obtained from Sigma Chemical Co (St. Louis, Missouri, USA). All other reagents were analytical grade from commercial sources.

2.3 METHODS

2.3.1 Culture media for the experiment

All media were in liquid form. The composition of the medium used for growing *Ochromonas* cells was described in detail by Ford (1953). The minimal medium used later in the experiment was Ford's medium less glucose, casein hydrolysate, tryptophan, methionine, cysteine, triammonium citrate and Tween 80.

2.3.2 Maintenance and growing of *Ochromonas danica*

Ochromonas danica (ATCC 30004) was maintained by serial transfer at two weekly intervals in the medium of Ford (1953). Growth was at room temperature (15 - 25^{O}C) in daylight.

2.3.3 Growing *Ochromonas danica* for experiments

For the experiments described here, the organism was grown in the dark at 20^{O}C on a rotary shaker (90 rpm) in 750 ml of Ford medium in 2 litre conical flasks fitted with sampling ports (Fig.4) or 2.8 litre fernbach flasks. Inocula for these cultures were prepared by transferring approximately 0.5 ml of maintenance culture to 100 ml of Ford's medium in 250 ml conical flasks which were left growing in the dark for 5 days as before.

Samples from 750 ml cultures were collected aseptically into bijou bottles using a "Pirbright" sampling head (LH Engineering Co., Stoke Poges, UK.) connected to a long needle piercing a suba seal septum in the side arm of the culture flask (Fig.4). Samples were collected from 100 ml cultures by syringe with needle.

2.3.4 Measurement of dry cell mass

Dry cell mass was determined by collection of cells from measured volumes of culture on tared 0.45 μm filters and reweighing the filters after drying overnight in a desiccator under vacuum, using silica gel as siccative.

2.3.5 Measurement of chrysolaminarin content of cells

The cells in measured volumes of culture were collected by centrifuging at 5000x g for 20 minutes and washed twice by resuspension in distilled water and recentrifuging. Next, the washed cell pellets were freeze-dried. Lipids were removed by first suspending the dry cell mass in 0.01 M EDTA diNa pH 7.0 (6ml/g cells) and then mixing the suspension with 10 volumes of chloroform/methanol (2/1, v/v). After phase separation, the lower phase was removed and the upper phase, including the suspended cell mass, was re-rextracted with fresh lower phase. The lipid-extracted cell suspension was then diluted to 10 ml with water and heated at 90^{O}C for 20 minutes. The hot water extract was separated from the cell mass by centrifuging at 5000x g for 20 minutes. This hot water extraction step was repeated three times. Chrysolaminarin was

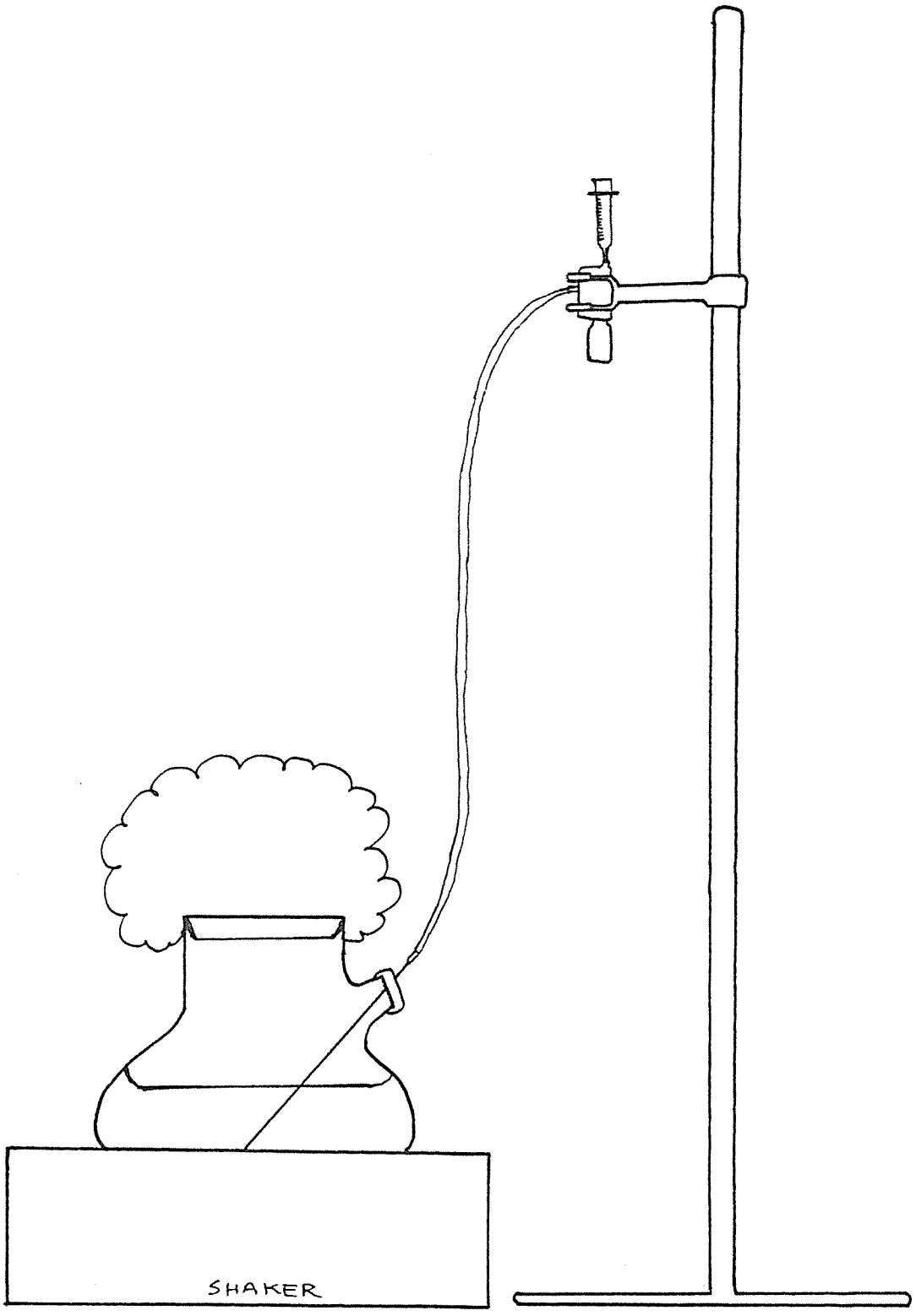


Figure 4 Arrangement for sampling *Ochromonas* cultures

Samples were sucked into bijou bottles through a "Pirbright" sampling head.

precipitated from the combined, cooled water extracts by addition of 5 volumes of 95 % ethanol and cooling overnight at 4°C. The precipitate was collected by centrifuging and washed by suspension and centrifuging two times in 95 % ethanol followed by two times in diethyl ether. The solid remaining was dried overnight in a desiccator under vacuum over silica gel and weighed.

The recovery of polysaccharide was tested by adding seaweed laminarin (250 mg), as a model poly β-1,3 glucoside for chrysolaminarin, to about 1.0g of freeze-dried *Ochromonas* cells and extracting as before. Total carbohydrate was assayed by the resorcinol sulphuric micromethod of Monsigny (1988) (Appendix 1), which is insensitive to proteins. A standard graph was constructed using laminarin solution (10 mg/ml) (Figure 5).

2.3.6 Demonstration that the material isolated from the water extract was a poly β 1,3-glucoside

0.5 ml samples of solutions of laminarin and water-soluble polysaccharide extracted from *Ochromonas* (2mg/ml) were treated separately with an equal volume of laminarinase (1 unit / ml) in sodium acetate buffer, pH 4.8, for 30 minutes at 50°C. The buffer for laminarinase hydrolysis is given in Appendix 1. Duplicate 0.5 ml aliquots were taken to determine the free reducing sugar content which was measured by the dinitrosalicylic acid method (Bernfeld, 1955) (Appendix 1) using glucose as standard. The standard curve is shown in Figure 6.

2.3.7 The effect of ethanol on the growth of *Ochromonas danica*

To test the ethanol tolerance of *Ochromonas*, the organism was grown for 5 days in 100 ml of Ford's medium in 250 ml conical flasks, as before, and in the presence of a range of ethanol concentrations up to 60 mM. 95 % ethanol was sterilised by filtration through a 0.45 μm filter and added to sterile media. Growth was measured by light scattering at 480 nm.

2.3.8 The effect of ethanol on chrysolaminarin catabolism

Ochromonas danica grown in 750 ml of Ford's medium in 2.8 litre fernbach flasks in the dark for 6 days, as before, was harvested by centrifuging at 5000x g for 20 minutes. The cell pellet was washed twice by suspension in sterile water and centrifuging in sterile bijou bottles.

Figure 5 Standard curve for chrysolaminarin assay

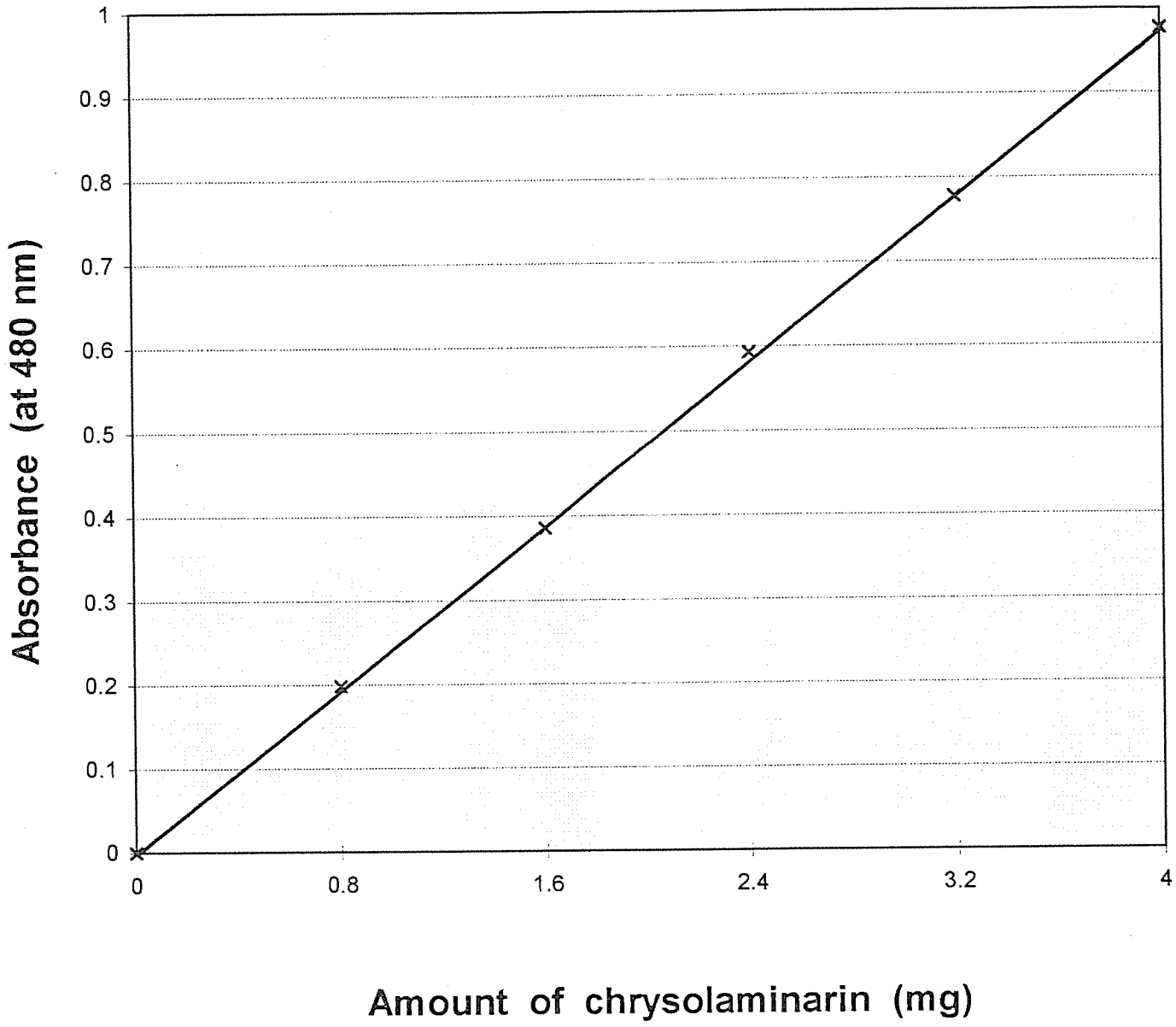
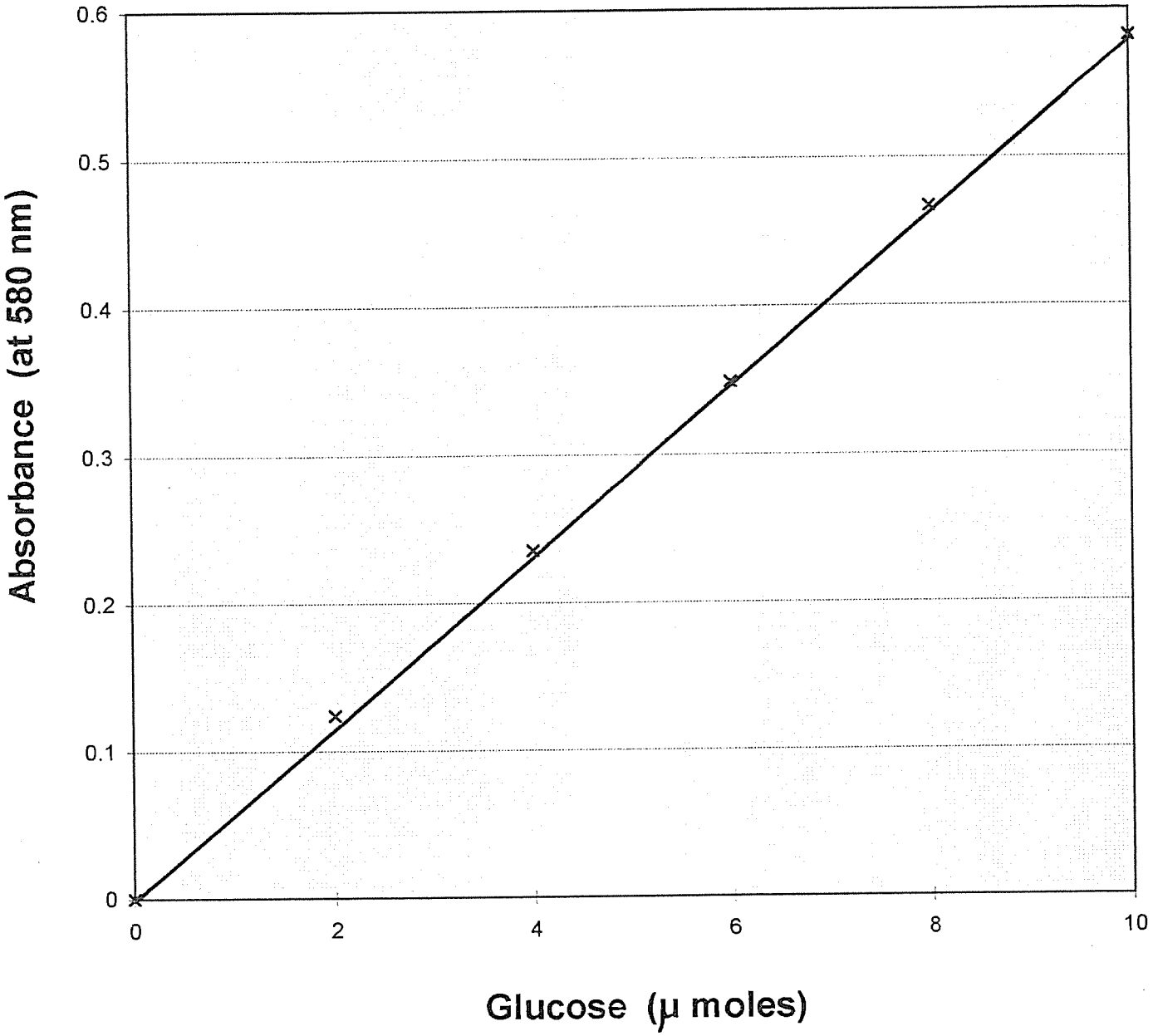


Figure 6 Standard curve for free reducing sugar assayed by the method of Bernfeld (1955)



Minimal medium (Ford's medium less glucose, casein hydrolysate, tryptophan, methionine, cysteine, triammonium citrate and Tween 80) was made isosmolar with Ford's medium by the addition of 282 gL^{-1} of polyethylene glycol (average molecular weight 2000). (Osmolarity was measured using an advanced instruments model 3D2 freezing point osmometer against aqueous NaCl standards). The washed cells were suspended in PEG/minimal medium and 100 ml of the suspension was added to sterile 250 ml conical flasks to which sterile 95 % ethanol was added to give final ethanol concentrations of 0, 5, 10, 15 and 20 mM. The cotton plugged flasks were incubated in darkness at 20°C on a shaker at 90 rpm for 5 days. Samples were removed daily for measurement of dry cell mass and chrysolaminarin content as before.

2.4 RESULTS & DISCUSSION

2.4.1 The growth of *Ochromonas danica*

The cell mass and cell chrysolaminarin content were measured daily and the results are shown in Table 1.

The results show that after day 4, cell mass and chrysolaminarin content increase dramatically. The results also show that chrysolaminarin production per cell mass reached a maximum at about day 5 and remained steady until about day 8. After then, cell mass decreased rapidly, presumably as nutrients were depleted. Chrysolaminarin contents per cell also decreased after day 8. Therefore, only cells from cultures day 7 or younger were suitable for studies.

2.4.2 The efficiency of the polysaccharide extraction procedure

Because a lengthy fractionation procedure was required to separate the subtypes of chrysolaminarin for structure determination (Archibald et al., 1963) and since, for the purposes of these experiments, it was only necessary to measure the total chrysolaminarin content of the cells, a simplified procedure, involving delipidation of freeze-dried cells followed by hot water extraction, was devised to isolate soluble reserve polysaccharide from Ochromonas. Since this was a novel procedure, it was necessary to show that the product isolated was chrysolaminarin and that the procedure was efficient.

TABLE 1

Growth characteristics of *Ochromonas danica* in Ford's medium

Days	Dry Cell Mass (mg)	Extracted Chrysolaminarin (mg)	Chrysolaminarin/ Cell mass (mg/mg)
0	0	0	0
1	3.6	0	0
2	3.4	0.1	0.029
3	4.3	0.5	0.116
4	6.4	2.3	0.359
5	9.7	3.8	0.400
6	35.0	14.7	0.420
7	25.0	11.9	0.476
8	13.0	5.3	0.408
9	11.5	3.0	0.261
10	6.0	1.5	0.250
11	5.6	1.5	0.270
12	5.7	1.6	0.286

Ochromonas cultures were grown for 12 days in Ford's medium as described in section 2.3.1.

The recovery of carbohydrate from cells after the hot water extraction is shown in Table 2.

Although the recovery of laminarin added to *Ochromonas* cells using the simplified extraction procedure was 91.6 %, this was sufficiently high to be confident that the changes in chrysolaminarin content of cells observed in later experiments do reflect accurately the cellular content of the polysaccharide.

2.4.3 The extracted polysaccharide from *Ochromonas* cells

2.4.3.1 The identity of the extracted polysaccharide

The identity of the product obtained using the simplified extraction procedure was checked by testing the susceptibility of the product to degradation by Penicillium laminarinase (EC 3.2.1.6) which is an endo 1,3-glucanase that attacks β-1,3 glucosidic bonds and β-1,4 glucosidic bonds adjacent to a β-1,3 glucosidic bond (Reese and Mandels, 1959). Seaweed laminarin was used as a control. The products of laminarinase activity were analysed as reducing sugar using the dinitrosalicylic acid method. The net quantity of reducing sugar released would indicate the chrysolaminarin content present in the extracted polysaccharide. The standard curve for this assay is shown in Figure 6 and the results of treatment of sea weed laminarin with laminarinase are shown in Table 3.

There would be a reducing group at the end of the chains of both laminarin and chrysolaminarin, but the number of reducing groups per glucose residue would depend on the degree of polymerisation of the polysaccharide chains. Laminaria digitata laminarin was said to have a degree of polymerisation of about 25 (Manners & Sturgeon, 1982). Assuming a degree of polymerisation of 25 and hence an average residue weight of 163.1, this would amount to 0.306 µmoles of reducing residues per mg laminarin. Hence the small quantity of free reducing sugar detected (~0.315 µmoles) in the untreated laminarin solution was probably due to reducing sugar residues at the end of chains.

After treatment with laminarinase, 1 mg of standard gave 6.1 µmoles glucose. Using an average residue weight of 162g (assumed degree of polymerisation of 25), this corresponds to 0.99 mg of untreated laminarin. Thus, under the conditions used, Penicillium laminarinase catalysed complete depolymerisation of the β1,3-linked glucan, laminarin, from seaweed.

TABLE 2

Recovery of polysaccharide by hot water extraction procedure

	Freeze-dried cells alone	Freeze-dried cells + 250 mg laminarin
cell weight (g)	1.0818	1.3383
mg laminarin added/g cells	____	186.8
mg carbohydrate recovered/g cells	307	477
mg laminarin recovered/g cells (%)		170 (91)

Freeze-dried O. danica cells and freeze-dried cells supplemented with 250 mg of laminarin were extracted as described in Methods section 2.3.5 and the extracts were assayed for carbohydrate by the method of Monsigny (1988).

TABLE 3

The effect of laminarinase on sea weed laminarin

Samples	Absorbance (at 580 nm)	µmoles Glucose (from standard graph)
Untreated laminarin	0.015	0.270
	0.020	0.360
		average = 0.315
Laminarinase treated laminarin	0.335	6.04
	0.342	6.16
		average = 6.10

2 mg/ml of standard laminarin solution was assayed for reducing sugar by the dinitrosalicylic acid method, before and after laminarinase treatment.

The results from treatment of the polysaccharide extracted from *Ochromonas* cells with laminarinase are shown in Table 4.

TABLE 4

The effect of laminarinase on the polysaccharide extracted from *Ochromonas*

Samples	Absorbance (at 580 nm)	µmoles Glucose (from standard graph)
Untreated polysaccharide	0.025	0.450
	0.030	0.540
		average = 0.495
Laminarinase treated polysaccharide	0.316	5.69
	0.308	5.55
		average = 5.62

2 mg/ml of *Ochromonas* polysaccharide solution was assayed for reducing sugar by the dinitrosalicylic acid method, before and after laminarinase treatment.

Assuming a degree of polymerisation for chrysolaminarin of 34 (Archibald et al., 1963), then the average residue weight would be 162.7 and the average molecular weight of the chains would be 5532.

Hence there would be 0.181 µmoles of reducing sugar equivalent per mg of polysaccharide, due to terminal sugar residues with a free reducing sugar group. The observed value of 0.495 µmoles was significantly larger than this. This suggests the presence of an additional source of reducing sugar in the chrysolaminarin isolate. Taking this additional reducing sugar into account (0.495-0.181 µmoles), the laminarinase-treated polysaccharide yielded 5.31 µmoles of reducing sugar. This corresponds to 0.86 mg of untreated polysaccharide. Hence 86% of the material isolated was poly β1,3-glucoside.

Because seaweed laminarin is polydisperse, the published degree of polymerisation of about 25 is an average value. Further, seaweed

laminarin includes some chains terminated by non-reducing mannitol residues and has branches consisting of single glucose residues linked β1,6 to the main chain to varying extent (data summarised in Manners and Sturgeon, 1982). Chrysolaminarin has a degree of polymerisation of about 35, a small percentage of branch glucose residues linked β1,6, but no mannitiol (Archibald et al., 1963). Consequently, it is not possible to determine the purity of laminarin or chrysolaminarin with great accuracy by means of enzymatic degradation. Assuming degrees of polymerisation of 25 and 34 and assuming there is no branching or mannitol, 99% and 86% of laminarin and the water-soluble polysaccharide from Ochromonas, respectively, were accounted for as glucose released by laminarinase activity. Therefore a major portion of the polysaccharide in the water extract of Ochromonas was β1,3-linked polysaccharide with possibly, a small portion of unidentified reducing material.

2.4.4 The effect of ethanol on chrysolaminarin catabolism

2.4.4.1 The effect of ethanol on the growth of *Ochromonas danica*

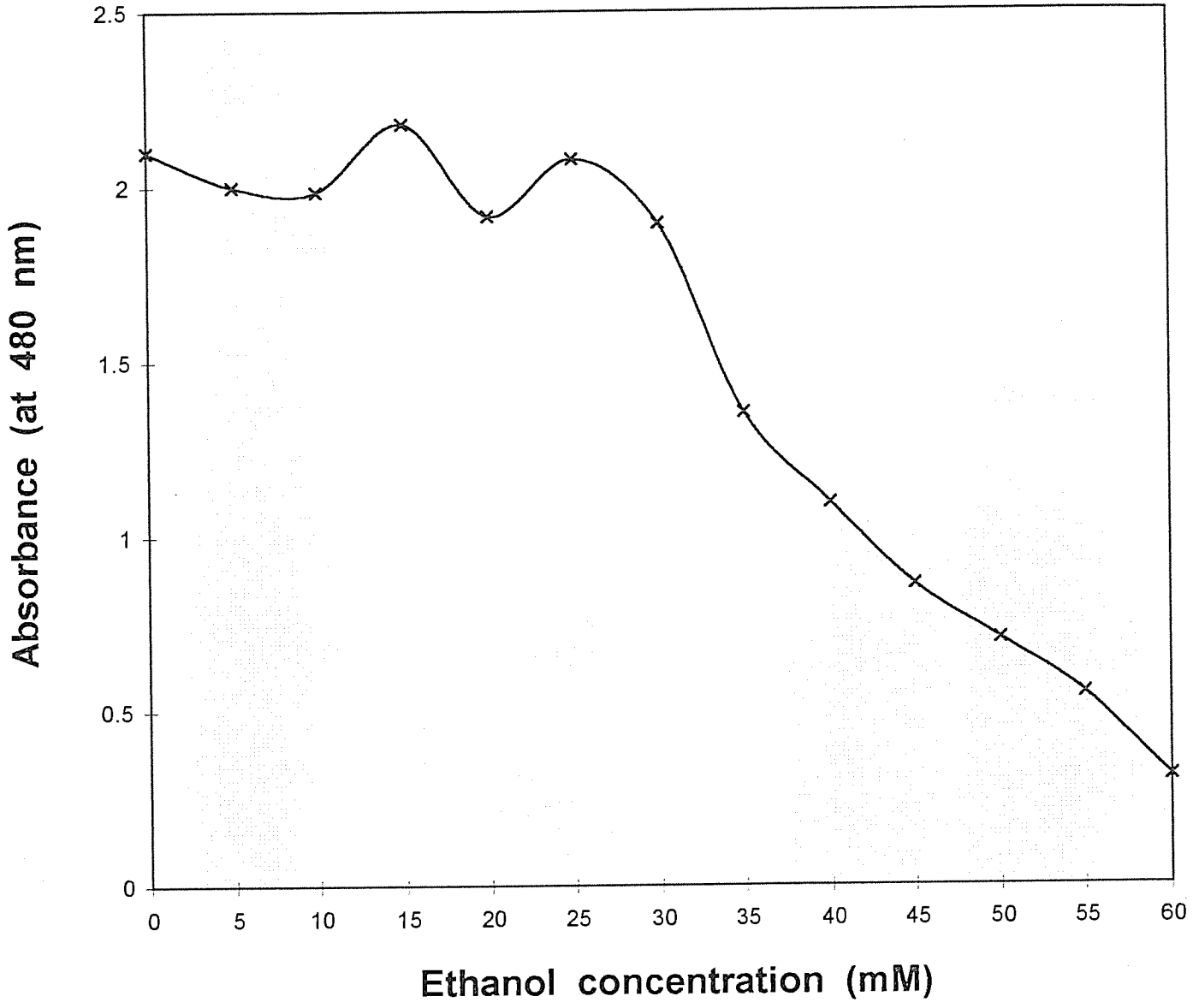
In order to investigate the effect of ethanol on catabolism of chrysolaminarin in *Ochromonas*, it was necessary to determine the maximum ethanol concentration the organism would tolerate before it affected its growth. The results of an experiment showing the effect of ethanol on cell growth, measured as light scattering by the cells, after 5 days growth are shown in Figure 7.

Although the relationship between cell mass and light scattered by the cell suspension at 480 nm is not linear, it is readily apparent from the results that the growth of *Ochromonas* was not significantly affected by ethanol up to a concentration of 25 mM. At higher concentrations ethanol inhibited growth as reflected by a decline in absorbance. Therefore a series of media containing up to 20 mM ethanol was used to test the effect of ethanol on chrysolaminarin catabolism.

2.4.4.2 The maintanance of osmotic pressure in Ochromonas cells

Ochromonas danica is a wall-less fresh water organism which is subject to osmotic stress in its natural environment. One mechanism used by *Ochromonas* for osmotic acclimation is the synthesis and degradation of an intracellular osmolyte, isofloridoside, identified as O-α-D-galactopyranosyl-1,1-glycerol (Kauss, 1979). Studies of the changes that occurred when *Ochromonas* was transferred to hyper-osmolar or

Figure 7 Effect of ethanol on growth of *Ochromonas danica*



hypo-osmolar media showed that carbon for isofloridoside synthesis could be derived from chrysolaminarin reserves and that the products of isofloridoside degradation were converted to chrysolaminarin, respectively (Kauss, 1967).

To study the effects of ethanol on chrysolaminarin catabolism, it would be necessary to control the osmolarity of the medium to avoid confounding changes in chrysolaminarin content due to osmotic stress. To stimulate catabolism of chrysolaminarin reserves, Ochromonas was transferred from a complete medium to a derived, minimal medium, lacking the major carbon substrates of the complete medium. The osmolarity of the minimal medium was restored to iso-osmolarity with the complete medium (Table 5) by the addition of the non-metabolised osmolyte polyethylene glycol (PEG). The amount of PEG required to increase the osmolarity to that of the complete medium was determined using the curve in Figure 8.

TABLE 5

Osmolarity of complete medium and minimal medium

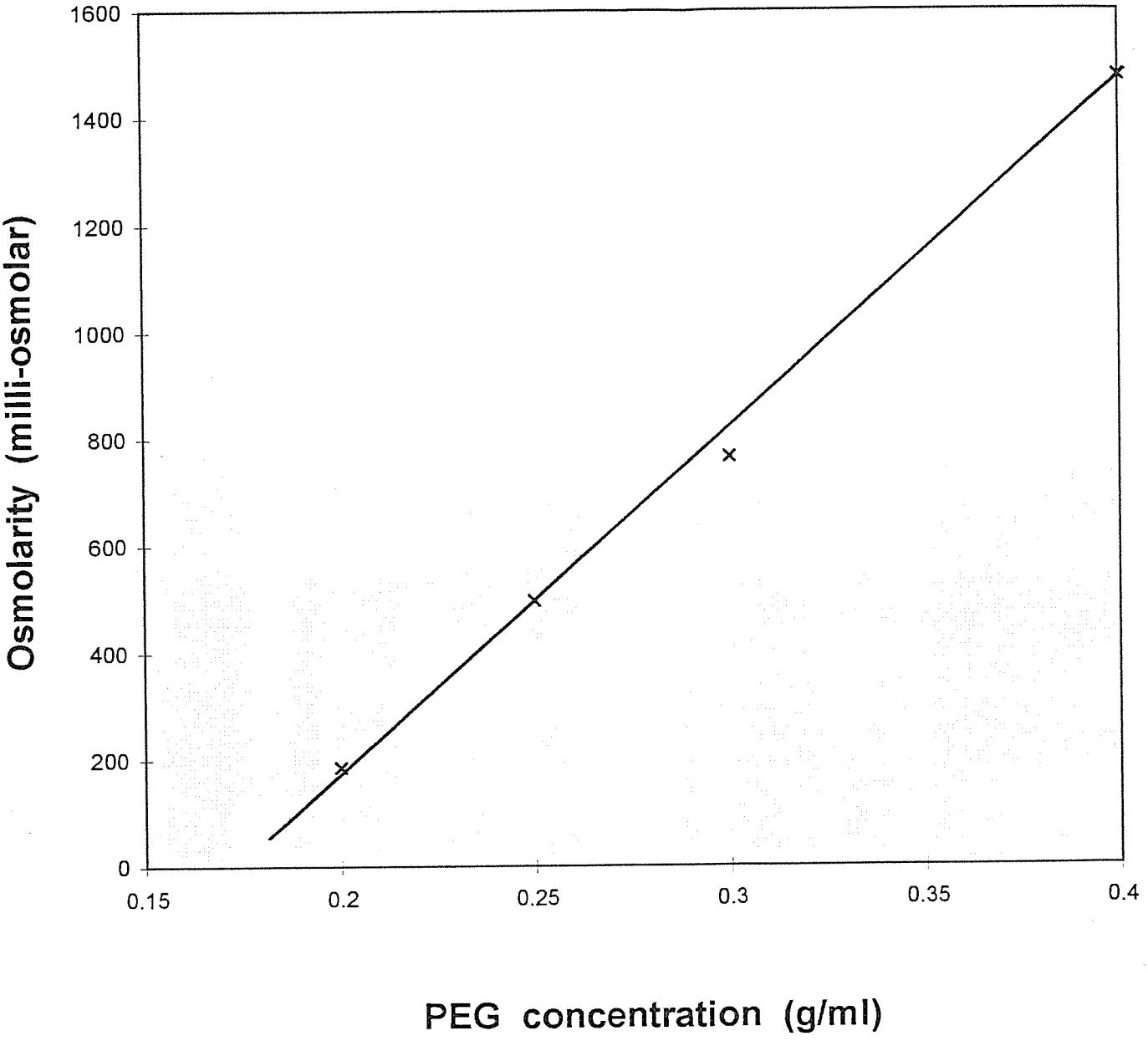
	m osmol/litre
Complete medium	734
Minimal medium	22

The complete medium was that of Ford (1953). The minimal medium was Ford's medium minus glucose, casein hydrolysate, tryptophan, methionine, cysteine, triammonium citrate and Tween 80.

2.4.4.3 Effect of ethanol on catabolism of chrysolaminarin by *Ochromonas danica*

The changes in cell mass and chrysolaminarin content that occurred when *O. danica* was transferred from a complete medium to an iso-osmotic PEG-minimal medium are shown in Figures 9 and 10. On the first day, in the presence or absence of ethanol, the cell mass increased. After day 1 cell mass declined with time. In contrast the chrysolaminarin content decreased from day 1. On the first day ethanol accelerated the

Figure 8 The relationship between polyethylene glycol (PEG) concentration and osmolarity



rate of chrysolaminarin catabolism at all concentrations tested. However, there was no linear relationship between the rate of chrysolaminarin catabolism and ethanol concentration, over the range of ethanol concentrations tested. After day 1 the rate of chrysolaminarin catabolism in the presence of 5 and 10 mM ethanol slowed to be about the same rate as that in the untreated cells. In contrast, after day 1 the rate of chrysolaminarin catabolism in the presence of 15 and 20 mM ethanol, slowed almost to zero by day 3 and it is unclear why this happens.

Thus ethanol does accelerate chrysolaminarin catabolism initially but, as the chrysolaminarin content of the cells is depleted this trend is reversed and the latter effect is greatest at higher ethanol concentrations.

The experiment using *Ochromonas danica* as a model shows that ethanol could cause a decrease in carbohydrate stores of an organism in an absence of hormones and it is suggested that the effect of ethanol on stored carbohydrate could be a direct effect of ethanol.

Figure 9 Effect of ethanol on dry cell mass

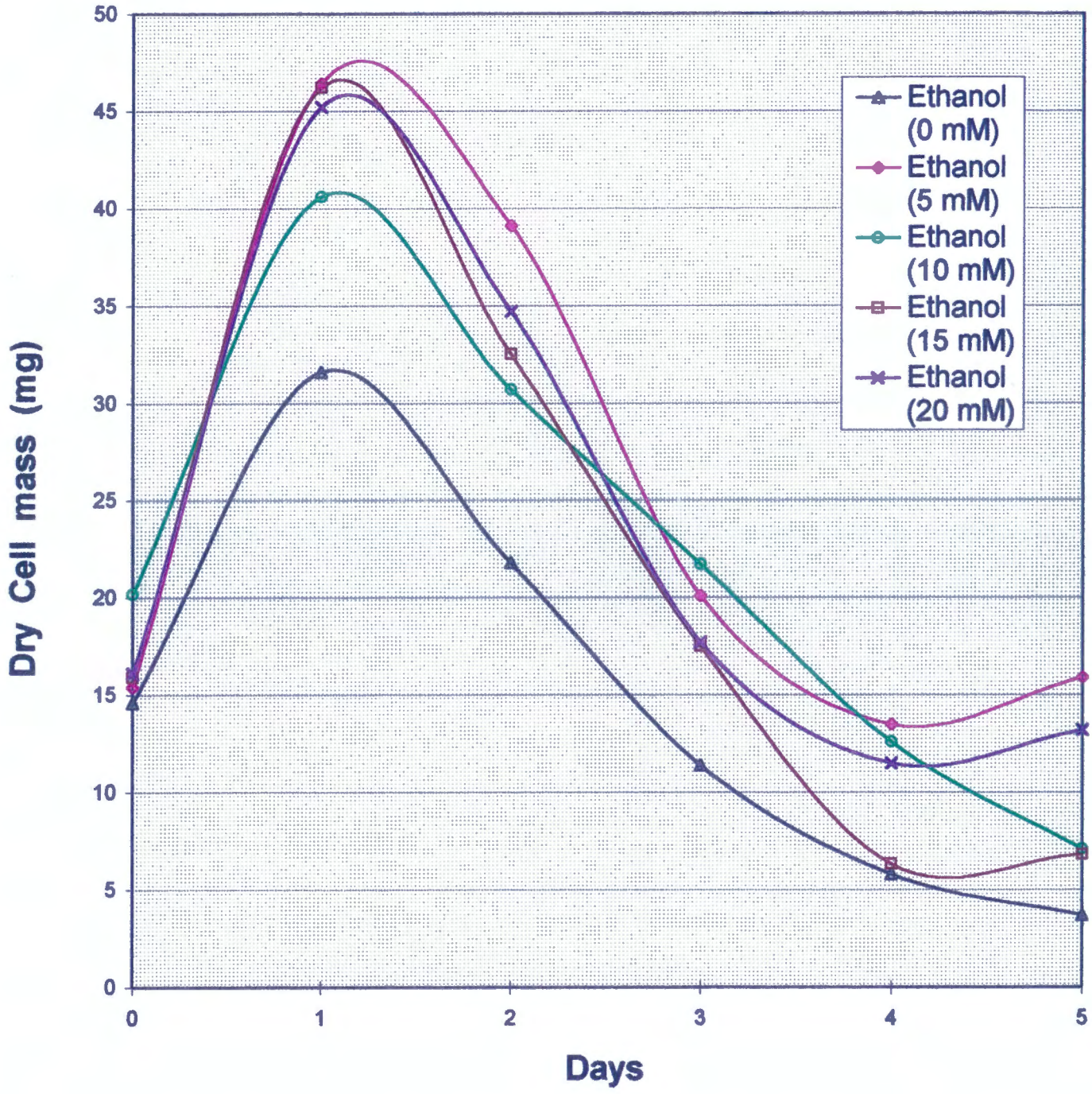
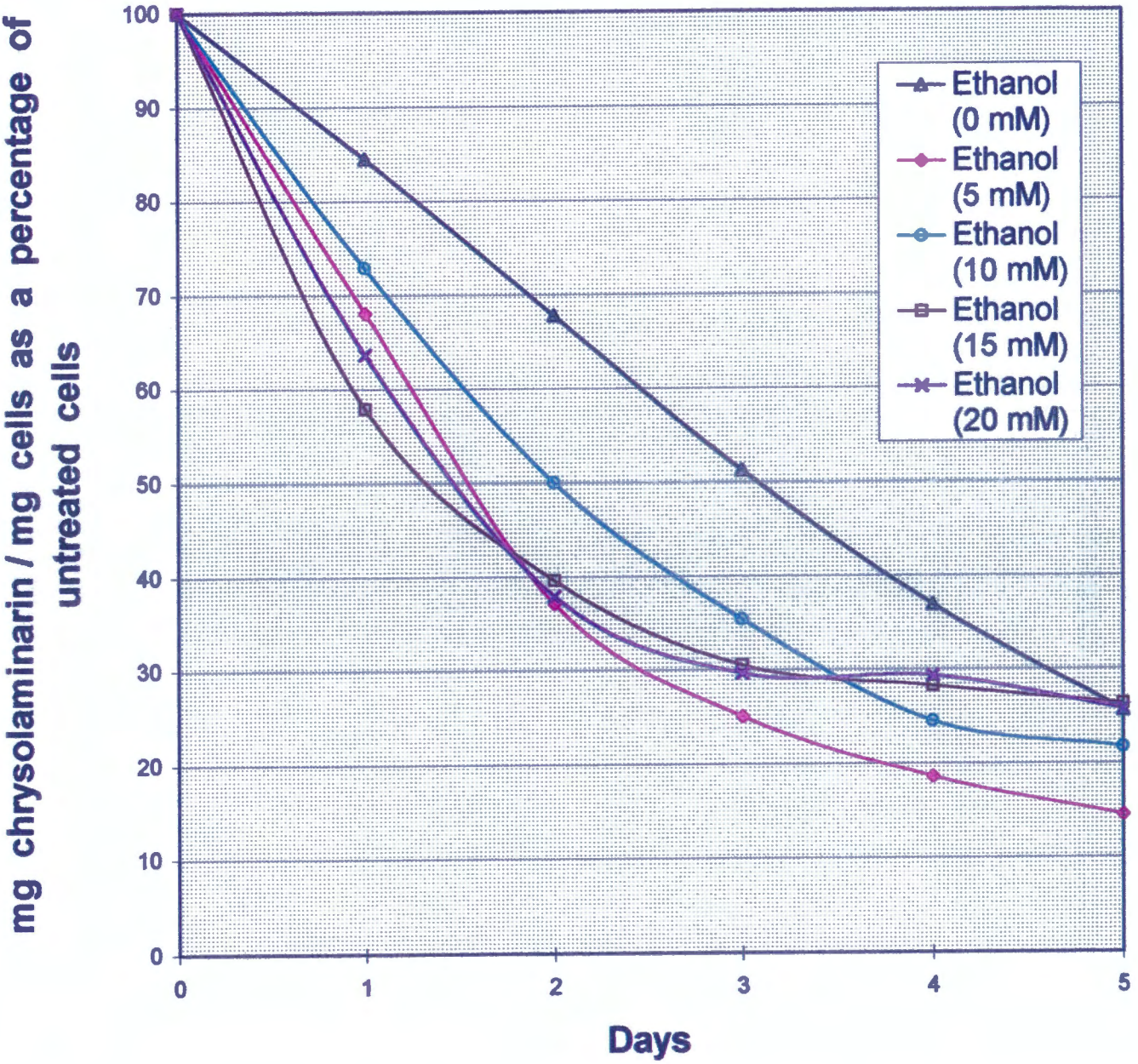


Figure 10 Effect of ethanol on chrysolaminarin content



Chapter 3

EFFECTS OF ETHANOL ON GLYCOGEN, GLUCOSE AND ATP CONCENTRATIONS IN LIVER AND OTHER TISSUES

3.1 INTRODUCTION

Results reported in the literature regarding the effects of ethanol on carbohydrate metabolism in fed animals are varied. Plasma glucose concentrations have been found to be similar in ethanol and control groups by one worker (Kubota et al., 1992), while other workers showed that plasma glucose concentrations were elevated by ethanol (Potter and Morris, 1979; Topping et al., 1979). The results in the literature for effects of ethanol on liver glycogen content however, agree well. Liver glycogen levels have been shown to decrease in all studies, although to different extents (Mirone, 1965; Mirone, 1966; Kubota et al., 1992; Jeyarathan, unpublished data). The present study was to clarify the effects of ethanol in fed animals on a number of parameters including liver glycogen content, free glucose concentrations and ATP content. In addition, the effects of ethanol on these metabolites in other tissues such as heart, kidney and muscle were investigated.

3.2 METHODS

3.2.1 Animals and materials

Adult male and female Sprague-Dawley rats were obtained from the Massey University Small Animal Production Unit.

The following chemicals were obtained from Sigma Chemical Company (St. Louis, Missouri, USA);
NADP, NAD^+, ATP, glycogen, glucose, bovine serum albumin and enzymes including glucose 6-phosphate dehydrogenase (EC 1.1.1.49), hexokinase (EC 2.7.1.1), amyloglucosidase (EC 3.2.1.3) and yeast alcohol dehydrogenase (EC 1.1.1.1). All other chemicals were reagent-grade products.

3.2.2 Experimental protocol

The protocol for this study was approved by the Massey University Animal Ethics Committee.

All experiments were carried out at an environmental temperature of 25°C on both male and female Spraque-Dawley rats which had free access to standard rat chow and water before the experiments. Both sexes were used since sex-linked differences in ethanol metabolism might lead to important differences in metabolic effects. The animals were killed by cervical dislocation without the use of anaesthetic, as the use of anaesthetics could cause changes in liver glucose and glycogen content. The experiment was conducted on a time-course basis. In addition, the whole experimental protocol was repeated twice. In the first experiment, two control and two treated animals were used at each time interval. The results from this experiment were unexpectedly different from those of previous experiments carried out by the research group, so the protocol was repeated, using three control and three treated rats at each time interval.

The experimental approach involved administration of a high dose of ethanol (6g/kg) to fed rats by stomach tube. Control animals were given water. This particular ethanol concentration was chosen based on previous work which showed that the effect of ethanol depended on its concentration and that a dose of 6g/kg gave a maximal effect on liver glycogen without adverse effects on the rats (Jeyarathan, unpublished data). The quantity of ethanol given depended on the exact weight of the animal but the dosage was always in the ratio of 6 g of ethanol per kg of weight. The procedure of applying ethanol by stomach tube has the advantage that it is similar to the process of drinking alcohol in humans. After treatment, the rats were placed in separate cages and were sacrificed at 45 minutes, 90 minutes and 3 hours. Immediately, the tissues were freeze-clamped using a pair of aluminium tongs precooled in liquid nitrogen. The tissue samples were then powdered at liquid nitrogen temperatures and stored at -70°C until later analysis.

Details of the experimental methods used and the results obtained are given in the following sections.

3.2.3 Metabolite assay methods

3.2.3.1 Enzymatic Analysis of Tissue Glycogen Content

This method is based on that described by Dietrich Keppler and Karl Decker (1984) using an enzymatic method to assay glycogen content. Because of specificity and sensitivity to glycogen, enzymatic hydrolysis is preferable to acid hydrolysis. This method also eliminates the inconvenience of isolating the glycogen. In fact alkaline extraction of glycogen from tissues, followed by ethanol precipitation, acid hydrolysis and colourimetric glucose determination is a conventional and less specific procedure which can result in a partial loss of glycogen (Passonneau et al., 1967). The reagents and preparation of solutions are given in detail in Appendix 2.

Principle of the glycogen assay

Tissue glycogen is hydrolysed in situ in a neutralised perchloric acid homogenate, and the glucose released is assayed enzymatically.

1) $\text{Glycogen} + (H_2O)_{n-1} \xrightarrow[\text{pH 4.8}]{\text{amyloglucosidase}} (\text{Glucose})_n$

2) $\text{Glucose} + \text{ATP} \xrightarrow[\text{pH 7.5}]{\text{hexokinase}} \text{ADP} + \text{Glucose 6-phosphate (G 6-P)}$

3) $\text{G6-P} + \text{NADP}^+ \xrightarrow{\text{G6-P dehydrogenase}} \text{6-phosphogluconolactone} + \text{NADPH} + H^+$

The glucose liberated after hydrolysis of glycogen is proportional to the increase of NADPH measured by the absorbance change at 340 nm. The glucose formed is specifically determined with hexokinase, HK (ATP: D-hexose-6-phosphotransferase, EC 2.7.1.1) and glucose 6-phosphate dehydrogenase, G6-PD (D-Glucose 6-phosphate: NADP 1-oxidoreductase, EC 1.1.1.49) (Barthelmai and Klin, 1962).

The enzyme used in hydrolysing glycogen is amyloglucosidase from *Aspergillus niger* (1,4-α-D-Glucan glucohydrolase, EC 3.2.1.3) (Fleming, 1965). It is an exoglucosidase which hydrolyses the α-D-(1→4) and α-D-(1→6)-linkages of glycogen (Pazur, 1960).

To obtain a quantitative hydrolysis of glycogen with amyloglucosidase, the pH is maintained between 4 and 5 (Keppler and Decker, 1984). The enzyme is fully active between 30°C and 50°C, and at 65°C two-thirds of the maximal activity still remains. The optimal conditions are pH 4.8, 40°C and a two hour incubation, with constant shaking in order to prevent the sedimentation of glycogen-protein aggregates.

The most economic commercial source of amyloglucosidase available from Sigma is supplied in a powder form. The enzyme is not fully purified and contains starch. Since starch can be readily hydrolysed by amyloglucosidase, the use of this impure enzyme would give a high glucose background because of glucose liberated from the starch. Therefore it is necessary to purify the enzyme before use. Starch can be removed by dialysis after hydrolysis by the enzyme. The enzyme is retained in the dialysis tube as small glucose molecules diffuse out to the surrounding solution of acetate buffer. The purification procedure for amyloglucosidase is described in the following section.

Preparation of amyloglucosidase (1mg/ml in acetate buffer)

5.012 g of starch-containing amyloglucosidase was added to 16.7 ml of acetate buffer (0.2 M; pH 4.8). The addition of the amyloglucosidase to the acetate buffer was accompanied by slow and constant stirring using a magnetic stirrer. The solution was left to stir for about two hours to ensure that the enzyme and starch had dissolved completely. The solution was then centrifuged at speed 4 in a Gallenkamp bench centrifuge for 15 minutes and the precipitate was discarded.

Dialysis tubing was prepared by boiling it twice in distilled water. The amyloglucosidase supernatant was dialysed against excess acetate buffer overnight to hydrolyse and remove the starch that was present in solution. The solution left was now concentrated amyloglucosidase which had to be diluted in acetate buffer for use at 1 mg/ml. The concentrated amyloglucosidase solution could be stored frozen at -20 °C to prevent any appreciable loss of activity.

Once the concentrated enzyme solution was prepared, the protein content was determined. The protein was determined by the Biuret method using bovine serum albumin (10 mg/ml) as a protein standard. The method is shown in Appendix 1. The concentration of the amyloglucosidase solution was about 5 mg/ml after dialysis, hence a 5-fold dilution was needed to give a final concentration of 1 mg/ml as required by the method (Keppler and Decker, 1984).

Preparation of tissue samples for glycogen hydrolysis

1. Deproteinisation

Deep-frozen tissue samples obtained by freeze clamping (section 3.2.2) were weighed and thoroughly homogenised with 5 parts by weight of ice-cold perchloric acid (PCA) solution. The homogenisation step was done manually with a ground glass rod grinding the tissue in a test tube. This took 5-10 min. Immediately after homogenisation, duplicate 0.2 ml samples of the homogenate were pipetted into clean plastic centrifuge tubes and kept in an ice-bath. These aliquots were used for glycogen hydrolysis. The remainder of the homogenate was centrifuged and also kept in an ice-bath. This portion was used for the determination of tissue glucose and ethanol. For ATP assays, fresh homogenates were used, because ATP degrades very rapidly once tissue homogenates have been prepared.

A summary of the procedures used in preparation of tissue samples for metabolite assays is shown in Figure 11.

2. Enzymatic hydrolysis

Duplicate 0.2 ml homogenates in PCA were incubated with 0.1 ml $KHCO_3$ and 2 ml amyloglucosidase solution (in acetate buffer, 1 mg/ml) in a shaking water bath at $40^{O}C$ for 2 hours (Details of the reagents are given in Appendix 2). The hydrolysis was stopped by addition of 1 ml PCA which inactivated the amyloglucosidase. After centrifugation at 12350x g for 10 min (SM 24 rotor), the pellet was discarded and the supernatant was neutralised with 3 M KOH. The neutralised supernatant was ready to be assayed for glucose by the method described below. Assay of this sample would give a total glucose content present in the tissue (ie.free glucose + glucose from glycogen). To determine free glucose in tissues, the samples were assayed, without undergoing the glycogen hydrolysis procedure, by the same enzymatic glucose assay.

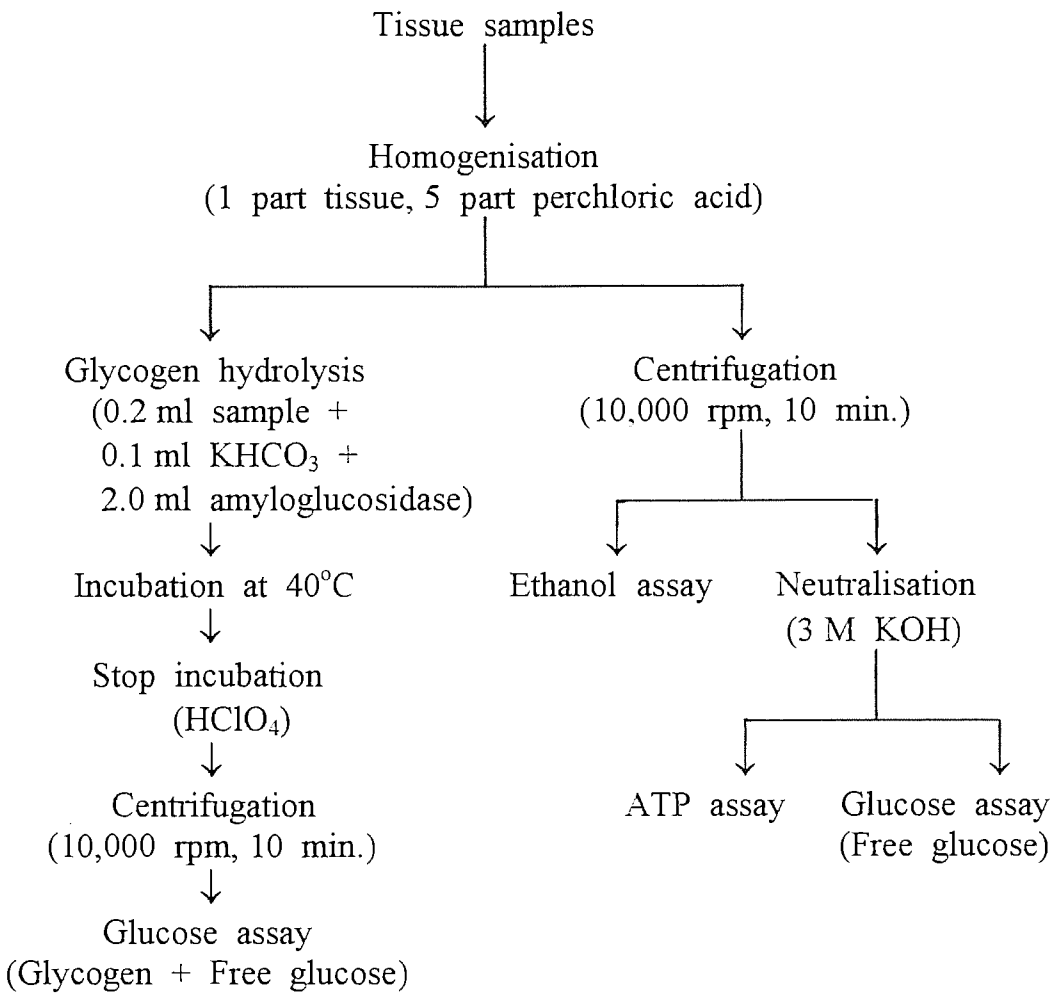


Fig.11 Summary of procedures in treatment of samples

Only samples used for ethanol assay were unneutralised, other samples had to be neutralised with 3 M KOH before any assays (glucose and ATP) were done.

3.2.3.2 Glucose Assay

Glucose was assayed using a spectrophotometric enzymatic assay as described by Lowry and Passonneau (1972). Glucose was assayed in neutralised supernatants of tissue samples homogenised in perchloric acid as described in section 3.2.3.1, using glucose 6-phosphate dehydrogenase and hexokinase. This was the same assay as was used to measure glucose after amyloglucosidase hydrolysis of samples for measurement of glycogen content (section 3.2.3.1). The details of the assay are given in Appendix 2.

3.2.3.3 ATP Assay

ATP was assayed enzymically as described by Lamprecht and Trautschold (1974). The homogenates were assayed immediately after centrifugation and neutralisation, because ATP was not a stable metabolite and tended to degrade very quickly once frozen samples thawed. In the present study it was demonstrated that tissues could lose half of their ATP content within the first hour after thawing (see section 3.3.3.2). This meant that the neutralised tissue samples that had been used for glucose assays could not be used for the ATP assay.

ATP was assayed using the same enzymes as for the glucose assay. The reaction cocktail was almost identical to the glucose cocktail except that ATP was replaced by glucose. The details of the assay are given in Appendix 2.

3.2.3.4 Ethanol Assay

Ethanol was measured according to the method of Cornell & Veech (1983). Ethanol was measured in unneutralised supernatants from perchloric acid treated tissue samples using alcohol dehydrogenase (ADH). The reaction produced NADH which was detected spectrophotometrically at 340 nm. The purpose of this assay was to detect and show the level of ethanol present in tissue samples after the ethanol dose given. The details of the assay are given in Appendix 2.

3.3 RESULTS & DISCUSSION

3.3.1 Glycogen Recovery

To ascertain complete hydrolysis and recovery of glycogen, a commercially available purified glycogen was used. Recovery was tested for both a solution of glycogen in perchloric acid and for glycogen added to a liver sample.

3.3.1.1 Recovery from glycogen standards made up in perchloric acid.

Two standards were prepared, one containing 0.5 mg of glycogen, the other 2.5 mg. The weighed glycogen was dissolved in 0.1 ml of water, then 0.5 ml of PCA was added. Details are shown in Table 6. These solutions were then used instead of homogenates in the glycogen hydrolysis. A glucose standard (1 mM) was included to assure that the assay was working. A PCA blank without glycogen was also included to act as a control. The results for glycogen recovery from PCA solutions are given in Table 7.

TABLE 6

Preparation of Standard Glycogen Solutions

Standard Solution	Glycogen (mg)	Volume of Water (ml)	Perchloric Acid added (ml)	Concentration (mg / ml)	Final mg in 0.2 ml
1	1.8	0.1	0.5	3	0.6
2	7.8	0.1	0.5	13	2.6

An 0.2 ml sample of each standard glycogen solution was used for glycogen determination by the method described in section 3.2.3.1 and Appendix 2.

TABLE 7

Recovery for glycogen / PCA solutions

Sample Assayed	mM Glucose in samples (average)	Net Glucose from glycogen	µ mol Glucose / 0.2 ml Sample	mg of Glycogen	% Recovery of Glucose or Glycogen
PCA Blank	0.128	——	——	——	——
Glucose Standard	0.910	——	——	——	91%
Glycogen Standard 1	20.45	20.32	4.06	0.66	110 %
Glycogen Standard 2	70.81	70.69	14.14	2.29	88%

The glycogen solutions were prepared as indicated in Table 6. The samples were assayed for glycogen as described in section 3.2.3.1. After enzyme hydrolysis, both standards and PCA blank were neutralised and then assayed for glucose as described in section 3.2.3.2. The PCA blank contained water and perchloric acid (PCA) in the same ratio as the samples and was incubated. It was used to measure free glucose content contributed by the enzyme solution and reagents. A glucose standard (1 mM) was included to show the enzymic glucose assay was working. The glucose and glycogen concentrations were calculated as shown in Appendix 3.

Since no glycogen was added to the PCA blank, the small glucose reading in the blank indicates that a small amount of glucose was present in the amyloglucosidase solution. This was essentially negligible in relation to the glucose from glycogen. Although recovery from the higher glycogen standard was 88%, it was acceptable. The result suggested that the majority of glycogen was hydrolysed and recovered from the solution. In fact the accuracy of this method has been established previously (Burchard et al., 1968) by reference to the weight of highly purified and extensively dried glycogen. Recoveries were between 97 and 100%. Therefore the current recoveries agreed well with the reference. Precision has also been determined and the standard deviations were only 2.5% to 3.5% (Burchard et al., 1968).

3.3.1.2 Recovery of glycogen from liver samples

Preparation of tissue sample with added glycogen

25 mg glycogen was weighed and dissolved in 5 ml of PCA. From this solution, 1 ml was pipetted into a given liver sample which was homogenised with 5 parts of PCA per gram of tissue. The sample now contained an extra 5 mg of glycogen. 0.2 ml of this homogenate was withdrawn for glycogen hydrolysis. The same liver sample without added glycogen was also assayed for glycogen. The glycogen assay for both samples was carried out as described in section 3.2.3.1 and Appendix 2. The results are given in Table 8.

The liver sample without added glycogen weighed 0.2826g (Sample 1), while the other liver sample containing added glycogen weighed 0.2635g (Sample 2). Therefore the amount of glycogen recovered from Sample 2 represented its liver glycogen plus 5 mg added glycogen.

Homogenate perchloric acid blank (HPCA) preparation

The homogenate blank contained the same ratio of perchloric acid to tissue homogenates except that it was not hydrolysed by amyloglucosidase. It therefore was used to measure free glucose content in tissue homogenates.

TABLE 8

Recovery of glycogen from liver samples

Samples Assayed	mM Glucose Concentration (average)	Net Glucose from glycogen (μM)	μ mol Glucose / 0.2 ml Sample	mg of Glycogen	% Recovery
Glucose Standard	0.968	——	——	——	97%
Glycogen Standard	131.73	——	26.35	4.27	85%
Sample 1 - HPCA Blank	4.21	——	——	——	——
- Glycogen	91.73	87.52	17.50	2.84	
Sample 2 - HPCA Blank	4.28	——	——	——	
- Glycogen	98.67	94.39	18.88	3.06	105%

1 mM glucose standard was used as glucose control and 25 mg/ml glycogen standard was used as glycogen control. Sample 1 and 2 were liver samples except that the latter contained an extra 5 mg of glycogen. HPCA blank was an unhydrolysed tissue homogenate which was used for measuring free glucose in samples.

Glycogen standard preparation

A glycogen standard was included as a control and was prepared as follows: 30 mg glycogen was dissolved in a 1.2 ml mixture containing 1 part water and 5 parts PCA. Withdrawal of 0.2 ml from final solution gave 5 mg glycogen.

The results for recovery of glycogen from liver samples are shown in Table 8. The recovery of standard glycogen was 85%. This recovery is lower than that in the previous section. It is interesting to note that at higher glycogen content, the recovery is decreased. It was suggested that the detection of tissue glycogen would be decreased if the concentrations of glycogen solution were increased (Pazur and Ando, 1972). Presumably this is due to incomplete hydrolysis of glycogen at high concentrations. However inadequate deproteinisation can also result in glycogen breakdown, yielding glucose not detected as coming from glycogen in this assay procedure (Keppler and Decker, 1984).

The glycogen obtained from liver Sample 1 was 2.84 mg. This represented the amount of glycogen present in 0.2826 g of liver tissue. Therefore in proportion, a 0.2634 g sample of the same liver would contain 2.65 mg glycogen. The difference between 3.06 mg, the assayed value for Sample 2 with added glycogen and 2.65 mg gave 0.41 mg which represented the amount of added glycogen recovered from 0.2 ml of homogenate. As the total volume = 2.58 ml, the corresponding amount of glycogen = 0.41 mg/0.2 ml x 2.58 ml = 5.3 mg. Hence the recovery of given glycogen from liver sample = 5.3/5 mg = 105%. This is a good recovery which indicates that all of the added glycogen is recovered from enzymic hydrolysis in liver samples and the hydrolysis is complete in two hours.

3.3.2 Effects of Ethanol on Tissue Glycogen Content

3.3.2.1 Effect of ethanol on liver glycogen content

The weight of each individual rat used in the first and second experiments is given in Appendix 4.

a) Results for the first experiment

The liver glycogen content of the experimental and control animals at given time intervals is shown in Table 9 and Figure 12a.

TABLE 9

Effects of ethanol on liver glycogen content
in male and female rats
First Experiment

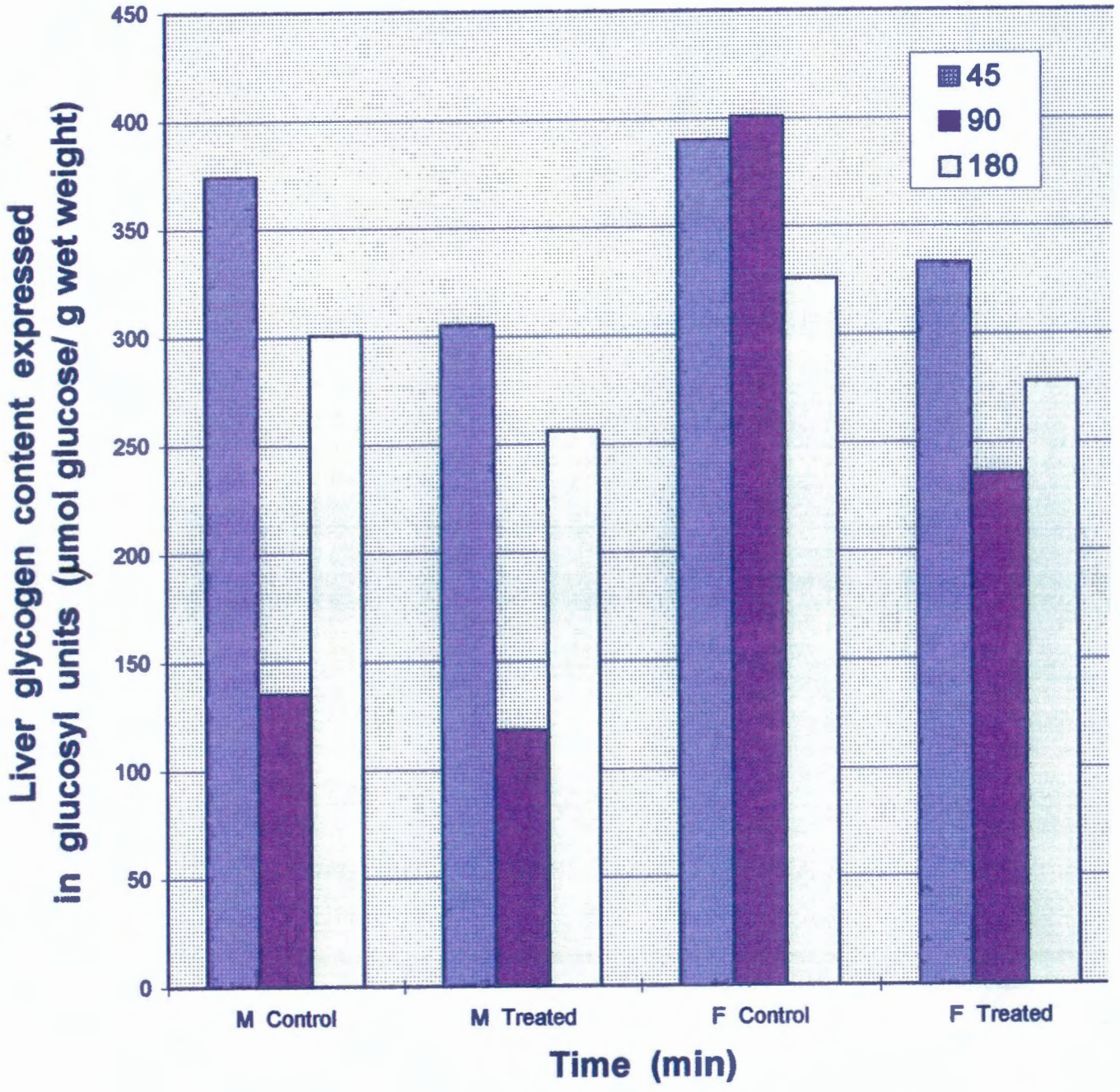
Time (min)		Liver glycogen content (μmol glucosyl units/g wet weight) Male	Female
45	Control	369	386
	Treated	300	328
	% relative to control	81.3	85.0
90	Control	129	397
	Treated	111	350
	% relative to control	86.0	88.2
180	Control	304	322
	Treated	250	274
	% relative to control	82.2	85.1

Two rats were used for control and treated at each given time. The values of liver glycogen are means, expressed in μmol glucosyl units/g wet weight. The glycogen content was analysed by enzymatic hydrolysis as described in section 3.2.3.1.

The liver glycogen content of the male and female controls at 45 minutes as expressed in glucosyl units is 369 and 386 μmoles/g respectively. According to literature, the normal liver glycogen contents in fed rats are in the range of 300-400 μmoles/g (Winder et. al., 1995; Calder and Geddes, 1992; Boyd et. al., 1981). The measured glycogen is in this range, therefore the result confirms the fed state of the animals used in the experiment.

In control animals, liver glycogen values at 180 minutes were lower than those at 45 minutes. Glycogen would be continuously broken down as the animals were removed from the food source immediately after

Figure 12a Liver glycogen content of male and female rats at given time after ethanol treatment (First experiment)



The values are average liver glycogen content of two animals.
M: male, F: female

ethanol administration. However, the result indicated a very low glycogen value at 90 minutes in the male control compared to that at 45 and 180 minutes. This was probably due to the small size of animals used for this group. (refer to Appendix 4 which has listed the body weight and liver weight of individual rats.) The 45 and 180 minute male groups contained rats which weighed at least 200 g while the 90 minute rats weighed merely 170 g.

To investigate this matter further, the glycogen values and liver weights of individual rats were plotted against the body weights as shown in Fig.13a and 13b. The data indicate that when an animal became bigger, both its liver weight and glycogen content increased. Most importantly, in the male rats, the data showed that a small increase in liver weight would lead to a dramatic increase in glycogen content. For instance, as the liver increased from 6 g to 9 g in weight, its glycogen content would rise from 110 μmoles/g to 300 μmoles/g, nearly 200% increase in liver glycogen content. This happened especially when the animal weights varied from 167 g to 200 g. However, for those which weighed more than 200 g, the changes in liver weight and glycogen were very small.

There is some evidence in the literature that glycogen content would vary more in using smaller animals. Kaminsky and Kosenko (1986) used 100-110 g rats and the liver glycogen was reported as only 200 μmoles/g. Winston and Reitz (1979) used rats weighing 140-160 g and liver glycogen was 230 μmoles/g. However, Sugden et al. (1992) using 200-250 g rats reported that glycogen was 419 μmoles/g. On the other hand, Winder et al. (1995) used animals weighing about 300 g and the glycogen was around 370 μmoles/g. Therefore the variation in liver glycogen would be greatest for rats weighing below 200 g. The result suggests that the glycogen content per g wet weight of liver becomes stable when rats are more than 200 g in weight. As a result, it was important to choose animals which had about the same weight, and those which weighed more than 200g would be preferred since their variation in glycogen content would be minimal.

Compared to the controls, there was a consistent decrease in liver glycogen in ethanol-treated animals. In treated males, glycogen was decreased by 19% at 45 minutes and by 18% at 180 minutes. For treated females, it was decreased by 15% at 45 minutes and by 12% at 90 minutes and by 15% at 180 minutes.

Figure 13a Relationship between liver glycogen content and animal body weight

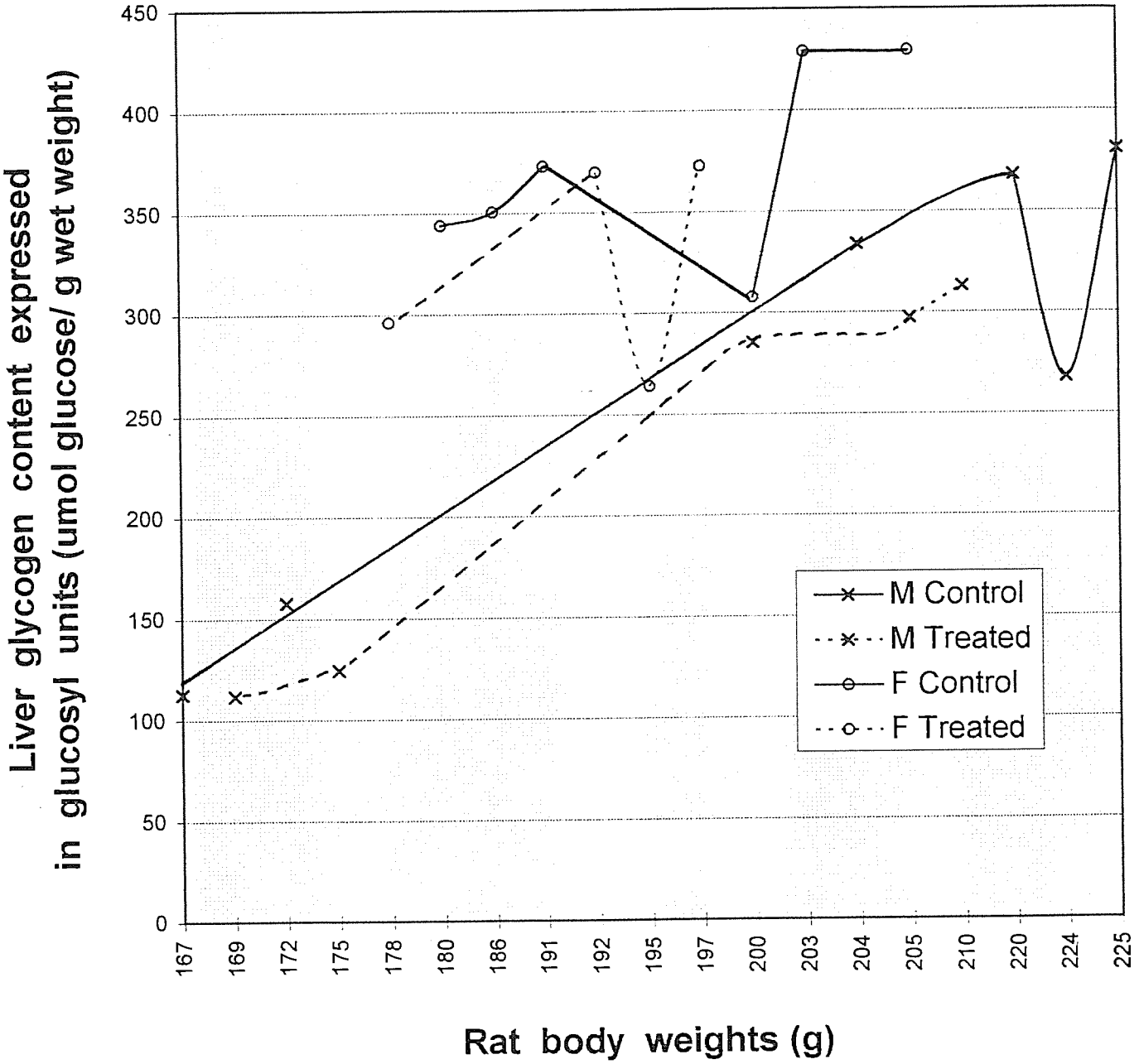
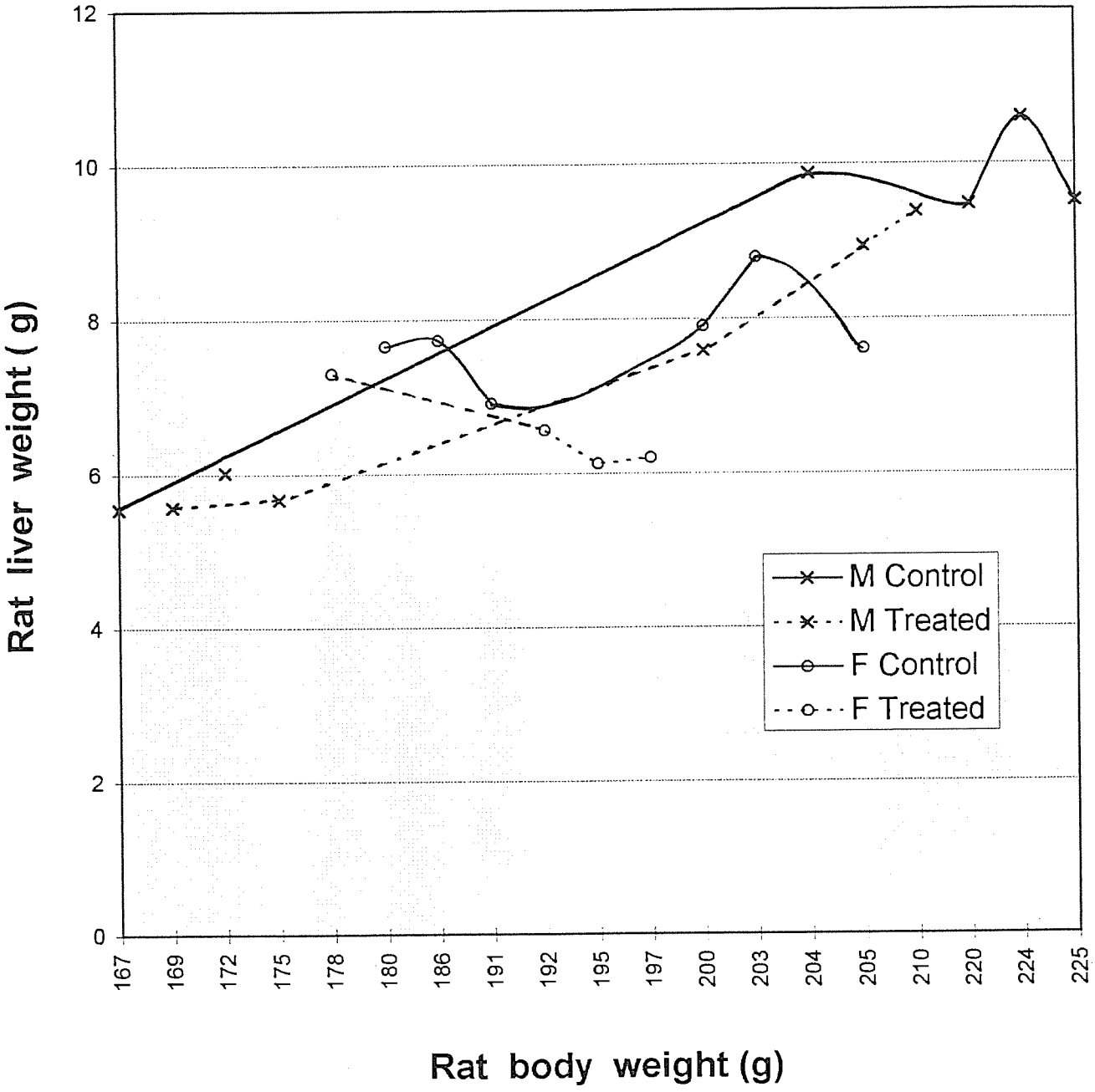


Figure 13b Relationship between liver weight and animal body weight



There was little or no difference observed between males and females regarding the percentage of decrease in liver glycogen induced by ethanol. Both ethanol-treated male and female rats showed about 15-20% less liver glycogen than in controls at any given time after ethanol consumption. However, the amount of decrease was much less than that observed from other work in this laboratory (Jeyarathan, unpublished data) which has shown that more than 60% decrease occurred in liver glycogen using the same ethanol dose (6g/kg) and the same strain of animals. Because of the difference between these results and the earlier ones, and the problem with small rats in the male group, the whole experimental protocol was repeated again. The second experiment would strictly use animals that weighed close to or more than 200g.

b) Results for the second experiment

The weights of the rats used in the second experiment are given in Appendix 4 and the results for the effect of ethanol on liver glycogen content in the second experiment are shown in Table 10 and Fig.12b.

Again, in the second experiment, the ethanol-treated rats showed lower liver glycogen content than controls at 45, 90 and 180 minutes. In males the decrease was 28% ($P<0.01$) at 90 minutes and 33% (P.0.001) at 180 minutes; whereas in females the decrease was 19% ($P<0.05$) at 45 minutes, 23% ($P<0.02$) at 90 minutes and 18% ($P<0.05$) at 180 minutes.

At 3 hours, the treated males showed more than 30% decrease in liver glycogen. Although the decrease was slightly higher than that seen in the first experiment (15-20 %), it is still less than the changes observed in previous work in this laboratory. The treated females showed as much as 23% of decrease in liver glycogen. In both control and treated animals, the glycogen level after 3 hours was also found to be less than that at 45 minutes because liver glycogen would have been continuously broken down but not regenerating as the rats were removed from the food source once ethanol was given. The only difference between this and the first experiment was that the decrease in liver glycogen content in treated rats was greater in this experiment.

The control male livers contained up to 530 μmoles/g of glycogen and the female had as much as 500 μmoles/g compared with 45 and 90 minute values of about 400 μmoles/g in the first experiment. This was probably due to the larger animals used in the second experiment. As discussed before, in the present experiments larger animals appeared to have a higher capacity for the accumulation of glycogen. In the second experiment, the animals chosen were all a bit heavier than those used in

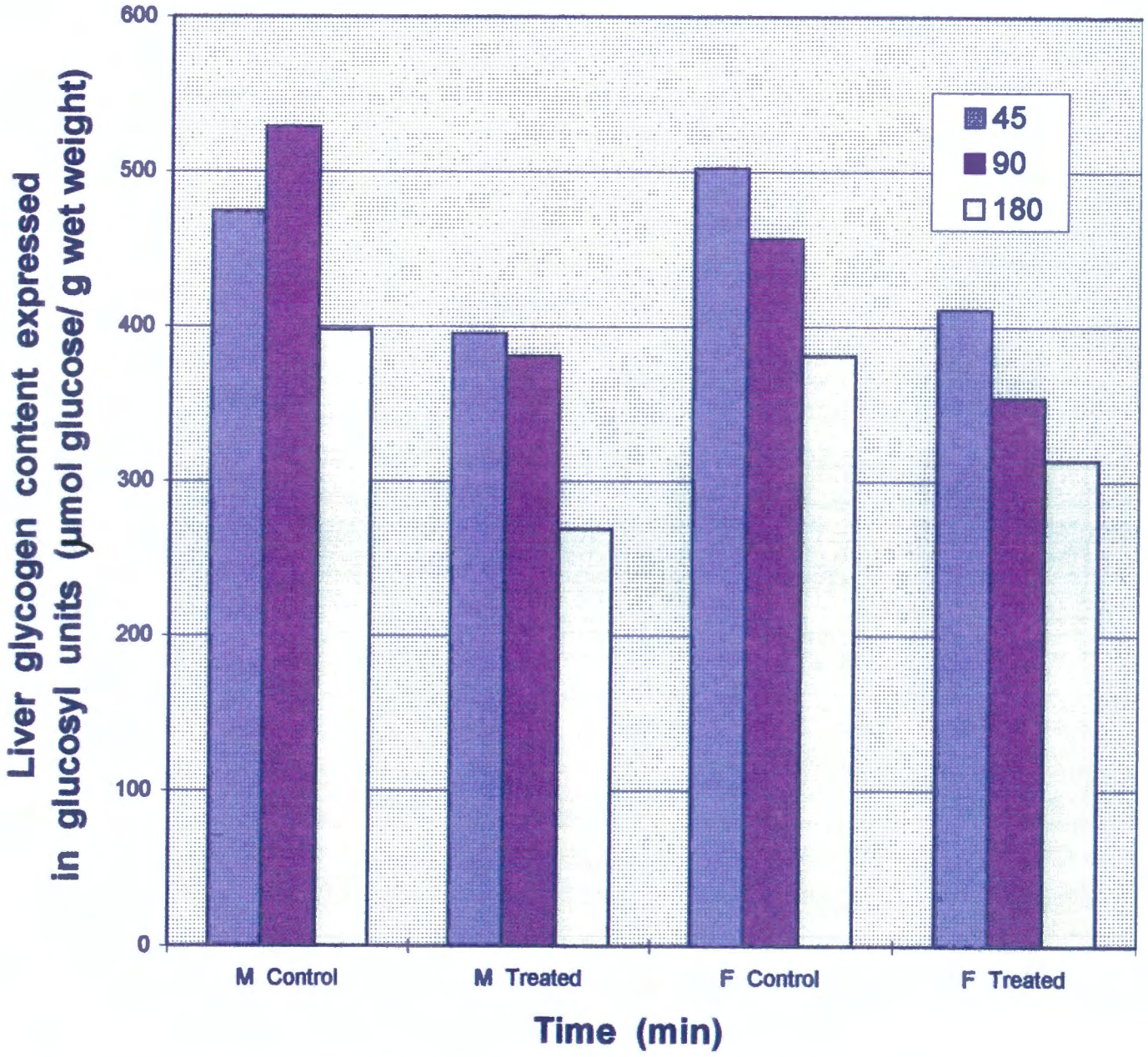
TABLE 10

Effects of ethanol on liver glycogen content
in male and female rats
Second Experiment

Time (min)		Liver glycogen content (μmol glucosyl units/g wet weight) Male	Female
45	Control	474 ± 34	502 ± 35
	Treated	395 ± 18*	411 ± 25
	% relative to control	83.3	81.80
90	Control	528 ± 5.2	456 ± 22
	Treated	381 ± 34*	354 ± 18*
	% relative to control	72.2	77.6
180	Control	398 ± 8.5	381 ± 21
	Treated	269 ± 12*	313 ± 16*
	% relative to control	67.6	82.2

Three rats were used for control and treated at each given time except the 180 minutes male control used four animals. The values of glycogen are means ± SEM, expressed in μmol glucosyl units/g wet weight. The glycogen content was analysed by enzymatic hydrolysis as described in section 3.2.3.1. *Significantly different from controls.

Figure 12b Liver glycogen content of male and female rats at given time after ethanol treatment (Second experiment)



The values are average liver glycogen content of three animals except the 180 minute male which used four animals.
M: male, F: female

the first set. The smaller animals weighed around 200g but the bigger ones weighed as much as up to 245g. The male group mainly contained slightly bigger animals (210-245g) than the female (190-212g), and the male liver glycogen content, on average, was a bit higher.

Collectively, the first and second results were similar in both liver glycogen content and in the amount of decrease in liver glycogen stimulated by ethanol.

There is general agreement in the findings that ethanol causes a decrease in glycogen content in liver. However, the amount of the decrease in glycogen content in the presence of an acute dose of ethanol has varied considerably in different studies (Kubota et al.,1992; Jeyarathan, unpublished data). From the current study, the liver glycogen content of treated male and female rats was found to be about 25-30 % less than in controls, whereas in other studies, it was found to be 61-78 % less at 90 minutes (Jeyarathan, unpublished data) and 75 % less in 4 hours after ethanol ingestion (Kubota et al., 1992). The discrepancies between results may arise for a number of reasons. There were differences between these studies in terms of the procedure of ethanol administration, assay methods of the liver glycogen and strains of the animals used in the experiments.

The present experimental approach involved administration of one single dose of ethanol (6 g/kg) by stomach tube, while Kubota used an infusion technique in which catheters were surgically inserted into the carotid artery and connected to infusion pumps. Jeyarathan also used oral administration as in the present experiment. The intake of ethanol via stomach tube required absorption from the stomach and small intestine, but by infusion, ethanol was fed directly into the rat via the circulation. The direct pumping of ethanol into the blood might produce a more potent effect than through absorption as oral ethanol administration does not necessarily ensure that all ethanol is absorbed into the body. Moreover, the continuous supply of ethanol into blood would help to maintain the dosage and hence prolong the effect of ethanol. There is evidence that blood ethanol concentration under oral administration is smaller than that given by intravenous application (DiPodova et al., 1987). Kubota and coworkers administered an acute dose of ethanol for 4 hours at a rate of 10 μl of 20% ethanol per minute (2g/kg of rat weight).

The ways in which glycogen was measured may also contibute to the differences in experimental results. The current experiment assayed glycogen in terms of glucose. In the present study, glycogen was first hydrolysed by amyloglucosidase and the liberated glucose moieties were subsequently analysed using glucose 6-phosphate dehydrogenase and

hexokinase. Jeyarathan measured glycogen directly by using the micro-method of Kemp and van Heijningen (1954) in which the tissue glycogen is extracted with methanol, then the glycogen is hydrolysed and measured in a colour reaction using phenol-sulphuric acid. It is possible that the extraction process with methanol might not be complete. Moreover, oligo- and small chain polyglucosides might be soluble and retained in the supernatant. Hence this might result in a partial loss of glycogen, which could be greater in ethanol-treated samples. On the other hand, Kubota and coworkers used alkaline glycogen extraction, followed by enzymatic hydrolysis and fluorometric determination. This method is a conventional but less specific procedure which could also lead to a partial loss of glycogen (Johnson and Fusaro, 1966; Passoneau et al., 1967). Therefore, it was possible that the lower glycogen content observed from their results might be caused by a partial loss of glycogen during the extraction process, which was accentuated in the ethanol-treated samples.

Using different strains of animals in the experiment might also lead to variations among results, since different strains have different response to ethanol. A single dose may have widely different effects within a population. The present study used Spraque-Dawley rats for investigation, but other researchers had used totally different strains. For instance, Kubota used Wistar rats for his studies. In the earlier work carried out in this laboratory, Jeyarathan used the same strain and source of rats as in the present experiments (supplied by the small animal unit of Massey University). However the original rats were discarded and replaced with completely new breeding stock when the present study started. Hence there might be some difference in the new stock of animals used for this experiment.

The hyperglycemic condition caused by ethanol which has been observed in the fed state in some studies (Shelmet et. al., 1988; Sneyd, 1989) may be due to ethanol-stimulated glycogenolysis which releases glucose molecules into the circulation. The present study supports this idea, as a decrease in liver glycogen content is seen, but the current study does not indicate the mechanism of the decrease in liver glycogen content. Although an increase in glycogenolysis is likely, the decrease could also be due to inhibition of glycogen synthesis. The mechanism by which glycogenolysis is stimulated by ethanol could be a direct effect of ethanol. This is based on the previous experiments (chap.2) that a decrease in stored carbohydrate caused by ethanol could be observed in an absence of hormones. In an earlier report, it has been shown that glycogen phosphorylase in liver tissue was stimulated at high ethanol concentrations (Langohr et. al., 1982). It was suggested that ethanol modified the properties of the proteins by inducing conformational change to the proteins, and as a result, glycogen phosphorylase was

activated (Cussó et al., 1989). Since glycogen phosphorylase catalyses the breakdown of glycogen, it is likely that ethanol stimulates glycogenolysis through activation of the enzymes involved in glycogen metabolism. By contrast, acetaldehyde does not show any effect on this enzyme (Cussó et al., 1989).

In recent years, research on glucose transporters has suggested a new mechanism for the effect of ethanol on carbohydrate metabolism. Transport of glucose across plasma membranes of cells is vital in all animal cells as glucose can be readily metabolised to produce energy to sustain life. There are various isoforms of transporters which have been numbered GLUT-1 through GLUT-5 (Fukumoto, 1989; Kayano et. al., 1990).

GLUT-1 and GLUT-3 are present in nearly all mammalian cells and they have a low K_m (~1 mM) that permits glucose uptake at blood glucose concentrations well below the normal fasting range (Unger, 1991). GLUT-4, with a K_m of about 5 mM, is believed to be the insulin-sensitive isoform of muscle and fat (Zorzano et. al., 1989). GLUT-5 present in the lining of small intestine with a K_m of about 1-2 mM, is responsible for glucose uptake from the gut. GLUT-2 however, is the only known facilitative glucose transporter with a high K_m. It has been found only in cells that participate in regulation of blood glucose homeostasis. This includes liver, pancreatic β cells and epithelial cells of small intestine (Unger, 1991).

In a recent report, it has been demonstrated that ethanol appeared to inhibit the function of the basal transporter GLUT-1 by interacting with highly specific active sites (Krauss et al., 1994). Moreover, ethanol was found to suppress glucose uptake by GLUT-1 but not uptake by any other transporters. It was suggested that a unique protein motif present in the GLUT-1 amino acid sequence but absent in others might confer ethanol sensitivity (Krauss et al., 1994). In a more recent report, ethanol was found to decrease GLUT-2 transporters at the level of transcription in hepatocytes (Nagamatsu et al., 1995). A substantial reduction in the levels of GLUT-2 mRNA was observed in the liver after 24 hours exposure of ethanol. Thus combining these two pieces of evidence, ethanol seems to promote liver glycogenolysis through an inhibitory effect on the levels of glucose transporters either directly by interaction or indirectly by transcription. Since liver glycogen is continuously breaking down, if glucose is not tranported into liver, the amount of glycogen would decrease as observed in Tables 9 and 10.

In addition to the effects of an acute dose of ethanol on liver glycogen content, some studies have shown that chronic ethanol treatment decreases liver glycogen content. Rats under prolonged exposure to ethanol (40-60 days) were reported to show a 60% decrease in liver glycogen compared with controls (Winston and Reitz, 1979). A gradual decrease of liver glycogen was also reported in experiments where treated animals had 50%, 45% and 70% less liver glycogen than controls after 3, 5 and 12 months of ethanol intake respectively (Mirone, 1965). These results suggest that a longer time exposure to ethanol produces a similar decrease in liver glycogen content to that seen in some other studies with an acute ethanol dose (Kubota et al., 1992; Jeyarathan; unpublished data).

3.3.2.2 Muscle glycogen

The data for the effects of ethanol on hind limb muscle glycogen content are shown in Tables 11 and 12, and Figures 14a and 14b.

From the literature, the muscle glycogen content for a 300 g male rat is around 40 μmoles/g wet tissue (Calder and Geddes, 1991; Fréminet and Leclerc, 1980). The male and female control animals in the first experiment generally have shown a similar value in muscle glycogen content except that the 90 minute female controls have a lower value of about 18 μmoles/g and it is unclear why this happens.

The male controls in the second experiment are also consistent with the value in the literature, but it is unclear why the 90 minute rats showed a high muscle glycogen value of 57 μmoles/g. The female controls on the other hand, showed a higher muscle glycogen content than the males in the second experiment. Since the females were slightly lighter than the males used in the experiments, it is interesting to see that the females would have a higher muscle glycogen value per g tissue. Perhaps the male rats were more active than the females during the experiments and therefore they tended to lose some muscle glycogen as a result of exercise.

In both experiments, an increase in muscle glycogen content was observed in the ethanol-treated male animals. In the first experiment, muscle glycogen of treated male rats was increased by 48% at 45 minutes and 31% at 90 minutes. The treated females showed an increase at 90 minutes but this might be due to the low value of controls. In the second experiment, the muscle glycogen in males was increased by 138% ($P<0.002$) at 45 minutes but was not increased at later time

TABLE 11

Effects of ethanol on muscle glycogen content in male and female rats

First Experiment

Time (min)		Muscle glycogen content (μmol glucosyl units/g wet weight) Male	Female
45	Control	27.5	34.2
	Treated	40.7	28.9
	% relative to control	148	84.5
90	Control	23.2	18.3
	Treated	30.4	22.5
	% relative to control	131	123
180	Control	33.4	35.5
	Treated	34.8	22.6
	% relative to control	104	63.7

Two rats were used for control and treated at each given time. The values of glycogen are means, expressed in μmol glucosyl units/g. The glycogen content was analysed by enzymatic hydrolysis as described in section 3.2.3.1.

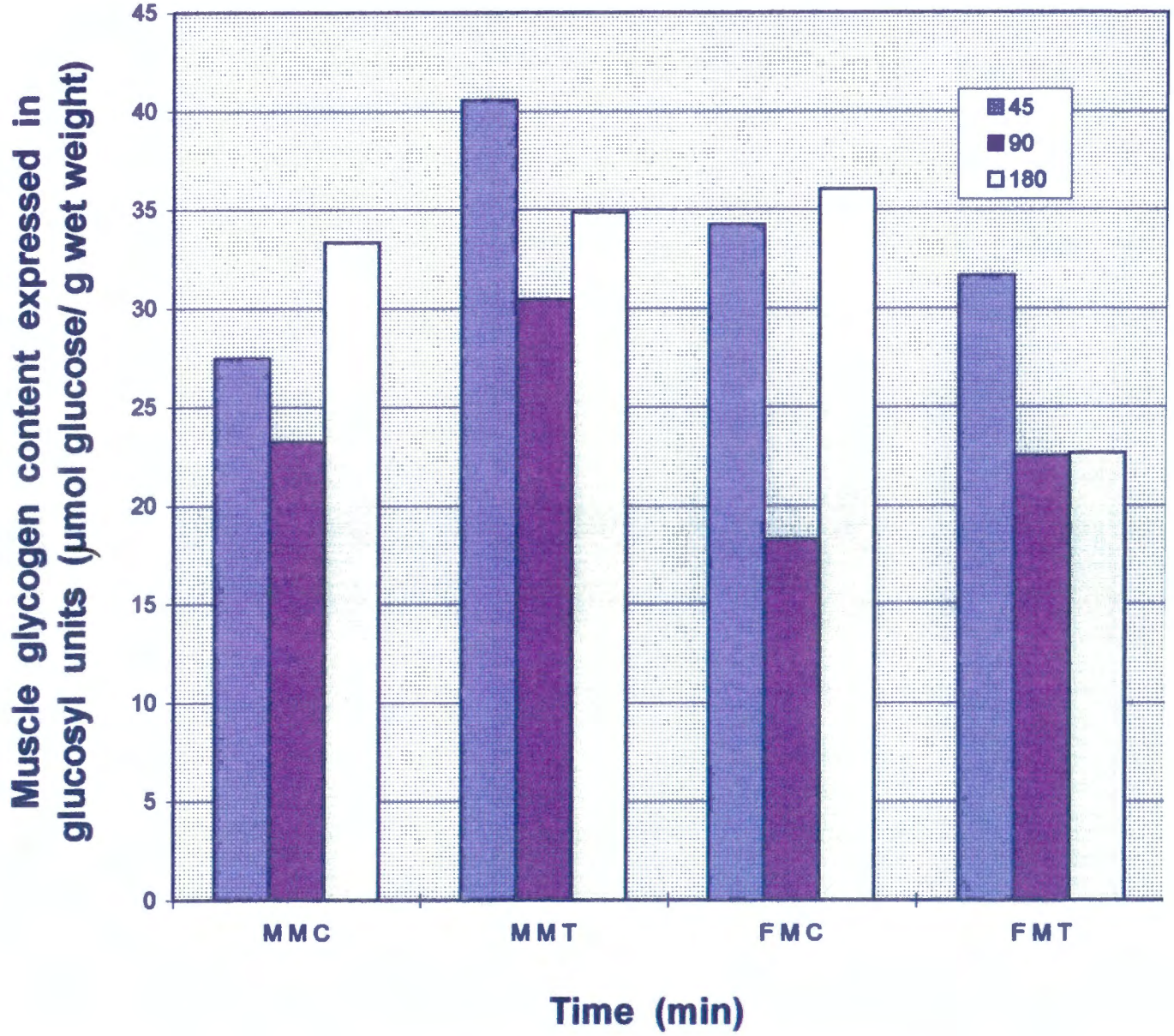
TABLE 12

Effects of ethanol on muscle glycogen content in male and female rats
Second Experiment

Time (min)		Muscle glycogen content (μmol glucosyl units/g wet weight) Male	Female
45	Control	32 ± 1.9	60 ± 8.5
	Treated	76 ± 5.9*	82 ± 8.3
	% relative to control	238	137
90	Control	57 ± 1.9	62 ± 0.32
	Treated	48 ± 6.7	60 ± 2.5
	% relative to control	84.2	96.8
180	Control	37 ± 5.5	60 ± 7.2
	Treated	36 ± 2.2	61 ± 5.1
	% relative to control	97.3	102

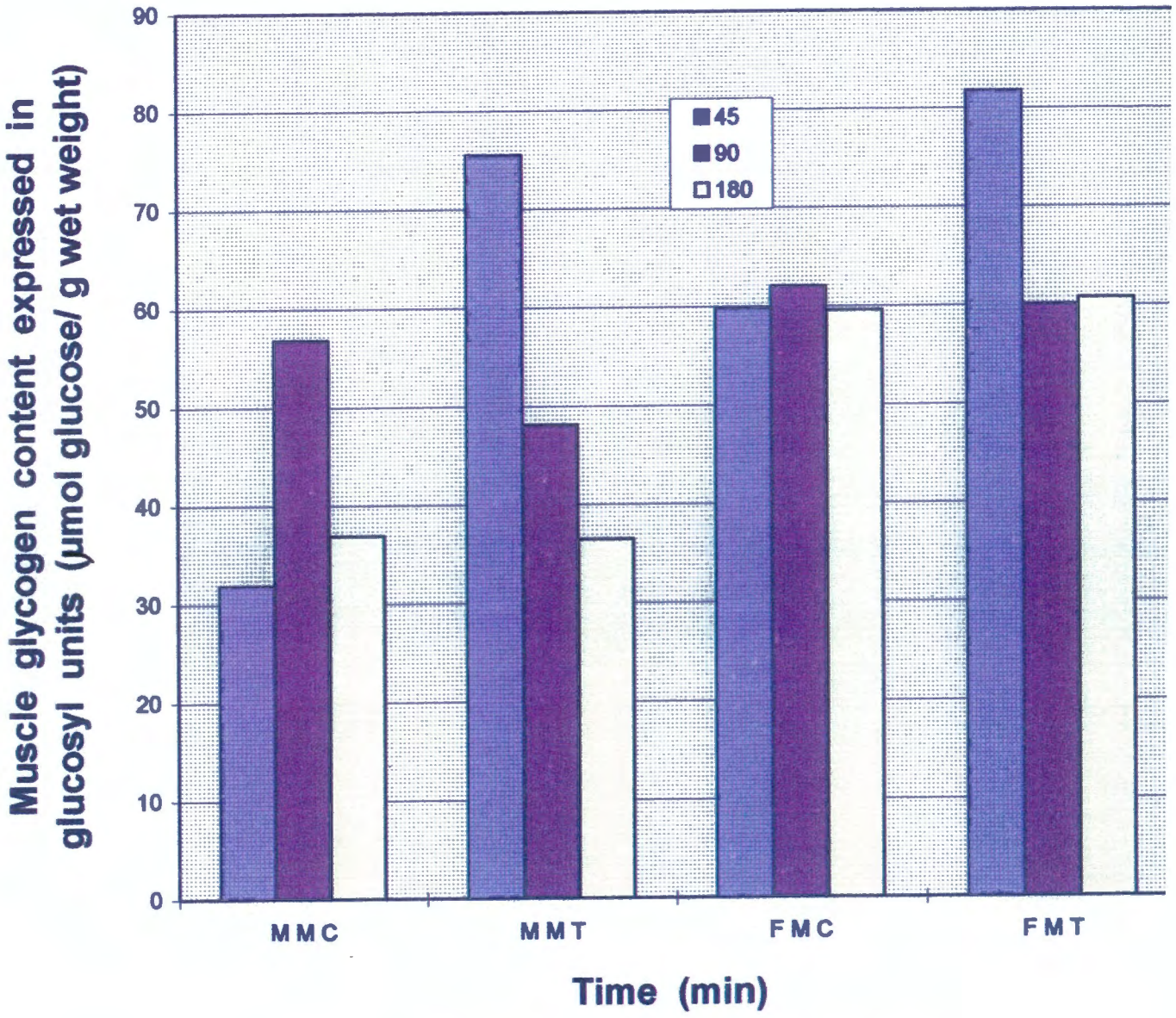
Three rats were used for control and treated at each given time except the 180 minutes male control used four animals. The values of glycogen are means ± SEM, expressed in μmol glucosyl units/g. The glycogen content was analysed by enzymatic hydrolysis as described in section 3.2.3.1. *Significantly different from controls.

Figure 14a Muscle glycogen content of male and female animals at given time after ethanol treatment (First experiment)



The values are average muscle glycogen content of two animals.
MMC: male muscle control
MMT: male muscle treated
FMC: female muscle control
FMT: female muscle treated

Figure 14b Muscle glycogen content of male and female animals at given time after ethanol treatment (Second experiment)



The values are average liver glycogen content of three animals except the 180 minute male which used four animals.
MMC: male muscle control
MMT: male muscle treated
FMC: female muscle control
FMT: female muscle treated

intervals. The treated female group at 45 minutes showed 37% increase, but this was not significant from students' t test.

The treated 45 minutes male rats in the second experiment showed a greater increase in muscle glycogen than the 45 minutes ones in the first experiment. The muscle glycogen content in treated males returned to about the same level as the controls at 180 minutes after ethanol administration.

Among the extrahepatic tissues, muscle has been shown to contain the highest total glycogen content in the body. In fact, about three quarters of all the glycogen in the body is stored in muscle (Cahill, 1976). The purpose of this glycogen is to provide a quick supply of glucose to muscle during muscular activities. The significant increase in muscle glycogen content observed in treated male rats might suggest that the glucose released from liver glycogenolysis was transported to muscle via circulation and was deposited as muscle glycogen. The ethanol-treated females, however, did not show any significant increase in glycogen content in the muscle compared with untreated controls, although they showed a similar decrease in liver glycogen content.

Hormonal mechanisms have been suggested to mediate the effect of ethanol since they act on adenylate cyclase, an enzyme catalysing the production of intracellular cAMP in hepatocytes (Erwin and Towell, 1983; Thurman et al., 1989). The experimental results indicate that glycogenolysis has taken place in liver but not in muscle and moreover in males, some glycogen deposition was observed in muscle. Since glucagon leads to glycogen breakdown in liver while adrenaline causes glycogenolysis in both liver and muscle (Dumm and Ralli, 1968), glucagon is more likely than adrenaline to have contributed to the effect of ethanol, if a hormonal response is involved.

3.3.2.3 Heart & Kidney glycogen

The results of the first and second experiments for the effects of ethanol on heart glycogen content are shown in Table 13.

The normal heart glycogen content in an adult male rat is about 20-30 μmoles/g wet weight (Kashiwaya et al., 1994; Fréminet et al., 1984). The male controls in the two experiments agree well with this value though the 90 minute group in the second experiment are a bit higher. The female controls tend to have higher heart glycogen content than males. The glycogen content in heart is less than that in liver and slightly less than in muscle because heart is not a main site for glucose storage

TABLE 13

Effects of ethanol on heart glycogen content in male and female rats

Time (min)			Heart glycogen content (μmol glucosyl units/g wet weight) Male	Female
45	1st	Control	12.7	19.7
		Treated	19.1	19.4
	2nd	Control	22 ± 6.3	45 ± 2.7
		Treated	40 ± 2.9*	51 ± 2.4*
90	1st	Control	30.2	19.0
		Treated	56.6	19.8
	2nd	Control	38 ± 4.5	44 ± 2.7
		Treated	31 ± 2.3	47 ± 3.5
180	1st	Control	25.7	39.2
		Treated	30.2	22.9
	2nd	Control	19 ± 4.5	49 ± 2.3
		Treated	26 ± 9.1	44 ± 5.3

At any given time, two rats were used in the first experiment and three rats were used in the second experiment except at 180 minutes in the latter where four rats were used. The values of heart glycogen are means for the first experiment and are means ± SEM for the second experiment, expressed in μmol glucosyl units/g. *Significantly different from controls.

and glycogen synthase activity is small compared to the other two tissues.

In the first experiment, the heart glycogen content in treated male rats showed a significant increase of 50% at 45 minutes, 87% at 90 minutes and 18% at 180 minutes, but the treated female rats did not show any difference from the controls at any given time. In the second experiment, a significant increase in heart glycogen was only observed at 45 minutes in treated male and female rats which were increased by 60% ($P<0.01$) and 13% ($P>0.05$) respectively. Overall the results for the effects of ethanol on heart glycogen content are similar to those for muscle glycogen content, in that ethanol treatment does not decrease the glycogen content, and, in some cases, appears to lead to an increase.

The results of the first and second experiments for the effects of ethanol on kidney glycogen content are shown in Table 14.

The kidney glycogen values obtained from the literature were very low, about 1-2 μmoles/g (Joseph and Subrahmanyam, 1972; Needleman et al., 1968). This value could vary with different portions of kidney being analysed, although the expected variation would be small. For instance, kidney cortex was recorded as having 1.6 μmoles/g and kidney medulla was reported as having 2.9 μmoles/g (Needleman et al., 1968). In this study, the entire kidney was freeze-clamped. The controls in the present experiments showed higher kidney glycogen contents (11-27 μmoles/g) than those given in this literature. It is possible that the difference is due to the effect of anaesthetic used by Needleman and the small animals (120-150g) used by Joseph (1972). In addition, because the detection limit of the current assay method for the determination of total glucose in the homogenate is about 6 μmoles of glycosyl units per g tissue wet weight (Keppler and Decker, 1984), the accuracy would be reduced for the small glycogen contents in kidney.

Generally, little variation in kidney glycogen contents was observed between controls and ethanol-treated animals at any given time. There was definitely no significant decrease in the tissue glycogen content, and if anything, as with muscle and heart glycogen, there was a slight increase in the male animals at some time intervals.

TABLE 14

Effects of ethanol on kidney glycogen content in male and female rats

Time (min)			Kidney glycogen content (μmol glucosyl units/g wet weight) Male	Female
45	1st	Control	13.0	24.6
		Treated	29.3	14.5
	2nd	Control	19 ± 14	26 ± 4.0
		Treated	35 ± 2.7	26 ± 4.7
90	1st	Control	17.9	11.1
		Treated	18.5	13.8
	2nd	Control	27 ± 9.0	25 ± 2.4
		Treated	25 ± 4.5	30 ± 2.9
180	1st	Control	21.6	12.7
		Treated	22.8	10.1
	2nd	Control	17 ± 2.8	26 ± 3.3
		Treated	18 ± 1.1	30 ± 9.2

At any given time, two rats were used in the first experiment and three rats were used in the second experiment except at 180 minutes in the latter where four rats were used. The values of kidney glycogen are means for the first experiment and are means ± SEM for the second experiment, expressed in μmol glucosyl units/g. *Significantly different from controls.

3.3.3 Effect of Ethanol on Other Metabolites

3.3.3.1 Tissue glucose contents

The results for the effects of ethanol on free glucose are shown in Tables 15 and 16 for male and female rats.

The normal glucose concentration of fed rats in liver, heart, kidney and muscle is about 7 (Kalkhoff et al., 1966), 2 (Kashiwaya et al., 1994), 4 (Needleman et al., 1968) and 2 μmoles/g (Williamson and Brosnan, 1974a) respectively. In the present results, both male and female controls showed free glucose values in these tissues similar to those in the literature.

From the results of earlier experiments, it has been shown that liver glycogen contents in ethanol-treated fed rats were significantly less than in controls. Hence it is suggested that glycogen breakdown might occur in liver. Based on this assumption, free glucose concentrations in liver might be expected to be elevated as glucose was released from liver glycogen. However, from the present results, there was no significant increase in free glucose in liver from either male or female rats. It is interesting that the free glucose contents in heart, kidney and muscle tissues were not generally increased as it is assumed that the free glucose released from liver as a result of liver glycogen breakdown might enter the circulation and translocate to other tissues like heart, kidney and muscle. There was only a small increase in glucose content in kidney and muscle in female rats at 45 minutes, and in muscle at 90 minutes. In all other cases, the tissue glucose content did not change.

It is interesting to note that little change was observed in the free glucose content of liver and other tissues when a decrease in liver glycogen content had occurred. From the literature, it has been reported that hyperglycemia is associated with ethanol treatment of fed animals (Potter and Morris, 1979). It seemed likely that an increase in tissue glucose content might also occur, but this study has shown that this is not the case. However, it cannot be concluded that the plasma glucose level in ethanol-treated rats was also unchanged, since in the present experiments the blood glucose concentration was not measured. Measurements of blood glucose were not included in the present study because it is difficult to sample blood while freeze clamping tissues. When the animals were cut open to remove tissues, blood clotted quickly and could not be sampled by syringe.

TABLE 15

Effects of ethanol on glucose content in different tissues from male rats

Time (min)		Tissue glucose content (μmol/g wet weight) Liver	Heart	Kidney	Muscle
45	1st Control	10.0	3.5	3.3	1.7
	Treated	5.6	4.2	4.9	1.7
	2nd Control	3.4 ± 0.2	2.3 ± 0.2	3.6 ± 0.3	2.0 ± 0.6
	Treated	4.2 ± 0.7	2.7 ± 0.4	3.6 ± 0.6	2.3 ± 0.3
90	1st Control	9.4	3.5	3.4	2.3
	Treated	12.4	5.6	4.3	1.7
	2nd Control	3.5 ± 0.2	2.9 ± 0.2	3.7 ± 0.8	2.7 ± 0.5
	Treated	3.6 ± 0.3	2.7 ± 0.4	3.8 ± 0.2	2.4 ± 0.1
180	1st Control	8.3	3.4	2.3	1.3
	Treated	9.7	3.9	4.9	1.4
	2nd Control	3.6 ± 0.1	2.9 ± 0.6	2.8 ± 0.4	2.0 ± 0.4
	Treated	3.2 ± 0.2	3.1 ± 1.0	3.3 ± 1.0	1.6 ± 0.2

At any given time, two rats were used in the first experiment and three rats were used in the second experiment except at 180 minutes in the latter where four rats were used. The free glucose contents are means for the first experiment and are means ± SEM for the second experiment. *Significantly different from controls.

TABLE 16

Effects of ethanol on glucose content in different tissues from female rats

Time (min)		Tissue glucose content (μmol/g wet weight) Liver	Heart	Kidney	Muscle
45	1st Control	6.1	2.7	3.1	2.0
	Treated	6.7	2.9	4.1	2.0
	2nd Control	2.1 ± 0.3	3.8 ± 0.1	3.3 ± 0.4	3.4 ± 0.2
	Treated	2.7 ± 0.3	3.4 ± 0.1	4.0 ± 0.1*	3.9 ± 0.1*
90	1st Control	5.6	2.6	2.6	2.4
	Treated	6.7	3.3	3.9	2.3
	2nd Control	3.3 ± 1.0	2.9 ± 0.5	3.8 ± 0.3	3.5 ± 0.2
	Treated	4.0 ± 0.2	3.5 ± 0.4	3.6 ± 0.2	4.0 ± 0.2*
180	1st Control	5.5	3.0	3.3	1.7
	Treated	7.0	3.0	3.8	3.5
	2nd Control	2.9 ± 1.0	3.2 ± 0.3	3.1 ± 0.4	3.5 ± 0.2
	Treated	3.1 ± 0.4	3.1 ± 0.1	3.2 ± 0.6	3.3 ± 0.3

At any given time, two rats were used in the first experiment and three rats were used in the second experiment except at 180 minutes in the latter where four rats were used. The free glucose contents are means for the first experiment and are means ± SEM for the second experiment. *Significantly different from controls.

3.3.3.2 Tissue ATP content

The results from male and female rats for the effects of ethanol on ATP are given in Table 17 and 18 respectively.

From the literature, the ATP content for fed rats of liver, heart and kidney tissues is 2.5 (Brosnan and Phil, 1968), 4.3 (Kraupp et al., 1967), and 1.4 (Brosnan and Phil, 1968) μmoles/g. Heart tissue has a high ATP content probably because it constantly requires energy to pump blood to all vital organs in the body. According to the literature, muscle ATP content ranges from 2.38 up to 6.0 μmoles/g (Marquez-Julio and French, 1967). Muscle ATP content was higher than in other tissues perhaps consonant with the need for a larger energy reserve in this tissue.

The liver ATP content in male controls from the two experiments were consistent with values given in the literature and there was little difference between male and female controls. However, the ATP values in heart and kidney of male and female controls were mostly lower than those in the literature. It is suggested that this is due to ATP degradation during the removal of tissues after killing. The ATP content for liver was not affected because liver was freeze-clamped first while heart and kidney were removed later. The other possible factor was that ATP started to degrade during analysis. ATP in male controls and the first set of female controls were analysed in an hour and individual samples were not assayed immediately after homogenisation but rather waited for a group. The ATP assays for second set of female controls however were completed in 15 minutes and individual samples were analysed immediately after homogenisation. Comparing the female controls in the first and second experiments, a difference in ATP content as much as 50% was observed. Therefore the time allowed to elapse before assaying ATP was critical for obtaining an accurate result. Once the tissues were thawed, enzymes in tissues might become active and start to degrade ATP. Thus it is desirable to complete the measurement as soon as possible preferably in 15-20 minutes.

It is interesting that the female controls in the second experiment showed a higher ATP content in muscle than described in the literature. Possibly these rats have a higher rate of metabolism in their muscle. From the present results, there are no significant differences in ATP levels between controls and ethanol-treated animals in any tissue at any given time, and this applied to both male and female rats. There was a tendency, however, for liver ATP content to be lower in treated animals. The lack of a significant change in tissue ATP contents is consistent with the literature. eg. Kubota et al. (1992) found that ATP

TABLE 17

Effects of ethanol on ATP content in different tissues from male rats

Time (min)		Tissue ATP content (μmol/g wet weight) Liver	Heart	Kidney	Muscle
45	1st Control	2.4	1.7	0.57	4.5
	Treated	2.2	2.0	0.59	6.0
	2nd Control	2.0 ± 1.4	0.38 ± 0.14	0.30 ± 0.02	3.9 ± 0.39
	Treated	0.8 ± 0.72	0.19 ± 0.09	0.34 ± 0.01	1.9 ± 0.01*
90	1st Control	1.6	1.9	0.54	6.5
	Treated	1.3	1.4	0.51	4.6
	2nd Control	1.4 ± 0.63	0.53 ± 0.08	0.62 ± 0.05	5.8 ± 0.28
	Treated	1.1 ± 0.38	0.47 ± 0.12	0.52 ± 0.05	6.0 ± 1.6
180	1st Control	1.7	1.4	0.48	5.2
	Treated	2.2	0.93	0.57	7.7
	2nd Control	1.7 ± 0.53	1.0 ± 0.87	0.53 ± 0.07	4.6 ± 2.56
	Treated	1.7 ± 0.37	0.44 ± 0.26	0.35 ± 0.10	4.8 ± 0.64

At any given time, two rats were used in the first experiment and three rats were used in the second experiment except at 180 minutes in the latter where four rats were used. The ATP values are means for the first experiment and are means ± SEM for the second experiment. *Significantly different from controls. Units in μmol/g.

TABLE 18

Effects of ethanol on ATP content in different tissues from female rats

Time (min)		Tissue ATP content (μmol/g wet weight) Liver	Heart	Kidney	Muscle
45	1st Control	2.0	0.79	0.17	5.8
	Treated	1.1	0.63	0.17	3.6
	2nd Control	3.6 ± 1.16	3.1 ± 0.62	1.7 ± 0.07	12 ± 0.75
	Treated	2.3 ± 0.45	2.0 ± 0.28	1.5 ± 0.33	10 ± 1.29
90	1st Control	2.1	1.2	0.2	2.5
	Treated	1.8	0.82	0.18	2.9
	2nd Control	3.3 ± 0.22	2.5 ± 0.24	2.4 ± 0.59	11 ± 0.45
	Treated	2.5 ± 0.70	2.9 ± 0.65	2.0 ± 0.53	9 ± 0.84*
180	1st Control	1.9	1.0	0.22	5.2
	Treated	1.4	0.65	0.18	2.2
	2nd Control	2.9 ± 1.18	3.1 ± 0.47	3.0 ± 0.26	10 ± 1.16
	Treated	2.7 ± 0.19	3.2 ± 0.29	2.2 ± 0.06	10 ± 0.82

At any given time, two rats were used in the first experiment and three rats were used in the second experiment except at 180 minutes in the latter where four rats were used. The ATP values are means for the first experiment and are means ± SEM for the second experiment. *Significantly different from controls. Units in μmol/g.

concentration was similar in both ethanol and saline groups after an acute dose of ethanol.

There is little difference in ATP concentration observed between controls and ethanol-treated animals in the two experiments, but from the literature, it has been reported that there is an increase in cellular ATP during acute ethanol intoxication (French, 1966; Connolly et al., 1962). Since the electron transport chain of mitochondria is involved in the production of ATP from the oxidation of NADH (Mitchell, 1979), and because the cellular increase in NADH (Connolly et al., 1962) from the cytosolic oxidation of ethanol results in an intramitochondrial increase via glutamate-aspartate shuttle mechanisms, it is possible that the cellular ATP concentration could increase after an acute dose of ethanol. This is not the case in the present experiment and it is unclear why such an increase is not detected. It is perhaps this is due to the small number of animals used in the present experiments. Other researcher, for instance, French (1966) used seventy rats compared to two or three animals used in the present study. Hence it is difficult to detect the small changes in ATP especially when ATP levels were low in tissue samples.

It is interesting that with chronic ethanol treatment, there is a marked decrease, rather than an increase, in cellular ATP (Spach et al., 1990; Gordon, 1973). In early literature, it was suggested that the decrease resulted from changes in mitochondrial structure (Kiessling and Tobe, 1964). In fact it is now well established that in mitochondria from chronically ethanol-fed rats, the activity and the content of ATPase and electron transport complexes (site I, II and III) are reduced (Spach et al., 1987; Rubin and Rottenberg, 1982; Thayer and Rubin, 1981). It has been suggested that ethanol might act as an uncoupler of the mitochondrial electron-transport chain, resulting in a lack of electrons flowing through the mitochondrial complexes (Ebina Nagai, 1980). As a result, no proton-motive force would be generated to drive ATP synthesis. A decrease in ATP would in turn stimulate glycolysis and glycogen breakdown. In a more recent report which dealt indirectly with this subject (Spach et al., 1990) showed that chronic ethanol consumption would cause a decrease in the hepatic energy state only at low oxygen tensions. Spach emphasized the importance of determining the hepatic energy state from ethanol-treated animals under conditions where the tissue was adequately oxygenated before being sampled for metabolite analyses, because under physiological oxygenation conditions, no significant ethanol-related alteration in the energy state of liver was observed. It was suggested that the decrease was related to the level of endogenous substrate available for energy metabolism (Miyamoto and French, 1988).

3.3.3.3 Ethanol concentrations in tissues of treated rats

The ethanol concentrations in tissues from fed male and female rats after an acute dose of ethanol are shown in Tables 19 and 20. The data from the first experiment are plotted in Figures 15a and 15b, and those from the second experiment are given in Figures 16a and 16b.

There is some ethanol detected in all tissues from control animals although the concentrations are low. This result is expected as endogenous ethanol which is produced by bacteria in the gut. Treated animals showed very high ethanol concentrations in all tissues at 45, 90 and, in some cases, 180 minutes, which indicated that these animals had absorbed a significant proportion of the ethanol dose.

The ethanol concentrations in treated male and female rats were generally highest at 45 minutes, followed by those at 90 minutes and then 180 minutes. This indicated that ethanol was oxidised and removed from the body over time. Compared to heart, kidney and muscle, liver generally showed the lowest ethanol concentration at 45, 90 and 180 minutes in the two experiments. This suggests that ethanol is oxidised at a faster rate in liver than in any other tissues. In fact it has been shown that liver tissue contains the most ADH activity in the body and this accounts for 90% of the total ADH activity in rats (Boleda et al., 1989, Duester, 1991). Generally, the ethanol concentration in male tissues was lower than that in female tissues in the first experiment at any given time. The second experiment however, showed similar ethanol concentration between male and female rats in liver, heart, kidney and muscle.

It is interesting that the ethanol concentrations returned to the basal levels in 3 hours in male rats in the first experiment. This is probably as a result of the size of animals used in the experiments. Braggins et al. (1981) had pointed out that larger rats tended to oxidise ethanol more slowly. It has been shown that a significant negative correlation exists between weight of animals and rates of ethanol clearance. Since the animals used in the first experiment were smaller than those in the second experiment (see Appendix 4), it is possible that ethanol might be cleared at a faster rate in small animals. In fact lower alcohol dehydrogenase activity has been reported in older rats (Rachamin et al., 1980). The decrease in ethanol oxidation rate together with alcohol dehydrogenase activity with increasing age have been demonstrated in spontaneously hypertensive rats (Rachamin et al., 1980). It was suggested that the decrease in alcohol dehydrogenase activity in older rats is due to an increase in testosterone levels in older rats (Braggins et al., 1981).

TABLE 19

Ethanol concentration in tissues from male rats

Time (min)		Ethanol concentration in tissues (μmol/g wet weight) Liver	Heart	Kidney	Muscle
45	1st Control	2.2	5	4.3	6.7
	Treated	16.6	46.2	56.2	5.7
	2nd Control	3.2 ± 0.08	3.0 ± 0.6	4.6 ± 1.1	3.7 ± 0.81
	Treated	39.1 ± 3.0	68.1 ± 5.2	52.4 ± 6.2	53.8 ± 2.3
90	1st Control	1.6	8.7	5.6	1.0
	Treated	18.7	44.0	50.4	2.0
	2nd Control	4.3 ± 1.2	3.7 ± 0.15	3.5 ± 0.22	3.2 ± 0.22
	Treated	35.7 ± 6.7	67.7 ± 32.7	43.3 ± 10.5	31.1 ± 3.7
180	1st Control	2.6	8.2	6.4	1.3
	Treated	2.7	11.4	6.3	1.3
	2nd Control	2.7 ± 0.2	3.0 ± 0.5	7.1 ± 3.1	3.9 ± 0.4
	Treated	34.9 ± 9.5	35.7 ± 6.6	38.0 ± 10.6	34.6 ± 9.8

At any given time, two rats were used in the first experiment and three rats were used in the second experiment except at 180 minutes in the latter which used four rats. The ethanol concentrations are means for the first experiment and are means ± SEM for the second experiment. *Significantly different from controls. Units in μmol/g.

TABLE 20

Ethanol concentration in tissues from female rats

Time (min)		Ethanol concentration in tissues (μmol/g wet weight) Liver	Heart	Kidney	Muscle
45	1st Control	6.5	4.7	4.4	6.5
	Treated	43	88.1	76.3	37.2
	2nd Control	4.4 ± 0.60	4.6 ± 0.08	5.8 ± 0.51	2.9 ± 0.82
	Treated	26.3 ± 2.0	60.9 ± 3.1	36.2 ± 4.0	44.5 ± 1.1
90	1st Control	5.5	6.3	4.1	7.0
	Treated	40.0	32.0	32.2	23.9
	2nd Control	4.4 ± 0.09	4.7 ± 0.15	5.8 ± 0.72	2.9 ± 0.17
	Treated	30.0 ± 1.9	34.2 ± 4.7	33.6 ± 2.7	34.9 ± 6.4
180	1st Control	5.1	5.7	4.4	6.5
	Treated	12.8	5.1	33.9	20.8
	2nd Control	4.2 ± 0.61	6.3 ± 1.1	4.9 ± 0.43	2.8 ± 0.2
	Treated	21.6 ± 4.9	28.9 ± 1.9	22.6 ± 5.3	23.6 ± 4.5

At any given time, two rats were used in the first experiment and three rats were used in the second experiment except at 180 minutes in the latter which used four rats. The ethanol concentrations are means for the first experiment and are means ± SEM for the second experiment. *Significantly different from controls. Units in μmol/g.

Figure 15a Ethanol concentrations of different tissues in male rats (First experiment)

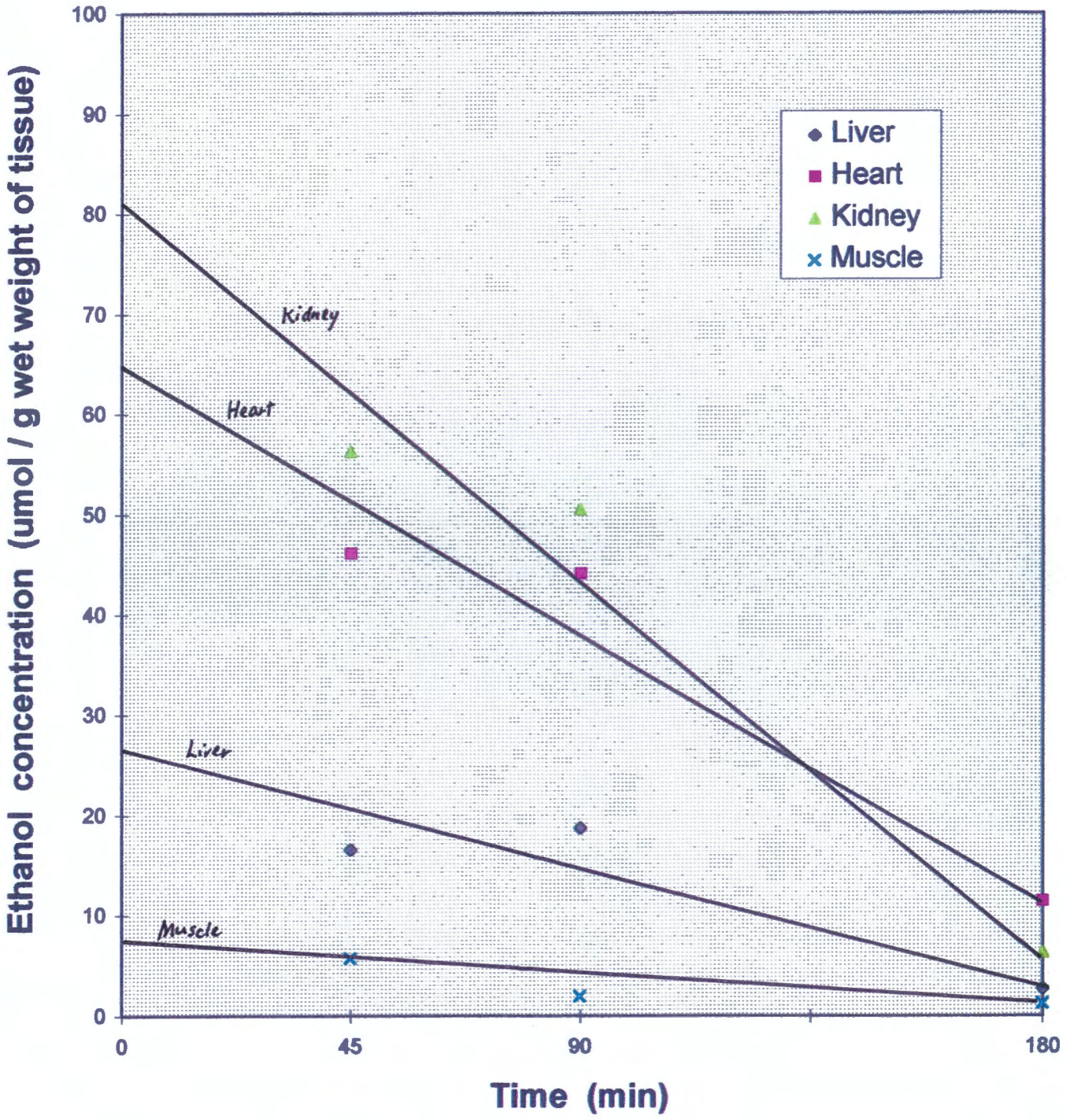


Figure 15b Ethanol concentrations of different tissues in female rats (First experiment)

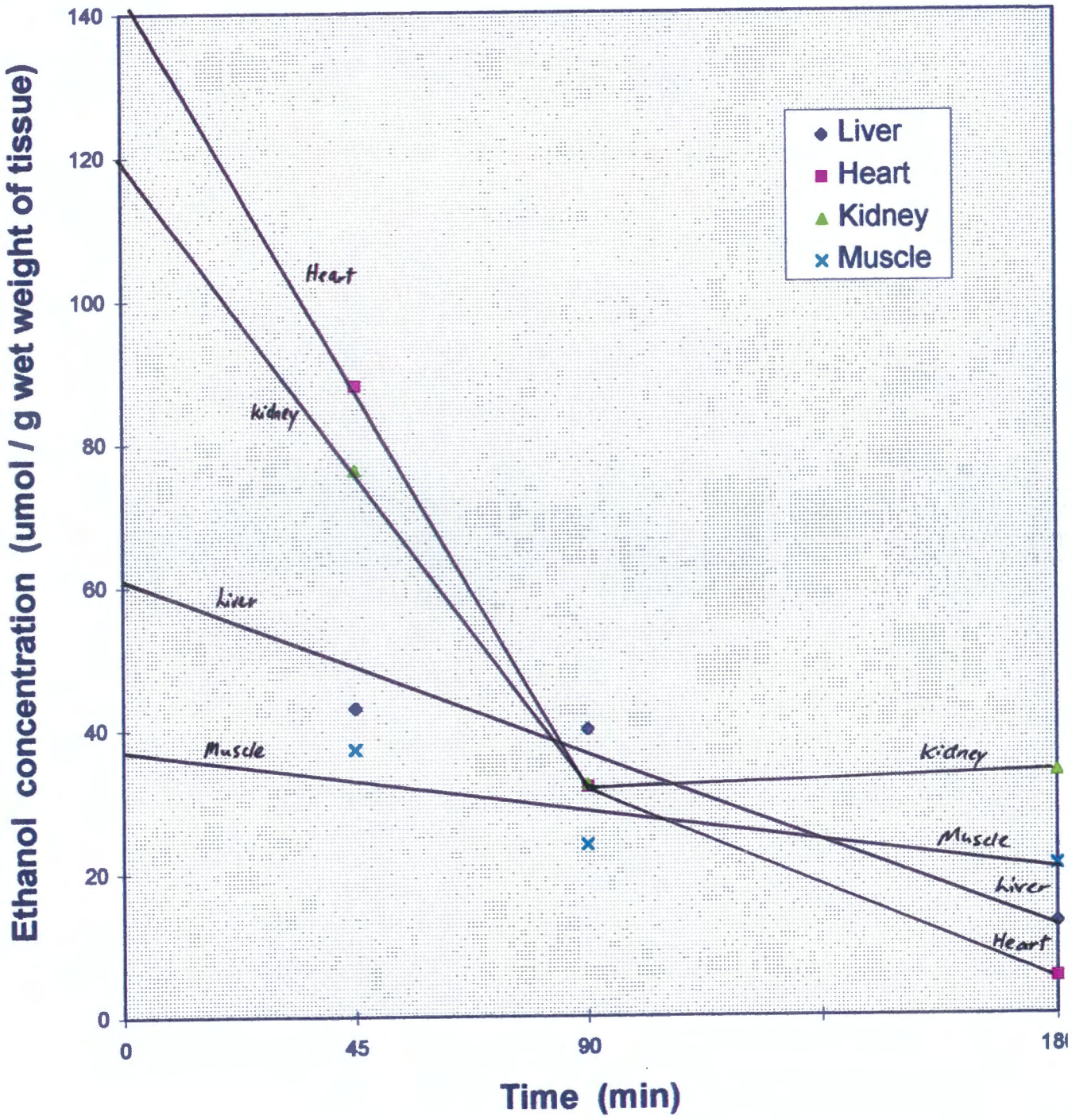


Figure 16a Ethanol concentrations of different tissues in male rats (Second experiment)

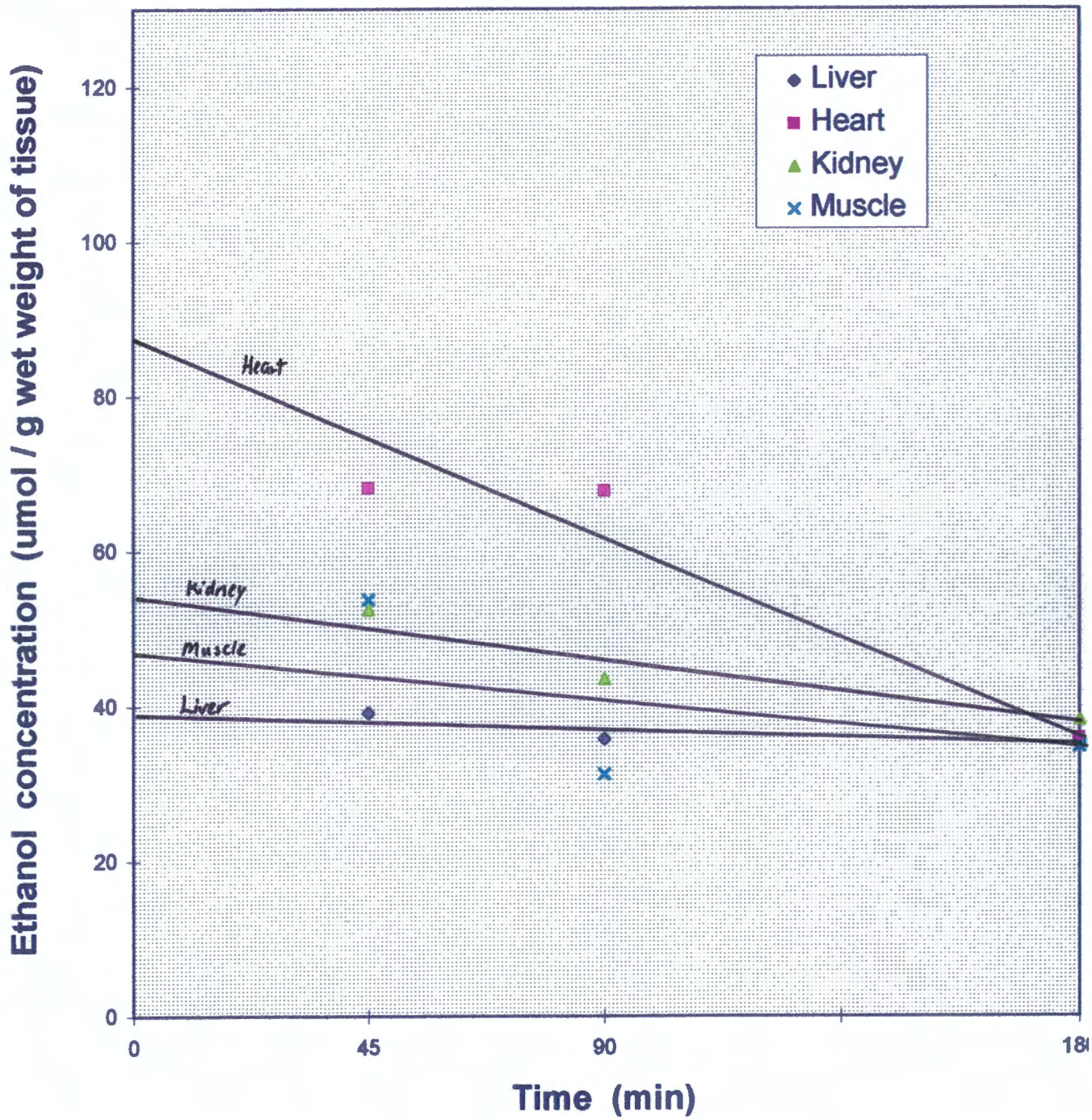
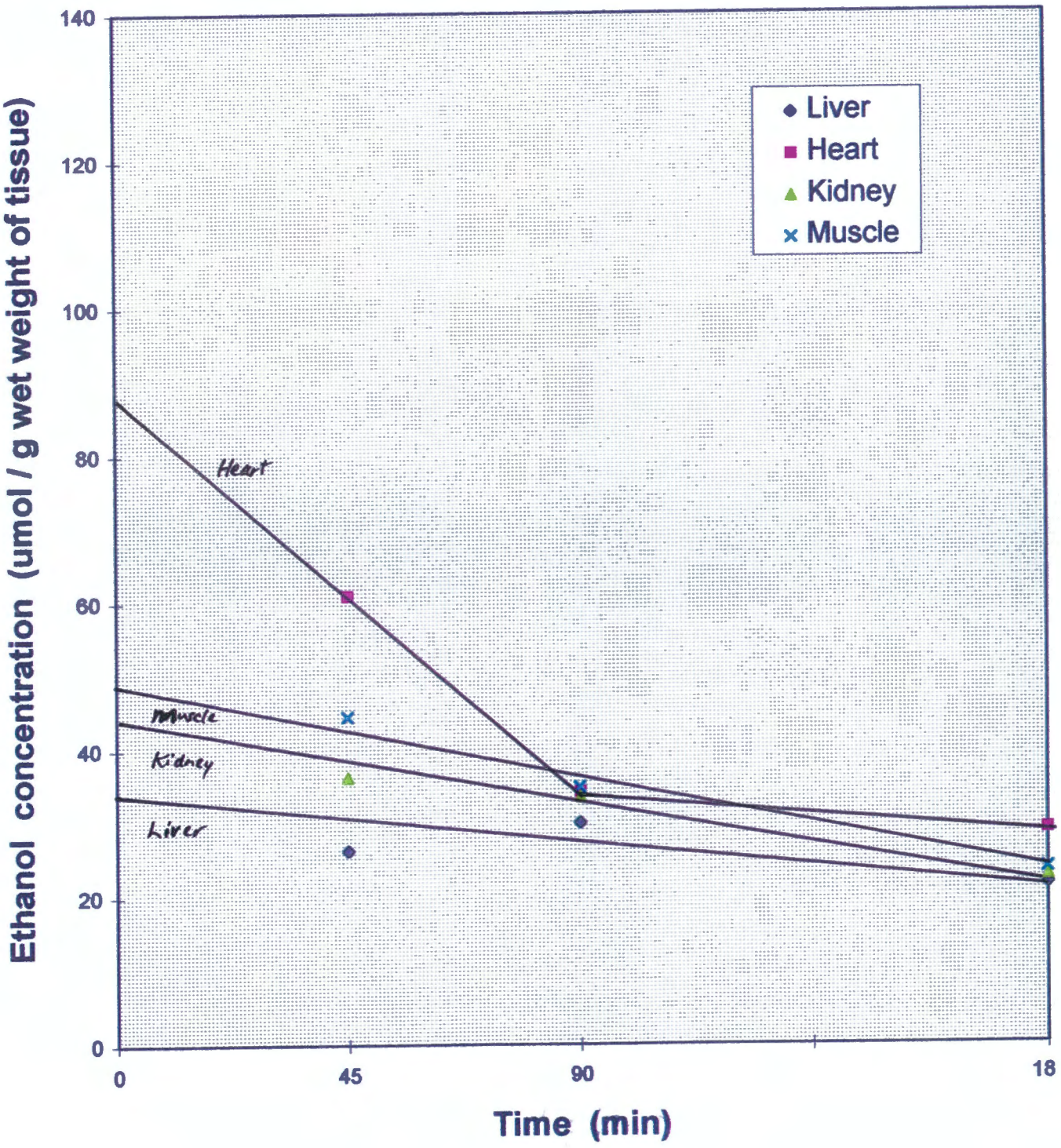


Figure 16b Ethanol concentrations of different tissues in female rats (Second experiment)



However, based on the rate of ethanol clearance data from Braggins et al. (1981), the small rats would still have been expected to take a longer time to oxidise all the ethanol they were given. For 200g rats, the rate of ethanol oxidation given by Braggins et al. was about 12 mmol/kg/h. Assuming all the ethanol (6g/kg = 130 mmol/kg) was taken into the body, the rats would require about 10 hours to oxidise all the ethanol. This did not happen in the first experiment. This could be due to a lack of absorption of ethanol into the body. For example, retention in the stomach might occur and the consequent delay in absorption could produce anomalous results in studies of ethanol clearance from the body. Some ethanol although in small quantities (< 10%) might be excreted in urine, expired air and sweat (Pawan, 1972). Another possible explanation is that the animals used in the first experiment had unusually high levels of liver alcohol dehydrogenase. In the second experiment, the rates of ethanol oxidation in the rats were obviously much lower, since a significant amount of ethanol was left in all tissues at 180 minutes after dosing.

In experimental studies, calculation of the theoretical initial blood alcohol concentration is useful because it provides an indication about the validity of the ethanol clearance curve for use in calculation of ethanol clearance rates. Extrapolation of the decreasing phase of the ethanol clearance curve to zero time should yield a blood alcohol concentration similar to that predicted on the basis of distribution of all the ethanol consumed in the body water of the animal. If extrapolation to zero time does not yield the expected blood ethanol concentration, it is an indication that the ethanol clearance curve is not linear and hence other factors such as delayed absorption or a first-pass effect (Lieber et al., 1994) might need to be considered.

The calculated theoretical value for initial ethanol concentration is 130 μmoles/g. In male rats in the first experiment and in both cases in the second experiment, back extrapolation of the ethanol clearance curves for any tissue gives an ethanol concentration at zero time of much less than 130 μmoles/g. The highest values are for heart (80 μmoles/g) and kidney (70 μmoles/g). Even these values are far away from the theoretical value of 130 μmoles/g which was calculated from the ethanol dose of 6 g/kg. This indicates that either all the ethanol given has not been absorbed, or that there has been significant first pass metabolism (Seitz et al., 1994). First pass metabolism occurs when some ethanol is oxidised before reaching general circulation. This may occur as ethanol is absorbed in the stomach, or on the first-pass of ethanol through the liver.

There is considerable evidence that ethanol is oxidised not only in liver but also in the gastrointestinal tract. Although this ethanol metabolism is less than that of liver, it has some important relevance with respect to first pass metabolism of alcohol and to ethanol induced tissue toxicity. Immunological studies have shown that ADH is present in the epithelial cells lining the gastrointestinal tract of men (Pestalozzi et al., 1983) and most activity was found in stomach and in colorectum (Boleda et al., 1989). It was proposed that stomach and possibly small intestine play the most significant role in gastrointestinal ethanol oxidation for several reasons. They have the largest ADH activity (Boleda et al., 1989), they represent the primary sites of ethanol absorption (Kirka and Clark, 1979), and they are exposed to high concentrations of ethanol for a long period of time. The contribution of other parts of the digestive tract is probably lower because ethanol absorption is minimal in the mouth and oesphagus (Kirka and Clark, 1979) while the concentration that reaches the large intestine is rather small (Krebs and Perkins, 1970).

At least three different forms of ADH are found in the stomach with either high, intermediate or low K_m values for ethanol (Moreno and Pares, 1991; Lieber et al., 1994). Besides class I ADH (the predominant ADH in liver), a newly described class IV ADH with a K_m of 41 mM at pH 7.4 (Moreno and Pares, 1991) has also been found in the stomach. This enzyme is responsible for 30-50% of the overall ADH activities in the stomach and is mainly responsible for the gastric first pass metabolism of alcohol (Seitz et al., 1994). It was suggested that because of the high gastric ethanol concentration after ethanol consumption, even the gastric ADH forms with a high K_m for ethanol become active, and significant gastric ethanol oxidation ensues (Seitz et al., 1994). There is evidence that greater first pass metabolism occurs at high ethanol concentrations (Lim et al., 1993). In addition to ADH activity, gastric emptying time was also reported to modulate first pass metabolism (Holt, 1981). It has been observed that slower stomach emptying increased first pass metabolism, probably due to a prolonged exposure of ethanol to gastric mucosal ADH (Pedrosa et al., 1994).

In the present study, back extrapolation of ethanol concentrations for heart and kidney tissue in female rats gaves a zero-time ethanol concentration close to the theoretical value. This indicates that the first pass effect may be less in female animals, which is consistent with evidence in the literature that female rats have a significantly lower gastrointestinal ADH activities than males (Frezza et al., 1990).

In all animals, the ethanol concentrations in liver tissue were lower than those in heart and kidney, especially at 45 and 90 minutes. The reason for this is not clear, but it may reflect the high level of ADH in liver

tissue. In addition, the muscle ethanol concentrations of both male and female rats from the two experiments were low. This is unlikely to be due to a high rate of ethanol metabolism, as muscle does not have high ADH activity.

Chapter 4

CONCLUSIONS AND FUTURE WORK

The present study has expanded the current knowledge of the effects of ethanol on carbohydrate metabolism, but further work is required to elucidate the mechanism by which ethanol acts on liver glycogen.

The use of *Ochromonas* as a model system has shown that ethanol might have a direct effect on stored carbohydrate, in which hormones are not involved. It appears that ethanol could cause a decrease in chrysolaminarin content of the organism and the effect is somehow concentration-dependent.

In experiments using rats, an acute dose of ethanol (6g/kg) was shown to cause a 15-30% decrease in liver glycogen content at 45, 90 and 180 minutes. There is general agreement between workers that ethanol causes a decrease in liver glycogen in fed animals, but the extent of the decrease in the present experiment was small compared with the results of other researchers. It is possible that the variation might be due to differences in methods of administering ethanol, as when ethanol is given orally, the amount of ethanol absorbed may vary, and the amount in circulation may be less than for the same dose given intravenously. Differences in methods of measuring glycogen content might also contribute to the variation since partial loss of glycogen might occur in some methods. Differences in the strains of animals used in experiments might also lead to variation in results.

At some time intervals, small increases in glycogen content were observed in muscle, but not heart and kidney, after an acute dose. The free glucose concentrations in liver, heart, kidney and muscle were unchanged though there was a tendency for glucose to increase in the extrahepatic tissues. This suggests that some glucose released from liver glycogen breakdown may have been taken up by other tissues. It is interesting that a decrease in glycogen content was observed only in liver and not in muscle. This implies that if release of hormones is involved in the effect of ethanol on tissue glycogen content, glucagon would be more likely to be involved than adrenaline as the latter causes glycogenolysis in both tissues.

In male rats, the zero time ethanol concentrations calculated by extrapolation were found to be lower than the theoretical value (130 μmoles/g), expected for the dose given. This implies that delayed

absorption or first-pass metabolism may have occurred. The latter suggests that alcohol dehydrogenase present in the epithelial cells lining the gastrointestinal tract, in particular the ones in the stomach, might participate in the metabolism of ethanol. Because of the high gastric ethanol concentration after ethanol consumption, even the gastric forms with a high K_m for ethanol become active, and significant gastric ethanol oxidation ensues. Female rats in the first experiment however, showed a zero time ethanol concentration calculated by extrapolation of results for kidney and heart tissue, similar to the predicted body water ethanol concentration. Female animals may have less first-pass effect, as it has been reported that ADH activity is lower in gastrointestinal tissues from female animals. The present experiments have shown that the ethanol concentration in liver, heart and kidney tissues was lower in treated male rats than in treated females at initial 45 minutes. It was suggested that this is due to higher ADH activity present in the epithelial cells lining the gastrointestinal tract of male animals and this contibutes to higher first pass metabolism. However the role of ADH in the gastrointestinal tract with respect to the overall metabolism of ethanol is still a matter of debate and whether first-pass metabolism may play a role in humans needs further investigation.

In future, experiments on the effects of ethanol on carbohydrate metabolism could be carried out in hepatocytes, where no hormonal effects are involved. The activity of enzymes participating in liver glycogen metabolism could be measured to see if they are affected by ethanol. If resources were available, more rats could be used to reduce the effects of individual variation and to obtain more reproducible results. The size of animals should be carefully controlled, as the present results suggest that tissue glycogen content may vary considerably with the size of the animal.

Appendix 1

COLORIMETRIC ASSAYS

Determination of free reducing sugar by a colorimetric procedure (Bernfeld, 1955)

Duplicate 1 ml diluted aliquots were pipetted into labelled test tubes. Next 2 ml of dinitrosalicylate reagent was added and mixed. The tubes were then heated in a boiling water bath for 15 minutes, followed by cooling with addition of 3 ml water. They were mixed thoroughly and the absorbance was read at 580 nm.

Determination of total reducing sugar by the dinitrosalicylate procedure (Massey University Biochemistry Practical Course Manual, Vol.I, 1993)

Duplicate 0.5 ml aliquots were pipetted and 0.5 ml of laminarinase was added to a give final volume of 1 ml. The solution was mixed and allowed to stand for 15 minutes. Then dinitrosalicylate reagent was added and the free glucose content was determined as above.

Determination of laminarin (Monsigny, 1988)

Reaction mixture: 200 µl sample
200 µl resorcinol (6 mg/ml)
1.0 ml sulphuric acid (75%)

The reaction mixture was mixed thoroughly by vortex. It was then heated in a 90°C water bath for 30 minutes, and cooled by placing in a cold bath for 30 minutes. The absorbance was read at 480 nm.

Preparation of laminarinase buffer

The sodium acetate buffer was prepared as in Appendix 2, reagent 5 under enzymatic analysis of tissue glycogen content.

Biuret Reaction for Determination of Amyloglucosidase Protein Concentration

Aliquots of protein-containing solution (0.7 ml) were mixed with 3.0 ml of Biuret reagent. The mixtures were left to stand for 20 minutes and the absorbance was read at 540 nm, using a reagent blank to zero the spectrophotometer. A standard curve was constructed using bovine serum albumin (10 mg/ml) as a standard protein.

Appendix 2

ASSAYS BY ENZYMATIC PROCEDURES

Enzymatic Analysis of Tissue Glycogen Content

Reagents:

1. Perchloric acid (PCA) (0.6 M)
   5.2 ml of 70 % $HCLO_4$ was made up to 100 ml with distilled water.

2. Potassium hydrogen carbonate (1 M)
   2 g potassium hydrogen carbonate was dissolved in distilled water and made up to 20 ml.

3. Potassium hydroxide (3 M)

4. Amyloglucosidase (AGS) in acetate buffer (1 mg/ml)
   See section "Preparation of amyloglucosidase", in section 3.2.3.1

5. Acetate buffer (0.2 M; pH 4.8)
   4.8 ml 96 % acetic acid and 9.75 g sodium acetate were made up to 1 litre with distilled water. pH was checked with a pH meter.

Glucose assay

Reaction cocktail (for 20 glucose assays) :

30 ml Tris buffer (adjusted with HCl to pH 7.6)
3.0 ml NADP (10 mg / ml)
0.6 ml ATP (0.1 M)
3.0 ml $MgCl_2$ (0.1 M)
6.0 ml H_2O

2 ml of cocktail was added to each cuvette, then the sample or glucose standard was added and the final volume was made up to 3 ml with water. 100 ul of 1 mM glucose was pipetted as a standard. The reaction mixture was mixed and absorbance was read at 340 nm. Next, G6-PDH was added and the absorbance was read when stable. Finally, HK was added. It took 5-10 min for the reaction to complete and the absorbance was read. The spectrophotometer was zeroed with a water blank

containing only water. The amount of glucose present in the original sample was proportional to the increase in NADPH measured by the absorbance change at 340 nm.

ATP assay

Reaction cocktail (for 30 ATP assays) :

22.5 ml Tris buffer (adjusted with HCl to pH 7.6)
4.5 ml NADP (10 mg / ml)
9.0 ml $MgCl_2$ (0.1 M)
9.0 ml Glucose (0.1 M)
22.5 ml H_2O

2.25 ml cocktail was pipetted into each cuvette. 200 ul of sample or 100 ul of 0.5 mM standard ATP solution was then added. The final volume was made up to 3 ml with water. The reaction mixture was mixed thoroughly and absorbance was read at 340 nm. Then G6-PD and HK were added and the absorbances read in the same way as described for the glucose assay.

Ethanol assay

Reaction cocktail (for 30 ethanol assays) :

45 mg NAD+
52 ml Tris/lysine buffer (0.6 M Tris - 12.11 g
0.4 M Lysine - 7.306 g
Each component was dissolved seperately and then combined; pH was adjusted to 9.7 with KOH pellets, then the solution was made up to 100 ml.)
52 ml H_2O

3 ml of cocktail was pipetted into clean cuvettes. 30 ul of sample or 20 ul of 10 mM standard ethanol was added. The reaction mixture was mixed and initial absorbance was taken. 20 ul of ADH (15 mg/ml) was then added and absorbance was read when stable.

Appendix 3

CALCULATION OF TISSUE GLYCOGEN CONTENT

(1) Glucose concentration (mM) =

$$\frac{\text{Change in absorbance}}{6.22} \times \frac{\text{Final volume in cuvette (= 3.01 ml)}}{\text{Volume of sample added}}$$

(2) Hydrolysis step

$$\text{Dilution factor (DF)} = \frac{\text{Total volume after hydrolysis}}{0.2 \text{ ml}} = \frac{3.3 \text{ ml}}{0.2 \text{ ml}}$$

$$= 16.5$$

(3)

$$\text{Neutralisation dilution factor (NDF)} = \frac{\text{Volume after hydrolysis}}{\text{Volume neutralised}}$$

(4) Therefore, total amount of glucose after hydrolysis =

glucose concentration in cuvette (mM) x DF x NDF x volume of sample (ml) x 1/1000 L

(5) The amount of free glucose from unhydrolysed homogenate (μmoles) =

glucose concentration x NDF x volume of sample (ml) x 1/1000 L

(6) Now, net amount of glucose (μmoles) released from glycogen = **(4) - (5)**

(7) To convert into glycogen/g wet weight, μmoles glycogen is divided by the corresponding weight (g) of sample.

Appendix 4

Experiment 1

Rat weights (g)			Liver weights (g)	
Samples	Males	Females	Males	Females
45 C1	225	186	9.51	7.73
45 C2	220	205	9.47	7.60
45 T1	205	178	8.94	7.30
45 T2	210	192	9.38	6.56
90 C1	172	191	6.02	6.91
90 C2	167	203	5.54	8.79
90 T1	175	192	5.67	6.71
90 T2	169	197	5.57	6.20
3h C1	224	180	10.60	7.65
3h C2	204	200	9.87	7.90
3h T1	200	192	7.59	5.70
3h T2	200	195	7.50	6.12

Experiment 2

Samples	Rat weights (g) Males	Females	Liver weights (g) Males	Females
45 C1	216	201	7.70	9.80
45 C2	220	190	9.20	5.80
45 C3	213	190	7.80	7.60
45 T1	225	212	8.20	5.50
45 T2	224	200	9.00	7.07
45 T3	250	192	10.60	6.07
90 C1	220	197	9.65	5.73
90 C2	245	210	10.22	7.10
90 C3	220	202	6.60	6.10
90 T1	210	210	7.50	6.40
90 T2	225	206	8.86	5.45
90 T3	226	210	8.84	7.30
3h C1	247	190	10.47	4.80
3h C2	216	207	9.90	5.89
3h C3	226	200	9.49	4.50
3h T1	240	212	10.47	5.70
3h T2	233	212	8.40	5.26
3h T3	226	195	8.56	7.50
3h T4	240		9.30	

Appendix 5

Statistical calculations

Mean: $x = \Sigma x / n$

Standard deviation: $s = \sqrt{\frac{1}{n-1} \Sigma (x_i - \bar{x})2}$

Standard error of the mean (SEM) $= s / \sqrt{n}$

To calculate significance,

assuming both controls and experimentals come from Normal distribution

$H_o : \mu_{control} = \mu_{treated}$ vs $H_1 : \mu_{control} < \mu_{treated}$

$$s_p = \sqrt{\frac{(n_1 - 1)\, s_1^2 + (n_1 - 1)\, s_1^2}{n_1 + n_2 - 2}}$$

$$\therefore \text{Under } H_o : t_{n-2} = \frac{x_1 - x_2}{sp \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

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