REGULATION OF ETHANOL METABOLISM IN ISOLATED RAT HEPATOCYTES

A thesis presented in partial fulfilment of the
requirements for the degree of
Doctor of Philosophy (BIOCHEMISTRY)
at Massey University

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1990

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ABSTRACT

The importance of an individual step in the regulation of a metabolic pathway can be quantitatively defined by determining a global coefficient called the flux control coefficient ($C^J_{E_i}$). The coefficient can be evaluated by altering the pathway flux in small increments using an enzyme-specific inhibitor. This modulation approach was applied to the study of the regulation of ethanol metabolism in isolated rat hepatocytes.

A procedure was developed for using isolated hepatocytes to measure the small decreases in rates of ethanol oxidation produced by increasing concentrations of inhibitor. The method, termed the two time point method, involved the incubation of replicate 1 ml or 2 ml samples in 10 ml erlenmeyer flasks containing cells, medium and substrates with varying inhibitor concentrations (including zero to provide a control rate).

Using this method, the apparent flux control coefficient of ADH in hepatocytes isolated from starved rats was determined using two inhibitors, tetramethylene sulphoxide (TMSO) and isobutyramide (IsB). Using the initial slope of the flux inhibition curve and the appropriate inhibition constant determined for isolated ADH, the apparent C^J_{ADH} was calculated to be 0.37 ± 0.09 (from inhibition by TMSO) or 0.43 ± 0.10 (from inhibition by IsB). The good agreement in the value obtained for the flux control coefficient of ADH, using two different inhibitors, showed that the method developed for measuring small changes in ethanol clearance rates was providing accurate, reproducible results.These flux control coefficients were calculated on the assumption that the ADH reaction is a one substrate, one product reaction. The apparent flux control coefficient of ADH was then corrected for the concentration effect of NAD^+, NADH and acetaldehyde. When substrate and product concentrations were considered, the value of the apparent C^J_{ADH} increased to a maximum of 0.53 (for TMSO inhibition) or 0.63 (for IsB inhibition). These results indicate that in starved rats the activity of the enzyme alcohol dehydrogenase is one of the major factors involved in regulation of ethanol metabolism.

The importance of ADH in fed rats was determined using TMSO. A range of values for the flux control coefficient of ADH was obtained with varying concentrations of acetaldehyde. The value of C^J_{ADH} was dependent on the concentration of acetaldehyde present in the isolated rat hepatocytes. For example the apparent flux control coefficient of ADH was about 0.02 when the acetaldehyde concentration was 138 μM and was approximately 0.5 when the concentration of acetaldehyde was 6 μM, which is analogous to conditions in starved rats. When the apparent C^J_{ADH} was corrected for the effect of substrate and product concentration the trend of decreasing

C^{J}_{ADH} with increasing acetaldehyde concentration was still observed. This indicates that the importance of alcohol dehydrogenase in regulation of ethanol metabolism, in hepatocytes isolated from fed rats, decreases with increasing acetaldehyde concentration. Obviously, as the significance of ADH in regulation of ethanol oxidation decreases, other enzymes in the ethanol pathway become more important. The most likely enzyme to have an increasing importance in the regulation of ethanol elimination, with increasing concentrations of acetaldehyde, is aldehyde dehydrogenase (AlDH).

Preliminary inhibitor studies were carried out on AlDH in hepatocytes isolated from starved rats, using the irreversible inhibitor disulfiram. The flux control coefficient of aldehyde dehydrogenase was calculated to be about 0.1. However, because of the problems encountered with the preparation of disulfiram stock solutions, an alternative inactivator of aldehyde dehydrogenase was required for the inhibitor titration experiments. Diethyldithiocarbamate (DDC), a metabolite of disulfiram, was used. The results showed that inhibition of AlDH occurred in the presence of DDC and that the minimum amount of DDC required for total inhibiton of aldehyde dehydrogenase was in the concentration range of 300 μM to 500 μM. Further studies should be carried out for determination of the initial slope of the flux inhibition curve, using DDC, so that the flux control coefficient of AlDH may be calculated.

In chapter 6, a theoretical approach for determining the importance of the shuttle systems and electron transport chain in regulation of ethanol oxidation in rat hepatocytes is discussed. A literature search on possible inhibitors for the malate-aspartate shuttle, α-glycerophosphate shuttle and electron transport chain was carried out.

The results from this study show that it is possible to evaluate the importance of some of the individual steps in the ethanol pathway by using the modulation approach devised by Kacser and Burns. However the two time point method developed for measuring the small decreases in flux through the ethanol pathway imposed by increasing concentrations of inhibitor is only sensitive enough to detect changes in flux as low as 8.0%. Enzymes that have low flux control coefficients will produce changes in flux of about 8.0%. Thus, the development of a more sensitive method for measuring the small changes in flux through the ethanol pathway is needed, or a method other than the modulation approach for calculating the flux control coefficient is required.

ACKNOWLEDGEMENTS

I would like to thank my supervisors, Dr K. E. Crow and Dr M. J. Hardman for their invaluable advice and encouragement when times were rough. Iwould like to say that I was lucky in having two very good supervisors. They were not only interested in the results produced during the course of my work but cared about my well being. Thanks Kathy and Mike.

I also wish to thank all my friends, especially Kathryn and John, for making my PhD years enjoyable.

Finally, Iwould like to thank my family, for their loving support, encouragement and help over the years. I could not have finished this thesis without it.

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CHAPTER 1

INTRODUCTION

1.1 METABOLISM OF ETHANOL

The main site of ethanol metabolism is the liver, although some other tissues such as kidney, muscle, lung, intestine and possibly even the brain may metabolise ethanol to a small extent (Pawan, 1972). Ethanol elimination involves the oxidation of ethanol to acetaldehyde, which in turn is oxidised to acetate. This last product enters the general metabolic pool through conversion to acetyl CoA.

Three different reaction mechanisms have been described for the first step in oxidation of ethanol in the liver. These are, the pathway catalysed by the NAD^+-dependent alcohol dehydrogenase (Theorell & Bonnichsen, 1951), the pathway involving hydrogen peroxide and catalase (Keilin & Hartree, 1945; Rouch *et al.* , 1969; Isselbacher & Carter, 1970) and the microsomal ethanol oxidising system (MEOS) involving NADPH and cytochrome P-450 (Orme-Johnson & Ziegler, 1965; Lieber & DeCarli, 1968, 1970).

Alcohol dehydrogenase, localised in the cytosolic compartment of the liver cell, is predominantly responsible for the transformation of ethanol to acetaldehyde (Hawkins & Kalant, 1972; Rognstad & Grunnet, 1979; Cederbaum, 1980). The combined contribution of the non-ADH pathways to the metabolism of ethanol appears to be less than 10% under normal conditions (Higgins, 1979; Rognstad & Grunnet, 1979; Williamson & Tischler, 1979). However, the role of the catalase and MEOS systems in the oxidation of ethanol is still the subject of debate.

Because MEOS has a relatively high K_m for ethanol of 8 to 10 mM (Lieber & DeCarli, 1972), compared to 0.5 to 2.0 mM for ADH (Cornell *et al.,* 1979; Crabb *et al.,* 1983), it may have a significant role in ethanol oxidation at high levels of ethanol or after chronic ethanol consumption (Lieber & DeCarli, 1968, 1970). Inhibitor studies have supported this idea (Lieber & DeCarli, 1970, 1972; Grunnet *et al.,* 1973; Matsuzaki *et al.,* 1981) but the evidence is not conclusive because the inhibitors used were not sufficiently specific. For example, the ADH inhibitor pyrazole will also inhibit MEOS (Teschke *et al.,* 1976; Takagi *et al.,* 1986).

The availability of a mutant deermouse strain that lacks ADH (ADH^-)but nevertheless actively oxidises ethanol (Burnett & Felder, 1978, 1980; Shigeta *et al.,* 1984) has stimulated research into the respective roles of catalase and MEOS in non-ADH mediated ethanol metabolism (Shigeta *et al.,* 1984 ; Takagi *et al.,* 1986; Handler *et al.,* 1986).

Intrinsic isotope effects (Cronholm, 1985) were used to calculate flux through the ADH, MEOS, and catalase pathways (Takagi *et al.,* 1986; Alderman *et al.,* 1987). When ADH^- deermice were treated with the catalase inhibitor 3-amino-1,2,4-triazole, there was no significant change in the rate of ethanol oxidation. This indicated that MEOS was the principal pathway for ethanol oxidation *in vivo* in ADH^- deermice. Even when ADH was present (ADH^+ strain) , the results showed that non-ADH pathways (mostly MEOS) participated significantly in ethanol metabolism at all concentrations tested and played a major role at high levels.

In rats, Vind and Grunnet (1985) determined the fate of ^3H from [1-^3H] ethanol and found that non-ADH pathways (primarily cyt P-450 dependent) made a significant contribution to hepatic ethanol oxidation, even in the presence of ADH.

From the above studies the involvement of catalase in hepatic ethanol metabolism appears to be minor. This can be attributed to the limited intracellular production of H_2O_2 (Boveris *et al.,* 1972). However it has been demonstrated recently that ADH^- deermice metabolise ethanol via catalase-H_2O_2 (Handler *et al.,* 1986; Handler & Thurman, 1988) in addition to the dehydrogenase systems present in both ADH^+ and ADH^- deermice (Norsten *et al.,* 1989).

The contradictions represented in the above studies concerning the importance of non-ADH pathways in ethanol oxidation, heighten the uncertainty of the physiological significance of MEOS and catalase pathways in the metabolism of ethanol. In view of this the quantitative importance of non-ADH pathways in the regulation of ethanol metabolism was not considered in this study.

The second step of ethanol elimination, which involves the conversion of acetaldehyde to acetate, is primarily catalysed by the NAD^+-dependent aldehyde dehydrogenases (Tottmar & Marchner, 1976; Svanas & Weiner, 1985; Harrington *et al.,* 1988). Depending on the species of animal, aldehyde dehydrogenases differ in their subcellular location, relative activity and kinetic constants (Sheppard *et al.,* 1970; Feldman & Weiner, 1972; Tottmar *et al.,* 1973; Crow *et al.,* 1974; Koivula & Koivusula, 1975; Greenfield & Pietruszko, 1977; Leicht *et al.,* 1978). In rat liver, acetaldehyde is oxidised predominantly in the matrix space of the mitochondria. This is because of the relatively high activity and very low K_m for acetaldehyde of the isozyme localised in this compartment (Eriksson *et al.,* 1975; Higgins, 1979; Weiner, 1979; Williamson & Tischler, 1979; Svanas & Weiner, 1985; Harrington *et al.,* 1988).

Acetaldehyde can be oxidised to acetate by aldehyde oxidase and xanthine oxidase (Rajagopalan, 1980). However these enzymes have K_m's for acetaldehyde in the order of 1-10 mM (Weiner, 1980) and are therefore unlikely to be of physiological significance in acetaldehyde metabolism

where concentrations of acetaldehyde reach maximum levels of 200 μM during ethanol oxidation (Braggins & Crow, 1981; Braggins *et al.,* 1980).

Ethanol metabolism to acetate is associated with the production of NADH in the cytosol (ADH reaction) and mitochondria (AlDH reaction), with a consequent decrease in the free cytosolic $\frac{[NAD^+]}{[NADH]}$ ratio (Veech *et al.,* 1972). To sustain ethanol oxidation, NADH must be reoxidised. Although some NADH may be oxidised directly in the cytosolic compartment, the majority of oxidation must occur in the mitochondria. Because the mitochondrial membrane is impermeable to NADH (Lehninger, 1951), reducing equivalents of NADH must be transferred into the mitochondria for eventual oxidation by the respiratory chain. This transfer is mediated by substrate shuttles, the malate-aspartate (Bucher & Klingenberg, 1958; Borst, 1963) and the α -glycerophosphate (Sactor & Dick, 1960; Klingenberg & Bucher, 1961) shuttles being proposed as the major pathways (Cederbaum *et al.,* 1977; Dawson, 1979; Williamson & Tischler, 1979).

Figure 1.1 shows the interactions between ethanol metabolism, the shuttle systems and the electron transport chain.

1.2 REGULATION OF ETHANOL METABOLISM

Over the past 20 years, two opposing views have arisen as to how the rate of ethanol metabolism is regulated. One hypothesis states that the rate of ethanol oxidation is determined by the rate at which NADH is reoxidised to NAD^+ (Hawkins & Kalant, 1972; Khanna & Israel, 1980). This was supported by experiments showing increased rates of ethanol clearance in the presence of substrates that increased the rate of NADH reoxidation (Israel & Khanna, 1970; Meijer *et al.,* 1975; Krebs & Stubbs, 1975). The observation that the free cytosolic $\frac{[NAD^+]}{[NADH]}$ ratio in liver cells decreases during ethanol metabolism (Christensen & Higgins, 1979) implied that either ADH activity was becoming limited by the supply of NAD^+ or ADH was being inhibited by NADH.

The second theory, stating that the amount of alcohol dehydrogenase is the crucial factor in regulation of ethanol metabolism (Crow *et al.,* 1977a,b; Cornell *et al.,* 1979) arose from the following findings:

1) Rat liver ADH operates at 50 to 80% of its maximum velocity during ethanol oxidation *in vivo* (Crow *et al.,* 1977a,b); therefore alcohol dehydrogenase is not in excess as assumed in the first theory (Hawkins & Kalant, 1972).

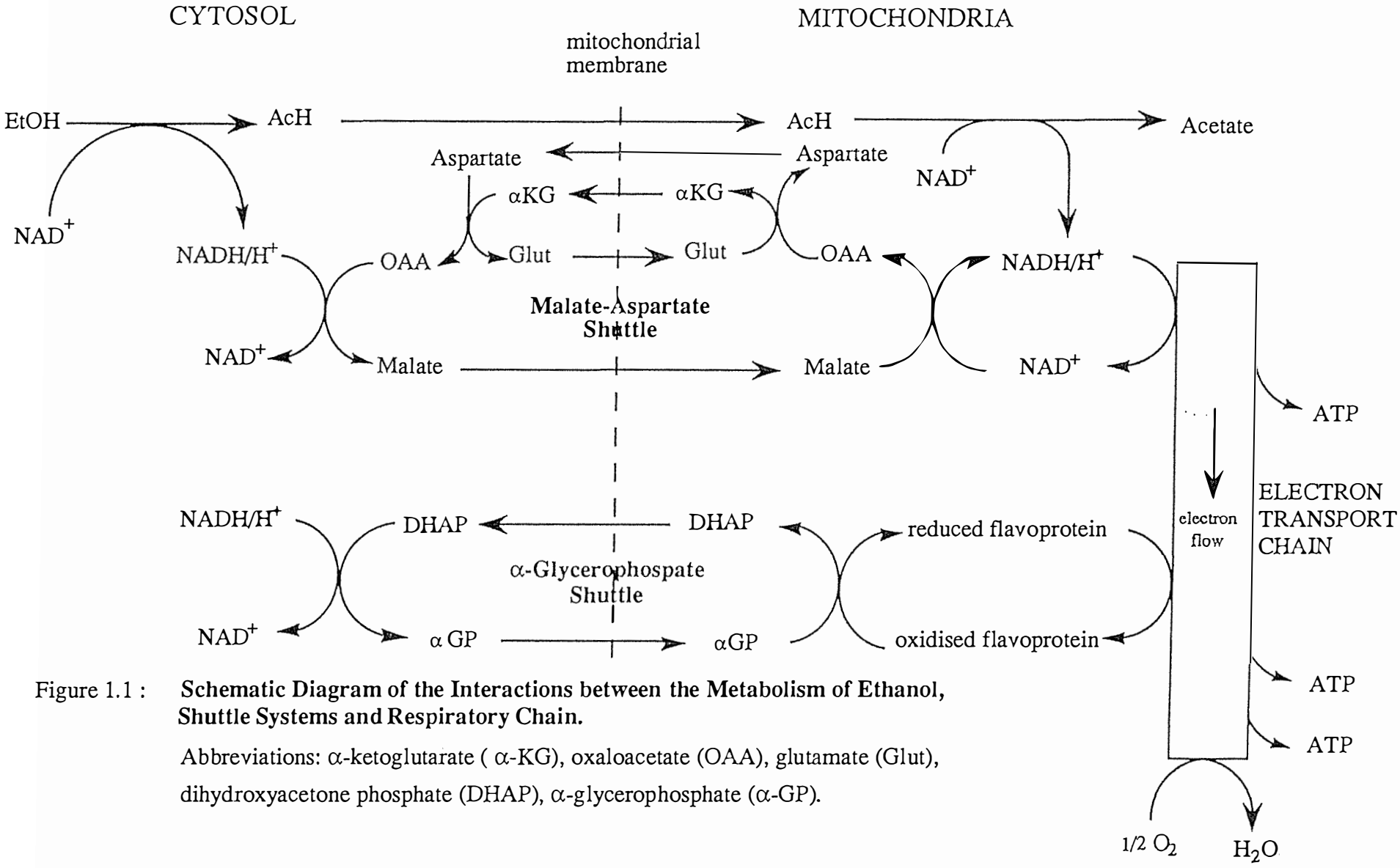


Figure 1.1 : **Schematic Diagram of the Interactions between the Metabolism of Ethanol, Shuttle Systems and Respiratory Chain.**

Abbreviations: α-ketoglutarate (α-KG), oxaloacetate (OAA), glutamate (Glut), dihydroxyacetone phosphate (DHAP), α-glycerophosphate (α-GP).

2) The liver cell does not become depleted of NAD^+ during ethanol metabolism (Crow *et al,* 1983a); hence the activity of ADH is not restricted by the availability of NAD^+. This is explained by referring to the actual concentrations of the metabolites (NAD^+ and NADH) in the liver cell and the change in the free cytosolic $\frac{[NAD^+]}{[NADH]}$ ratio (Crow, 1985). The free cytosolic $[NAD^+]$ is about 0.5 mM (Bucher *et al.,* 1972). During ethanol oxidation the ratio of free cytosolic $[NAD^+]$ to [NADH] drops from 1000 to 200-500 (Veech *et al.,* 1972). This drop can best be explained by an increase in [NADH] from 0.5 to 1-5 μM. The cytosolic NAD^+ concentration essentially remains unchanged during ethanol oxidation (Crow *et al.,* 1983a).

3) The concentrations of NADH occurring during ethanol oxidation *in vivo* will inhibit rat liver ADH by approximately 10-20% (Cornell *et al.,* 1979); therefore the rate of ethanol metabolism could only be increased by a maximum of 20% if the rate of NADH reoxidation was increased. Such an increase has not been demonstrated *in vivo*.

4) The change in cytosolic redox state may be explained by the kinetic properties of cytosolic malate dehydrogenase (Crow *et al.,* 1982, 1983b). Malate dehydrogenase (MDH) is not saturated with NADH at physiological concentrations and therefore the rate of oxidation of NADH by MDH rises with increasing [NADH] until it equals the rate of production of NADH and a new steady state is achieved.

The above experimental evidence has made the second theory more attractive. However, from the existing evidence, we can not state definitely that the level of ADH is the principal factor in regulation of the rate of ethanol oxidation.

A theoretical framework developed by Kacser and Burns (1973) and Heinrich and Rapoport (1974), called the metabolic control theory (see sections 1.3.2 and 1.4), discounts the idea that one particular element (enzyme or transport protein) in a metabolic pathway must be the only rate-controlling step. Thus, metabolic control in a pathway is shared by the enzymes, rather than confined to one rate-limiting enzyme per pathway. Therefore the assumption that the rate-limiting step for ethanol metabolism must be either the ADH level or rate of NADH reoxidation, is incorrect. Figures 1.1 and 1.2 illustrate the possible sites of control in the metabolism of ethanol. These are the enzymes alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (AlDH) and the reoxidation of NADH which involves the transfer of cytosolic NADH across the mitochondrial membrane (shuttle systems) and the oxidation of NADH by the electron transport chain.

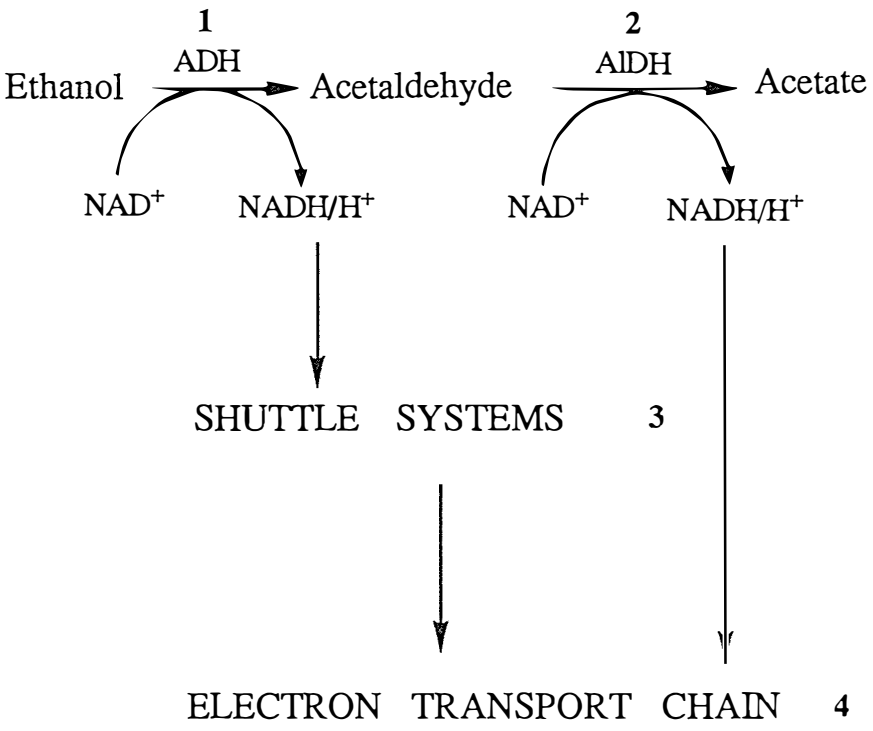


Figure 1.2: **Steps in the Metabolism of Ethanol.**

1 Ethanol oxidised to acetaldehyde by the enzyme alcohol dehydrogenase (ADH)

2 Acetaldehyde oxidised to acetate by the enzyme aldehyde dehydrogenase (AlDH)

3 Transfer of cytosolic NADH into the mitochondria by shuttle pathways

4 Oxidation of NADH via the electron transport chain

By applying the metabolic control theory, quantitative assessment of the relative importance of each potential control step in the regulation of ethanol metabolism may be achieved. This involves determining the flux control coefficients of all factors (enzymes and transport proteins) involved in the metabolism of ethanol.

1.3 THEORETICAL FRAMEWORKS FOR THE STUDY OF METABOLIC CONTROL

In recent years there has been a shift of interest from the qualitative concept of a "rate-limiting step " and one "controlling enzyme", to obtaining quantitative information concerning the control structure of a metabolic pathway.

Higgins (1965) was one of the first investigators to propose a quantitative expression for the influence of an enzyme on the flux through a pathway. This expression was termed the control strength. There have been several theoretical frameworks developed during the past two decades for the study of metabolic control. Following is a brief synopsis of the three main theories, biochemical systems theory (BST), metabolic control theory (MCT) and flux oriented theory (FOT), in chronological order of development.

1.3.1 Biochemical Systems Theory (BST)

This theory was formulated in the late 1960's by Michael Savageau (Savageau, 1969a,b, 1970, 1971a,b, 1972) and has been extended by the same author (Savageau, 1974, 1976, 1979; Voit & Savageau, 1986; Sorribas & Savageau, 1989c). The theory provides a general approach that takes into account the nonlinear dynamics of the metabolic system being analysed. In BST the fundamental equations that describe biochemical systems are written explicitly in terms of the Power-Law Formalism (Savageau, 1969b; Voit & Savageau, 1987).

There are several levels at which biochemical systems can be described by this formalism (Savageau, 1969a,b, 1979; Voit & Savageau, 1987). The representation that was explicitly selected for the development of biochemical systems theory (BST) was designated the S-systems variant (Savageau, 1969; Savageau *et al.,* 1987a,b). This variant results when one aggregates rate laws to obtain two functions, one representing the sum of the rate processes that lead to an increase (V_i) in a given system constituent (X_i) and the other representing the sum of the rate processes that lead to a decrease (V_{-i}) in a given system constituent (X_i). Each of these aggregate

rate laws is then represented as a single product of power law functions (equation 1.1).

$$\frac{dX_i}{dt} = V_i - V_{-i} = \alpha_i \prod_{j=1}^{n+m} X_j^{g_{ij}} - \beta_i \prod_{j=1}^{n+m} X_j^{h_{ij}} \qquad i = 1, ...n \qquad (1.1)$$

The dependent variables (n) may be thought of as variables "internal" to the system and the independent variables (m) may be considered external variables that are determined by factors outside the system of interest (e.g. by the experimentalist, the environment, or other systems within the same organism). These variables typically refer to concentrations, but may also refer to any other physical or chemical quantities. The symbol α_i is the rate constant (enzyme level) for the net increase (synthesis, import, concentration, etc) of X_i and g_{ij} is the kinetic order for the net increase of X_i with respect to variation in X_j. The symbol β_i is the rate constant (enzyme level) for the net decrease (degradation, export, dilution, etc) of X_i and h_{ij} is the kinetic order for the net decrease of X_i with respect to variation in X_j.

The steady state solution derived from equation (1.1) relates each concentration variable and flux within an arbitrary system to external concentration variables and parameters associated with the individual enzymes and processes of the system (Savageau, 1969b). This allows the complete characterisation (determination of logarithmic gains and parameter sensitivities) of the relationships between systemic and molecular properties in the system's steady state behaviour.

Many different types of biochemical systems have been analysed using the S-system representation of BST. These include feedback and feedforward mechanisms of control in biosynthetic pathways (Savageau, 1972, 1974, 1975, 1976), network regulation of immune responses (Irvine & Savageau, 1985a,b) and enzyme-enzyme interactions (Sorribas & Savageau, 1989a,b).

1.3.2 **Metabolic Control Theory (MCT)**

The metabolic control theory was developed approximately 16 years ago by two independent research groups (Kacser & Burns, 1973; Heinrich & Rapoport, 1974). This theory provided the theoretical framework for understanding the steady state behaviour of biochemical processes in living organisms as well as the experimental strategy required for its analysis.

The main concept of MCT is that metabolic control is shared by all enzymes in the metabolic system and there is rarely (or probably never) a single enzyme that is truly "rate-limiting". The two important parameters of MCT are the control coefficients and elasticity coefficients. These

coefficients define how metabolic control is distributed in the system and may be calculated indirectly by using the summation (Kacser & Burns, 1973; Heinrich & Rapoport, 1974) and connectivity (Kacser & Burns, 1973; Westerhoff *et al.*, 1984) theorems.

The elasticity coefficient (ε) defines the response of the rate of the 'isolated' species to changes in the concentration of metabolite that directly affects the response (Westerhoff *et al.*, 1984; Burns *et al.*, 1985a,b). The mathematical formula for the elasticity coefficient is given in equation 1.2.

$$\varepsilon_M^v = \frac{\partial v/v}{\partial M/M} \qquad (1.2)$$

where: v represents the rate of any functional entity 'isolated' from the system (enzyme, translocator, etc).
M represents any molecular species which affects the function directly (substrate, product, inhibitor, cofactor, etc).

The control coefficient (C) defines the response of the 'whole system', when the enzyme is embedded in and interacts with the rest of the system, to changes in any parameter of the enzyme (equation 1.3) (Westerhoff *et al.*, 1984; Burns *et al.*, 1985a,b):

$$C_P^V = \frac{\partial V/V}{\partial P/P} \qquad (1.3)$$

where: V represents any variable in the system (flux, concentration pool, free energy, etc).
P, an independent variable, represents any parameter (enzyme concentration, turnover number, etc) whose change causes the changes in V.

The control theory (Kacser & Burns, 1973; Heinrich & Rapoport, 1974) has subsequently been extended to analyse branched pathways (Heinrich *et al.*, 1977; Kacser, 1983; Fell & Sauro, 1985), substrate cycles (Fell & Sauro, 1985) and moiety-conserved cycles (Hofmeyer *et al.*, 1986). New theorems have been developed (Westerhoff & Chen, 1984), as well as a matrix algebra procedure used for determining flux control coefficients (Fell & Sauro, 1985) and concentration control coefficients (Sauro *et al.*, 1987).

Several reviews of control analysis have been published (Heinrich *et al.*, 1977; Kacser & Burns, 1979; Groen *et al.*, 1982c; Porteous, 1983; Westerhoff *et al.*, 1984; Derr, 1985, 1986; Kacser & Porteous, 1987a), as well as an agreed terminology (Westerhoff *et al.*, 1984; Burns *et al.*, 1985b).

Most importantly, the control theory has been experimentally applied to several metabolic systems: glycolysis in erythrocytes (Rapoport *et al.,* 1976), arginine biosynthesis in *Neurospora* (Flint *et al.,* 1980, 1981), respiration in mitochondria from rat liver (Duszynski *et al.,* 1982; Groen *et al.,* 1982b; Gellerich *et al.,* 1983; Tager *et al.,* 1983; Westerhoff *et al.,* 1983; Wanders *et al.,* 1984; Brand *et al.,* 1988) and yeast (Mazat *et al.,* 1986), amino acid metabolism (Groen *et al.,* 1982a; Pogson *et al.,* 1986; Salter *et al.,* 1986) and gluconeogenesis (Groen *et al.,* 1983, 1986; Pryor *et al.,* 1987) in rat liver cells, citrulline synthesis in isolated rat liver mitochondria (Wanders *et al.,* 1983), glycerol-3-phosphate production in soluble extracts from rat liver (Torres *et al.,* 1986, 1988a,b), photochemical reactions in bacteriorhodopsin (Westerhoff & Arents, 1984), ethanol metabolism in *Drosophila* (Middleton & Kacser, 1983) and horse liver (Derr & Derr, 1987), and the Calvin photosynthesis cycle in C_3 plants (Pettersson & Ryde-Pettersson, 1989).

1.3.3 **Flux Oriented Theory (FOT)**

Crabtree and Newsholme's quantitative approach to metabolic control is based on a simple measure of the response of a given system to a given regulator, which they termed "sensitivity"

There are two types of sensitivity in FOT that enable complex control systems to be analysed. The intrinsic sensitivities (S_i) are derived (using simple addition and product rules) from experimentally determined parameters such as kinetic response of enzymes and displacement from equilibrium. The overall (net) sensitivity of a system, flux or reaction, to effector *in situ* , is derived from the intrinsic sensitivities, by using a power approximation (Savageau, 1972) to the rate of a reaction i.e. the rate (v) is approximated by the product of the concentrations of all its effectors (substrate, product, activators, inhibitors -- X_1 ..... X_n), each raised to the power of its intrinsic sensitivity (equation 1.4):

$$v = K\,[X_1]^{Si\,(X1)}\,[X_2]^{Si\,(X2)} \ldots\ldots\ldots\ [X_n]^{Si\,(Xn)} \qquad (1.4)$$

The regulatory sequence is identified and the rate of the regulator reaction approximated by a power equation which includes the interaction of the regulator (s). Unwanted fluxes and effector concentrations are eliminated from this equation using power approximations for all the other reactions of the sequence. The end result is shown in equation 1.5:

$$\text{flux (J)} = \text{const. (K)}\ [\text{regulator (X)}]^{S} \qquad (1.5)$$

The net sensitivity of the flux to changes in the specified regulator via that sequence is then represented by the algebraic expression forming the index S.
The flux oriented approach has been used to analyse the sensitivity conferred by a substrate cycle

(Crabtree, 1976; Crabtree & Newsholme, 1985a), to evaluate the control of glycolysis by ATP, ADP and AMP acting together (Crabtree & Newsholme, 1985a, 1987a), and to analyse the importance of fluxes in determining the sensitivity at a branch point (Crabtree & Newsholme, 1985a).

1.3.4 **Comparison of Theories**

Until recently, these three approaches (BST, MCT and FOT) to metabolic control have not been compared. This has resulted in separate literatures with few cross-references between them, different terminologies for equivalent control parameters (see Table 1.1) and a great deal of confusion concerning the best theory for quantitative analysis of control (Burns *et al.*, 1985; Crabtree & Newsholme, 1985a, 1987, 1988; Porteous, 1985; Canela & Franco, 1987; Fell, 1987; Kacser & Porteous, 1987b; Savageau, 1987; Sorribas, 1987; Voit, 1987; Welch & Keleti, 1987; Groen & Tager, 1988).

TABLE 1.1

Terminolgy in BST, MCT, and FOT for Control Parameters

BST	MCT	FOT	References
logarithmic gain factor	response coefficient	*	Savageau *et al.* (1987b)
rate constant sensitivity	concentration control coefficient	net sensitivity	Westerhoff *et al.* (1984) Savageau *et al.* (1987b)
*	flux control coefficient	net sensitivity	Westerhoff *et al.* (1984) Burns *et al.* (1985b)
kinetic orders	elasticity coefficient	intrinsic sensitivity	Westerhoff *et al.* (1984) Burns *et al.* (1985b) Savageau *et al.* (1987a,b) Sorribas & Savageau (1989b)

* there is no equivalent terminology

Recent detailed comparisons of these three approaches (Savageau *et al.*, 1987a,b; Voit & Savageau, 1987; Sorribas & Savageau, 1989a,b) have shown that the metabolic control theory and the flux oriented theory are special cases of BST. They are based on the mathematical structure of BST, involving the explicit (FOT) or implicit (MCT) use of the power-law formalism developed by Savageau (Savageau, 1969a,b, 1970, 1971a,b, 1972).

Even though the concepts, theory and methodology of BST provide a very general framework for analysing complex biochemical systems, I elected to use Kacser and colleagues' approach to metabolic control.

MCT is valid in steady state systems since it shows which enzyme alteration leads to the most dramatic changes in flux or intermediate concentrations. The theory is the easiest to understand and the experimental methodology provided for determining the essential control parameters (elasticity and flux control coefficients) is clearly outlined. The experimental applications of MCT are numerous (see section 1.3.2) and the diverse number of research groups (see section 1.3.2) using MCT clearly show its popularity.

Some criticisms (Savageau, 1987a,b,c; Sorribas, 1987; Welch & Keleti, 1987; Sorribas & Savageau, 1989b) of the metabolic control theory are that:

1) The theory is not valid for cascades and enzyme-enzyme interactions. It has already been acknowledged (Kacser & Burns, 1979; Kacser & Porteous, 1987b) that possible modifications may be needed for particular cases and an algebraic formalism (Kacser & Porteous, 1987b) has been developed, which takes into account enzyme-enzyme interactions.

2) The theory is oversimplified. Elasticity coefficients and flux control coefficients are defined in a simplified manner to represent local and global changes in the system. This avoids the complexity of the system by omitting its dynamic nature. This simplification is perhaps one of the attractions of control analysis. For experimental application MCT would appear more straight forward than BST which is mathematically more complex.

The aim of this thesis was to use the metabolic control theory to evaluate the distribution of control in the pathway for metabolism of ethanol in hepatocytes isolated from fed and starved rats. This involved determination of the flux control coefficients of the individual steps in the ethanol pathway (see Figure 1.2).

1. 4 DETERMINATION OF FLUX CONTROL COEFFICIENTS

The flux control coefficient ($C^J_{E_i}$) of a step (i) in a metabolic pathway is defined as the fractional change in steady state (ss) flux (J) through the pathway induced by a fractional change in the activity of enzyme (E_i) under consideration (equation 1.6) (Kacser & Burns, 1973; Heinrich & Rapoport, 1974).

$$C^J_{E_i} = \left(\frac{dJ/J}{dE_i/E_i}\right)_{ss} \qquad (1.6)$$

The quantitative value of the flux control coefficient for an enzyme describes how sensitive the flux is to changes in that particular enzyme. In practise large changes that are imposed are uninformative and infinitesimal changes impracticable. Therefore the method of modulation (Kacser & Burns, 1973) must be applied. This modulation approach involves altering the activity of one enzyme by small amounts (∂E_i), and measuring the effect of this alteration on flux through the pathway. The enzyme activity may be increased in small amounts by addition of extra enzyme (Rapoport *et al.*, 1976; Groen *et al.*, 1982b; Westerhoff & Arents, 1984) or decreased by small amounts by titrating with enzyme-specific inhibitors (Duszynski *et al.*, 1982; Groen *et al.*, 1982b; Verhoeven *et al.*, 1985; Torres *et al.*, 1986). The latter approach was taken for determining the flux control coefficients of steps involved in the oxidation of ethanol (see Figures 1.1 and 1.2).

The effect of a change in the concentration of an external effector (x) on the pathway flux (J) is defined (Kacser & Burns, 1973; Groen *et al.*, 1982c ; Derr, 1986) in terms of the response coefficient, R (equation 1.7):

$$R^J_x = \frac{\partial J/J}{\partial x/x} \qquad (1.7)$$

The effect of a change in the concentration of the effector on the rate (v_i) of an 'isolated' enzyme is defined (Kacser & Burns, 1973; Groen *et al.*, 1982c ; Derr, 1986) in terms of the controllability coefficient or elasticity coefficient of an external effector (x) (equation 1.8):

$$\varepsilon^{E_i}_x = \frac{\partial v_i/v_i}{\partial x/x} \qquad (1.8)$$

The flux control coefficient of an enzyme can be calculated from the response coefficient and controllability coefficient (equation 1.9):

$$C^{J}_{E_i} = R^{J}_{x} / \varepsilon^{E_i}_{x} \qquad (1.9)$$

The external effector in this study is the enzyme-specific inhibitor. Therefore the response of the pathway flux (J) to the action of an inhibitor (I) on enzyme (E_i) is given by :

$$C^{J}_{E_i} = \frac{\left(\frac{dJ/J}{dI/I}\right)_{ss}}{\left(\frac{\partial v_i/v_i}{\partial I/I}\right)_{S_j P_j}} \qquad (1.10)$$

where: J = the pathway flux at steady state (ss).
I = the free concentration of inhibitor
v_i = velocity of the enzyme isolated from pathway flux but incubated with fixed concentrations of substrate (S_j) and product (P_j) equivalent to concentrations prevailing in pathway at flux J.

The flux control coefficient in absence of inhibitor (equation 1.11) can be obtained by taking the limit of equation 1.10 at I=0 (Groen *et al.*, 1982b,c; Derr, 1986).

$$C^{J}_{E_i} = \frac{\left(\frac{dJ/J}{dI}\right)_{I=0}}{\left(\frac{\partial v_i/v_i}{\partial I}\right)_{I=0}} \qquad (1.11)$$

The top term of equation 1.11 can be derived from the initial slope of the flux inhibition curve (flux (J) vs [I]). J is the flux at zero inhibitor concentration and $\left(\frac{dJ}{dI}\right)_{I=0}$ is the initial slope of the flux inhibition curve. Some researchers (Lemasters & Sowers, 1979; Rognstad, 1979; Yont & Harris, 1980) have assumed that the shape of the inhibition curve in itself provides quantitative information about the relative importance of the step under consideration. A step was said to be rate-controlling when on titration with an inhibitor a hyperbola-like curve is obtained and non rate-controlling when a sigmoidal inhibition curve is produced. Use of this criterion for evaluating the

importance of a step in regulation of the pathway can be misleading (Groen *et al.*, 1982c) because the nature of the inhibition and the amount of inhibitor used must be taken into consideration.

The denominator of equation 1.11 can be calculated from the inhibition kinetics of the enzyme. The mathematical formula of $C^J_{E_i}$ will differ with respect to the type of inhibitor used (Rapoport, 1977; Groen *et al.*, 1982c ; Derr, 1986). For instance, the influence of an uncompetitive inhibitor on the rate equation for an irreversible reaction is:

$$v = \frac{v_1 S}{K+S(1+\frac{I}{K_{ii}})} \qquad (1.12)$$

where: K represents the K_m for substrate; S is the substrate concentration; I is the inhibitor concentration and K_{ii} represents the intercept inhibition constant.

Differentiation of (1.12) leads to equation 1.13

$$\frac{dv}{vdI} = \frac{-S}{K_{ii}\,(K+S(1+\frac{I}{K_{ii}}))} \qquad (1.13)$$

Substitution of (1.13) into (1.11) gives equation (1.14) .

$$C^J_{E_i} = \frac{-dJ}{JdI}\,\frac{K_{ii}(K+S)}{S} \qquad (1.14)$$

Other important points to note on the use of specific inhibitors for determination of flux control coefficients are:

1) If the inhibitor has to cross a membrane to reach its site of action then it would be helpful to know the membrane's permeability to the inhibitor. The value of the flux control coefficient will be underestimated if the inhibitor is excluded whereas an accumulation of inhibitor will produce an overestimation of the $C^J_{E_i}$.

2) If an irreversible inhibitor is used, there should be insignificant binding to other proteins; otherwise the flux control coefficient will be underestimated.

3) When using competitive, noncompetitive, or mixed type inhibitors, knowledge of the kinetic constants and concentration of the metabolites interacting with the enzyme under consideration is required.

The best type of inhibitors to use are irreversible inhibitors and some noncompetitive inhibitors that do not require the measurement of substrate and product concentrations for the enzyme being studied (Groen *et al.*, 1982c; Derr, 1986). Irreversible inhibitors are extremely good because on titration the end point indicates the amount of enzyme in the system (Segel, 1975 pp 127-128). However specific irreversible and noncompetitive inhibitors are not very common.

It was important to find specific inhibitors for ADH, AlDH, malate-aspartate shuttle enzymes and enzymes of the respiratory chain, so that the flux control coefficient of these enzymes could be determined using the modulation approach (Kacser & Burns, 1973). A search for specific irreversible and noncompetitive inhibitors of the enzymes was the first step. When this failed competitive and uncompetitive inhibitors were found. The final inhibitors chosen for the inhibitor titration studies are described in chapters 4, 5, and 6.

Because the flux control coefficient is a global coefficient, the enzymes and proteins under study must be intact in their environment. This can be accomplished by using isolated hepatocytes. These cells, when well prepared, retain the metabolic capabilities of the intact liver (Krebs *et al.*, 1974; Cornell *et al.*, 1982b). For the inhibitor titration studies, the rates of ethanol oxidation were measured under physiological conditions in isolated hepatocytes. To be able to measure the small changes in rates of ethanol oxidation, brought about by the introduction of specific inhibitors, a new method for incubation of the isolated hepatocytes and measurement of ethanol clearance rates had to be developed. This is explained in detail in chapter 3.

The flux control coefficient is a quantitative measure of how much a given enzyme limits the flux through a metabolic pathway. The pioneers of the metabolic control theory (Kacser & Burns, 1973; Heinrich & Rapoport, 1974) have shown that the sum of all the flux control coefficients for any one flux in a steady state system is equal to unity (equation 1.16). This is called the flux control summation theorem.

$$\sum_{i=1}^{n} C_{E_i}^{J} = 1 \qquad (1.16)$$

The summation property has been demonstrated in a number of experimental systems (Groen *et al.*, 1982b, 1983; Salter *et al.*, 1986; Torres *et al.*, 1986). The implication of this theorem is that the enzyme with the greatest flux control coefficient exercises the most control in the metabolic system in question under the conditions tested. If one enzyme activity is changed by a substantial amount, its' flux control coefficient will change to a new value at the new level, and the flux control coefficients of all other enzymes in the metabolic pathway will also change, so that their sum still equals one. Hence the summation theorem will hold for any set of enzymes and external parameters but the internal distribution will alter with changing conditions. Because the flux control coefficient for a particular enzyme is not a fixed value and will change under different metabolic conditions, it was important to assess the regulation of ethanol metabolism in hepatocytes isolated from both fed and starved rats.

In summary, I have studied the distribution of control in the metabolism of ethanol in isolated rat hepatocytes, using Kacser and Burns (1973) modulation methodology. The method developed to apply the modulation approach to analysis of ethanol oxidation in rat hepatocytes is described in chapter 3. Determination of the flux control coefficient for alcohol dehydrogenase and aldehyde dehydrogenase is described in chapters 4 and 5 respectively. The data presented in chapter 5 represent preliminary work for determination of the flux control coefficient of AlDH. The reason for this work being incomplete is because I became allergic to the rats, chemicals and detergents and as a result developed contact dermatitis on my hands. This made it impossible to continue with the hepatocyte preparations and the inhibitor titration experiments. Also, titration experiments were not carried out on the possible steps in the shuttle systems and respiratory chain. Hence chapter 6 is a discussion on what could be done to determine the contribution of the individual steps in the hydrogen shuttle cycles and electron transport chain to regulation of ethanol oxidation in isolated rat hepatocytes. Chapter 7 is a discussion of all the results obtained in this thesis along with prospects for future work.

CHAPTER 2

MATERIALS AND METHODS

2.1 SOURCE OF MATERIALS AND REAGENTS

The list in Table 1.1 shows the source of all chemicals of analytical grade, enzymes, cofactors, inhibitors, and special equipment. All other chemicals mentioned in this thesis are reagent - grade products.

2.2 HYDROLYTIC ENZYMES

Berry and Friend (1969) were the first to prepare isolated hepatocytes by perfusing the liver with the digestive enzymes, collagenase and hyaluronidase. Perfusion exposes virtually every cell to the hydrolysing enzymes. Both of these enzymes were used in the preparation of isolated rat liver cells.

2.2.1 Collagenase

Collagenase was obtained from Boehringer Mannheim GmbH (West Germany) in 500 mg lots. Each lot was tested for cell yield (greater than 2 gram of cells from starved rats) and cell viability (greater than 85% viable) before continued use of that batch.

A crude collagenase preparation was used because the contaminating proteolytic enzymes aid digestion of the liver (Berry & Friend, 1974 ; Krebs *et al.*, 1974) . Optimum activity for collagenase is obtained at pH 7.4 (Seglen, 1976) . Maintenance of the pH was achieved by dissolving collagenase in Krebs-Henseleit buffer (see section 2.3) and gassing with 95% O_2: 5% CO_2.

The concentration of collagenase used for perfusion was dependent on the activity of the batch, since variation in the protease activity occurs between batches. The age of the rats was also important as younger rats require less collagenase for cell dispersion because of their lower collagen content (Seglen, 1976; Pogson, 1983). Typically 30 to 50 mg collagenase was used in the perfusion of liver from a 180 to 300 g rat.

TABLE 1.1

Materials and their Source

Category	Item	Source
Enzymes	ADH (Bakers Yeast)	Sigma Chemical Co. Ltd, U.S.A
	AlDH (Bakers Yeast)	Sigma Chemical Co. Ltd, U.S.A
	G-6-P-DH (Type Vll, Yeast)	Sigma Chemical Co. Ltd, U.S.A
	Hexokinase (Type C-130,Yeast)	Sigma Chemical Co. Ltd, U.S.A
	Hyaluronidase (Ty.V,Sheep Testes)	Sigma Chemical Co. Ltd, U.S.A
	Collagenase(Ty.lV,Cl.Histolyticum)	Boehringer Mannheim GmbH, FRG
Cofactors	ATP (grade ll)	Sigma Chemical Co. Ltd, U.S.A
	NAD^+ (grade lll, Yeast)	Sigma Chemical Co. Ltd, U.S.A
	$NADP^+$ (Yeast)	Sigma Chemical Co. Ltd, U.S.A
Inhibitors	Diethyldithiosodium carbamate	BDH Chemical Ltd, England
	Disulfiram	Sigma Chemical Co. Ltd, U.S.A
	Isobutyramide	Aldrich Chemical Co. Inc., U.S.A
	Tetramethylene sulphoxide	Aldrich Chemical Co. Inc., U.S.A
Chemicals and Reagents	BSA (fraction V)	Sigma Chemical Co. Ltd, U.S.A
	Glucose (anhydrous, grade lll)	Sigma Chemical Co. Ltd, U.S.A
	L(+) lactic acid	Sigma Chemical Co. Ltd, U.S.A
	lysine	Sigma Chemical Co. Ltd, U.S.A
	Trypan Blue	Sigma Chemical Co. Ltd, U.S.A
	Acetaldehyde	BDH Chemical Ltd, England
	KCl	BDH Chemical Ltd, England
	Sodium pyrophosphate	BDH Chemical Ltd, England
	ß-mercaptoethanol	J T Baker Chemical Co., NJ
	Perchloric acid (70%)	AJAX Chemicals PTY Ltd,Australia
	Ethanol (spectroscopic grade, 96%)	Fluka AG Buch, Switzerland
	Sodium pyruvate	Fluka AG Buch, Switzerland
	Nembutal (powder)	Abbott Lab. NZ Ltd, Auckland
	Nembutal (liquid)	Ceva Chemicals PTY Ltd, Australia
	Sagatal (liquid)	Hay & Baker NZ Ltd, Lower Hutt
	Heparin	NZP, Palmerston North, NZ
Equipment	2.2 ml Microcentrifuge Tubes	Gibco NZ Ltd, Auckland
	Abbocath-T 16x2 (19-G needle)	Abbott Lab. NZ Ltd, Auckland
	C_{6000} and C_{200} Gilson tips	John Morris Scientific Ltd, NZ

2.2.2 Hyaluronidase

Hyaluronidase was purchased from Sigma Chemical Company (St.Louis, MO, USA). Several researchers have found that this hydrolytic enzyme was not necessary for the dispersion of the whole liver cells (Seglen, 1973; Veneziale & Lohmar, 1973; Meijer *et al.*, 1975; Baur & Heldt, 1976; Pogson *et al.*, 1983) and that it can have an inhibitory effect at concentrations of 0.2 mg/ml or greater (Seglen, 1976).

Others have stated that hyaluronidase will increase the cell yield (Berry & Werner, 1974; Krebs *et al.*, 1974; Berry, 1976a). This is an important factor. The production of as many intact viable cells as possible is required for metabolic studies.

In this laboratory hyaluronidase has been used for many years and will continue to be used. The concentration (as high as 0.08 mg/ml) is noninhibitory, the possibility of greater cell yield is advantageous to our studies and with a consistent technique past results may be compared to present data.

2.3 KREBS-HENSELEIT (K-H) BUFFER

Krebs-Henseleit bicarbonate buffer (Krebs & Henseleit, 1932) with Ca^{2+} omitted, was utilised for perfusion of the rat liver. K-H buffer supplemented with 2.5% BSA was used as a medium for suspension and incubation of the cells. The buffer contained 118 mM NaCl, 4.75 mM KCl, 2.5 mM $CaCl_2.2H_2O$, 1.18 mM KH_2PO_4, 1.18 mM $MgSO_4.7H_2O$, and 25 mM $NaHCO_3$. It is a physiological saline solution that provides the Na^+: K^+ extracellular concentration ratio of 143: 6 (Cornell, 1983b) and is a good buffering system to maintain pH 7.4, when gassed with 5% CO_2 in O_2.

2.3.1 Perfusion Medium

The perfusion system was primed with 150 mls of calcium-free K-H buffer. If fed rats were used 20 mM glucose was added to the perfusion medium to maintain hepatic glycogen.

The liver must be perfused with calcium-free medium before any separation of intact parenchymal cells can be obtained (Seglen, 1976; Wanson, 1976). It appears that cleavage of desmosomes is the key element for liver cell separation and that calcium ions are essential for desmosomal integrity (Berry & Friend, 1969; Berry, 1976b; Seglen, 1976; Wanson, 1976; Berry *et al.*, 1983) Perfusion with a Ca^{2+} - free medium will flush out calcium ions and cause cell dispersion to occur. However Ca^{2+} ions are required for collagenase activity (Seifter & Harper, 1970;

Peterkofsky, 1982). Seglen (1976) proposed a two-step procedure, where calcium ions were removed by preperfusion of the liver with a Ca^{2+}- free medium and then calcium was introduced back into the system with collagenase. We and others (Johnson *et al.*, 1972; Cornell *et al.*, 1973; Krebs *et al.*, 1974; Pointer *et al.*, 1976; Birnbaum & Fain, 1977; Tolbert *et al.*, 1980; Pogson *et al.*, 1983) have found this second step to be unnecessary. Analysis of crude collagenase preparations have shown that the endogenous Ca^{2+} present was enough for enzymatic activity (Berry, 1976b; Pogson *et al.*, 1983). Collagenase has been suggested to aid cleavage of desmosomes by removing residual calcium ions (Berry, 1976b; Berry *et al.*, 1983) .

2.3.2 **Cell Suspension Medium**

This medium was K-H buffer supplemented with 2.5% dialysed BSA. The buffer was gassed with O_2+CO_2 (95:5) until the pH was about 7.4, then dialysed BSA (initial concentration about 10%) was added to make a 2.5% solution. Gassing (5% CO_2 in oxygen) of this mixture was continued during perfusion of the liver.

For each experiment a total volume of 150 ml K-H / 2.5% BSA medium was prepared and used for the washing and incubation of the cells.

2.4 BOVINE SERUM ALBUMIN (BSA) FOR HEPATOCYTES

Bovine Serum Albumin (BSA) was not present during perfusion of the rat liver because it could decrease the activity of the digestive enzymes, thereby decreasing the cell yield (Berry, 1974; Krebs *et al.*, 1974). However K-H buffer containing 2.5% dialysed BSA was used for washing and incubation of the isolated hepatocytes. Albumin offers some protection for the cells against mechanical stress, binds some toxic substances and most importantly limits cell aggregation (Krebs *et al.*, 1974; Seglen, 1976). A decrease in the metabolic activity is concomitant with clumping of cells (Krebs *et al.*, 1974).

2.4.1 **Dialysis of BSA**

Most Albumin preparations contain ethanol and acetic acid and these contaminants are readily metabolised by the liver. Before the BSA can be used in the cell suspension medium, the impurities must be removed by dialysis.

36 g BSA (fraction V) was dissolved in approximately 200 ml of K-H buffer. To minimise bacterial contamination the plastic 250 ml beaker was kept cold (4 ^{o}C) and covered except for the

times when the solution was stirred. It took several hours for BSA to dissolve completely in the bicarbonate buffer.

Dialysis tubing (25 mm in diameter) was cut into lengths of approximately 27 cm and washed in 3 changes of distilled water heated at 8O °C, to remove any impurities.

The BSA solution was dialysed against 3 changes of 4 L of K-H buffer. Dialysis was carried out in the cold room (4 °C) with the 5 L erlenmeyer flasks covered.
The saline buffer was continuously stirred (via magnetic stirrer) during dialysis of the BSA.

After 3 days the BSA solution was removed from the tubing, the total volume was recorded and then the protein concentration was determined.

2.4.2 **Determination of Protein Concentration**

The absorbance of a solution containing 0.1 ml dialysed BSA in 9.9 ml of 0.02 M HCl was measured at 278 nm. The blank contained 0.1 ml of K-H buffer in 9.9 ml of 0.02 M HCl.

The concentration of the dialysed BSA was determined assuming that the absorbance of a 0.1% BSA solution at 278 nm is 0.64 (Sober, 1970).

The BSA solution was stored frozen in plastic bottles in quantities appropriate for the preparation of 150 ml K-H / 2.5% BSA (see section 2.3.2).

2.5 PREPARATION OF ISOLATED HEPATOCYTES

Isolated hepatocytes were prepared by the method of Berry & Friend (1969) as modified by Cornell *et al.* (1973).

The procedure involved perfusion of the rat liver with collagenase and hyaluronidase to promote liver digestion and then removal of the liver for collection of the isolated liver cells.

It is technically quite difficult to obtain a high yield of isolated hepatocytes that are metabolically viable. Isolation of hepatocytes is an art which relies on judgement and experience for the preparation of a high yield of viable cells.

2.5.1 **Rats**

Male Sprague-Dawley rats, weighing 160 to 300 g, were obtained from the Massey University Small Animal Production Unit. They were housed in a temperature-regulated room (24°C) with artificial light providing a 12 hr light/dark cycle. The rats were fed a standard pellet diet, supplied *ad libitum* or starved 48 hrs prior to the experiment. In both cases there was free access to tap water.

Before the operation, the rats were injected intraperitoneally with nembutal (30 mg/ml of 0.9% saline). When we were unable to purchase powdered nembutal, Nembutal (liquid) or Sagatal in doses of around 0.5 ml/190 g rat was used.

2.5.2 **Operative Technique**

The principle of this operation was to insert a cannula into the hepatic portal vein and a cannula into the *vena cava* above the diaphragm, to allow recirculatory perfusion of the rat liver. The route of perfusion (Figure 2.1) would involve a flow of medium from the reservoir to the perfusion lung, through the perfusion lung to the liver via the portal cannula, and through the liver back into the reservoir by way of the *vena cava* cannula. The speed and precision of the operation were factors that contributed to the final yield and viability of the cell preparation.

2.5.2.1 ***Surgical Procedure***

The anaesthetised rat was placed on its back on an operating platform, where the limbs were secured. 0.1 ml heparin (5000 U/ ml of saline) was injected into the leg vein before commencement of the operation. A mid line incision was made through the skin from the abdomen to the neck. The abdomen was then opened by a mid line incision and 2 mid transverse incisions to the left and right of the mid line cut. The intestines were placed to the animals' left exposing the liver, portal vein, right kidney, and inferior *vena cava.* The thin strands of the connective tissue between the right lobe of the liver and the *vena cava* were cut and a loose ligature was tied around the *vena cava* above the right renal vein. Two loose ligatures were tied around the portal vein to aid the insertion of the portal cannula (16-G needle). The needle was removed, leaving the catheter (51 mm) secured in the vein by tying the two threads. An opening in the thorax was made to expose the heart and *vena cava* above the diaphragm, the cannula (see section 2.5.5) was inserted via the auricle and secured. The ligature around the *vena cava* above the renal vein was tied. The rat was now ready to be connected to the perfusion apparatus.

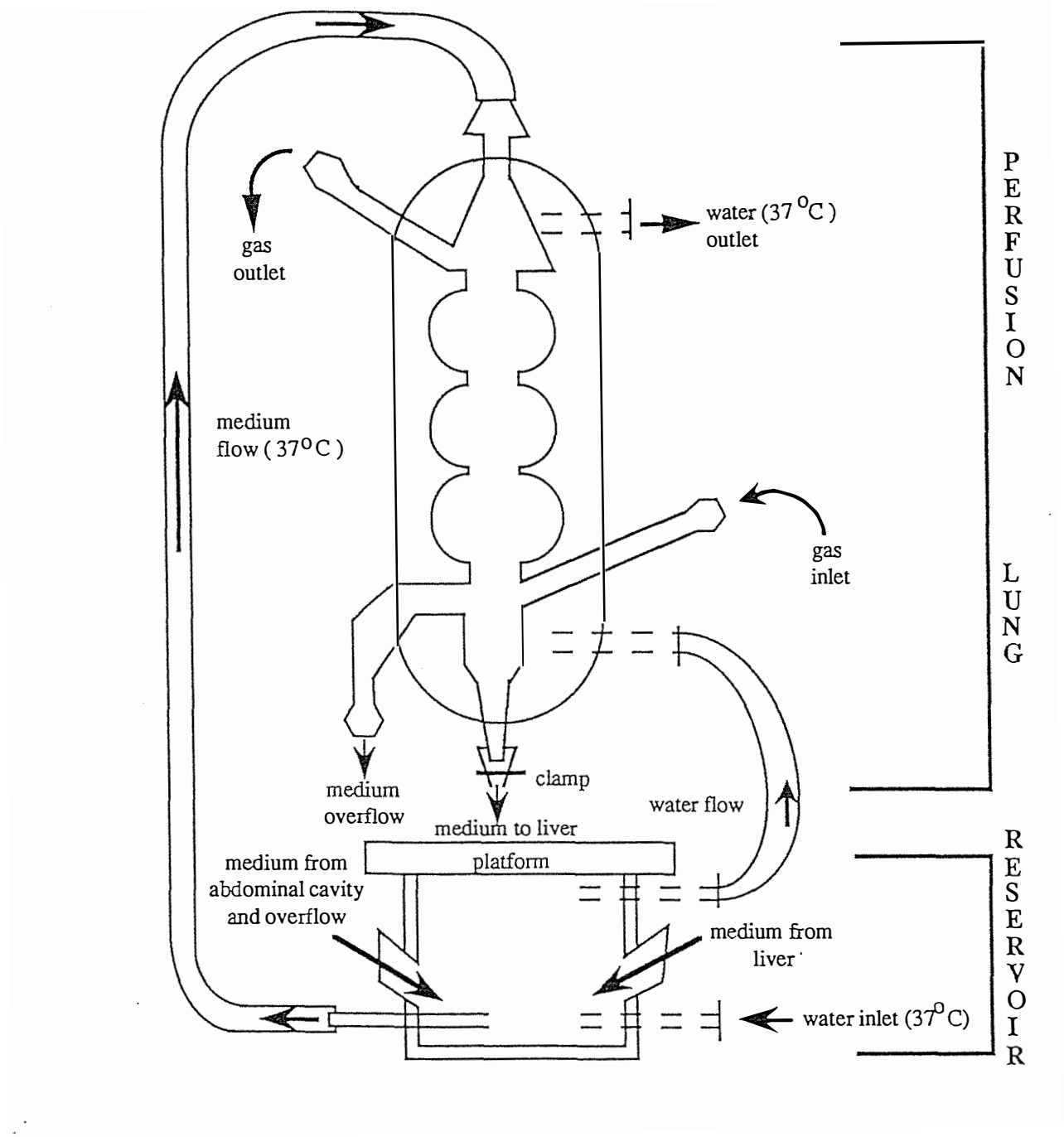


Figure 2.1: **Perfusion Apparatus**

The dotted lines represent connections on the reverse of the perfusion lung and reservoir. The clamp regulates the flow rate of the medium. The medium is gassed with 5% CO_2 in O_2.

2.5.3 **Perfusion**

The perfusion apparatus was based on Hems *et al.* (1966). A diagram of our perfusion system is shown in Figure 2.1.

The system was primed with 150 ml of enzyme-free perfusion medium. En route the medium was oxygenated by continual gassing with $O_2 + CO_2$ (95: 5) and maintained at a constant temperature of 37°C and pH of 7.4 (see section 2.3). The flow rate was adjusted to approximately 25 ml/min for a 200 g rat. Too high a flow rate led to disruption of the liver and a slow flow rate resulted in anoxia. It was necessary to clear the liver of all blood, after connection of the rat to the perfusion apparatus. Gentle massaging of the tissue facilitated this. During blanching of the liver, the *vena cava* cannula and hepatic cannula were adjusted to obtain a maximum flow rate. The appearance of the liver gave some indication of the flow of medium through the organ. With a uniform flow complete loss of the reddish colour of the liver was observed. An irregular flow produced patchiness (uneven colouring of the liver) where inadequate perfusion and digestion of the liver occurred in the dark coloured regions of the organ (areas retaining blood).

The first 40 to 50 ml of perfusate flowing from the *vena cava* cannula was discarded during the flushing of blood and adjustment of flow rate. The next 5 ml of medium was used to dissolve the digestive enzymes, then the cannula effluent was redirected into the reservoir.

Collagenase and hyaluronidase were added to the perfusion system via a suction tube. This tube returned perfusion medium exudated from the liver surface back into the reservoir. Perfusion was continued until the liver was very soft, massive fluid leakage from the liver surface was apparent and the flow rate from the *vena cava* cannula dropped considerably. After approximately 30 minutes perfusion was discontinued and the liver was removed for collection of the isolated hepatocytes.

2.5.4 **Collection Of Hepatocytes**

Once perfusion was stopped, the hepatic portal cannula was removed. The liver was carefully cut out and placed in a 250 ml plastic beaker containing some perfusion medium from the reservoir. The procedure for collection of hepatocytes, described below, involved several low speed centrifugation steps which were necessary for separating the parenchymal cells (hepatocytes) from subcellular debris, damaged cells, residual red blood cells and nonparenchymal cells (Krebs *et al.,* 1974; Seglen, 1976).

The liver was chopped up, while immersed in the reservoir medium, to aid in the separation of the liver cells. This suspension was then filtered through a nylon gauze sieve (see section 2.5.7) to remove cell debris and undissociated cells. The filtrate was centrifuged in two 50 ml glass centrifuge tubes for 2 minutes at 50 x g. The supernatant was poured off and the precipitated cells were resuspended in K-H / 2.5% BSA medium (see section 2.3.2) using a 10 ml wide tipped pipette. The suspension was filtered and recentrifuged in one glass centrifuge tube at 50 x g for 2 minutes. The precipitated cells were resuspended as before, placed in a tared centrifuge tube and spun for 2 minutes at 50 x g. The pellet was weighed and suspended in 10 volumes of wash medium (see section 2.3.2). The suspended cells were placed in a 250 ml erlenmeyer flask and gassed with 5% CO_2 in 0_2 (mixing occasionally to keep aerated) until ready for use.

For collection of isolated hepatocytes, gentle handling of the digested liver and cell suspension was required. Rapid pipetting through a narrow orifice, magnetic stirring and bubbling of 95% O_2: 5% CO_2 through the cell suspension are harsh techniques and should be avoided if intact cells are to be maintained.

2.5.5 *Vena Cava* Cannula

The cannula placed in the inferior *vena cava* above the diaphragm was made in the laboratory. Plastic tubing of inside diameter 2 mm was cut into lengths of 3.0 to 3.5 cm. The end was tapered (using a razor) to form a tip sharp enough to pierce the auricle. Once the cannula was pushed down the vein, enough tube protruded from the vein to allow for connection to the reservoir and manipulation for adjustment of the flow rate.

2.5.6 Perfusion Lung

The perfusion lung was stored in a Pyroneg/water solution, in a 10 L plastic container. Before each experiment the lung had to be cleaned thoroughly to remove all traces of detergent. The overall perfusion and digestion of the liver would be affected if detergent was present. Filming of the lung bubbles becomes difficult which leads to inadequate oxygenation of the medium, and detergent contamination of the perfusion medium would obstruct digestion of the liver and separation of cells.

Firstly the lung was washed several times with tap water to remove the bulk of detergent. This was followed by a wash with methanol which aided removal of residual detergent. The lung was then washed thoroughly with tap water and, finally, rinsed with distilled water.

The cleaned lung was connected to the water pump and reservoir. The perfusion medium was pumped through the lung. All lung bubbles should be covered, or filmed, with the medium to ensure good oxygenation and perfusion of the rat liver.

After many experiments, inadequate filming of the lung occurred. The lung was then soaked overnight in chromic acid (35 ml saturated $Na_2Cr_2O_7$ to 1 L conc. H_2SO_4) or in an alkaline solvent (NaOH solution in saturated $KMnO_4$) .

2.5.7 Nylon Gauze Sieve

The sieve was made in the laboratory. A 100 ml disposable plastic beaker (with the bottom removed) was heated on a metal plate. When the plastic had melted it was pressed firmly on a nylon cloth (mesh size 0.5 mm x 0.3 mm) on a cool smooth surface. The final step involved trimming the gauze around the beaker once the seal was formed between the mesh and plastic.

2.6 CELL WET WEIGHT

Metabolite content (µmole/g) and metabolic rates (µmole/min/g) were expressed on the basis of cell wet weight.

The cell wet weight was determined from the dry weight as described by Krebs *et al.*(1974). The dry weight of the isolated cells was obtained by placing 2 ml cells and medium (in duplicate) and 2 ml medium (in duplicate) in preweighed vials and drying to constant weight at 100 °C. The difference in averaged weights represented the dry weight of cells collected. The wet wt/dry wt ratio of the perfused organ was 3.7 (Krebs *et al.*, 1974). This factor was used to convert from dry weight into wet weight of cells.

2.7 CRITERIA FOR CELL INTEGRITY

The metabolic performance of cells with damaged membranes is markedly decreased (Cornell, 1983b; Dickson & Pogson, 1977) therefore intact cells are required for metabolic studies.

The physiological quality of the hepatocyte preparation was assessed by examination of the cell morphology (Trypan Blue Exclusion Test) and measurement of the ATP content.

2.7.1 Trypan Blue Exclusion Test

The Trypan Blue Exclusion Test (Phillips, 1973) was used to visualise the integrity of the cells as well as estimating the viability of the hepatocyte preparation.

50 µl of cell suspension was added to a solution containing 0.1 ml of 0.1% aqueous Trypan Blue and 0.9 mls of 0.9% saline. A small amount of this mixture was placed on a Neubauer counting chamber and viewed under a light microscope (Nikon) at 10x magnification. The number of stained cells (dead cells) and nonstained cells (viable cells) were counted and the percentage of viable cells was calculated. Cell preparations with a viability count of 85% or greater were used in metabolic experiments.

Under the light microscope intact cells were readily recognised by their roundness and refractility (Berry, 1974; Berry & Werner, 1974; Krebs *et al.*, 1974; Seglen, 1976). Clumps of cells or bleb formation easily observed under the microscope indicated a decrease in the metabolic performance of the isolated liver cells.

2.7.2 ATP Content

The measurement of ATP (see section 2.11) is a convenient and reliable method for evaluating cell viability (Krebs *et al.*, 1974 ; Cornell, 1983b; Pogson *et al.*, 1983). The relationship between membrane damage and maintenance of cellular ATP is reflected in the corresponding association between ATP levels and intracellular enzyme leakage (Dickson & Pogson, 1977; Cornell, 1983b).

Cornell (1983b) stated that the level of ATP provided a useful minimum criterion for determining cell quality. Hepatocyte preparations with ATP content 2 µmole/g wet wt cells or greater were used in metabolic experiments. When ATP levels dropped below 2 µmole/g wet wt cells intracellular enzyme leakage became pronounced (Cornell, 1983b) .

2.8 INCUBATION OF ISOLATED HEPATOCYTES

In all experiments, the isolated liver cells were incubated in K-H buffer containing 2.5% BSA, with 13 mM ethanol, 10 mM lactate, 1 mM pyruvate, and inhibitor at the appropriate concentration. Incubations were carried out at 37°C and were stopped by the addition of 0.05 ml of 60% $HClO_4$ per ml of incubation mixture. The sample was then centrifuged at 13500 rpm for 4.5 minutes. The acidic supernatant was used for measurement of metabolite concentrations which were corrected for the $HClO_4$ dilution.

Details of procedures for incubation of the isolated hepatocytes are described in chapter 3.

2.9 ETHANOL ASSAY

Ethanol was measured in unneutralised acid samples using the yeast alcohol dehydrogenase method of Dickinson & Dalziel (1967) as modified by Cornell & Veech (1983). NADH produced in the ADH reaction is measured via absorption at 340 nm.

Only 20 µl of acidic supernatant was required for measurement of ethanol concentration. Each sample was assayed in triplicate and the ethanol assay had to be completed on the day of the experiment because loss of ethanol in the samples was apparent if they were kept overnight.

The yeast alcohol dehydrogenase solution was freshly prepared. A concentration of 0.1 mg/ml was required per reaction mixture. This enzyme will catalyse the oxidation of alcohols other than ethanol. These alcohols (e.g. propanol, butanol, ethylene glycol) interfere with the quantitative detection of ethanol; therefore it is important that the room where the assay is being performed is free of all possible alcohol contaminants. A reagent blank is prepared and any change in absorbance in the reagent blank is subtracted from the change in absorbance in the samples.

The NAD^+ (grade lll) was mixed with Tris/lysine buffer and deionised water within an hour before carrying out the ethanol assay, to prevent increased absorbance due to the decomposition products of NAD^+ (Lowry & Passonneau, 1972) .

The ethanol content of samples was calculated from the spectrophotometric measurement of NADH, using 6220 $mol^{-1}\,l\,cm^{-1}$ as the molar absorption coefficient of NADH.

2.10 ACETALDEHYDE ASSAY

Acetaldehyde (AcH) was enzymically measured using an automated distillation and fluorometric technique developed by Stowell *et al.* (1978). Acetaldehyde is oxidised to acetate by yeast aldehyde dehydrogenase with production of NADH. NADH is then measured by fluorescence, with excitation at 350 nm and emission at 460 nm.

2.10.1 Standard Acetaldehyde Solutions

Glassware (beakers, volumetric flasks, pipettes) and distilled water were kept cold for preparing stock and standard solutions of acetaldehyde. Because acetaldehyde is very volatile any preparation should be performed in the cold room (4 °C).

The acetaldehyde stock solution was prepared by a 1/100 dilution of re-distilled acetaldehyde. This solution was stable for several months at 4 °C.

A further 1/100 dilution into ice-cold distilled water provided a suitable acetaldehyde stock solution for preparing standards for the fluorometer in a concentration range of 5 μM to 150 μM.

2.10.2 Assay Reagents

Stock solutions of the 100 mM pyrophosphate buffer adjusted to pH 8.0 using 10 M HCl, were kept in the fridge. One litre was enough for approximately 90 assays.

KCl (analytical grade) at a concentration of 700 mM was included in the NAD^+ solution (1.5 mM). This solution was freshly prepared. 100 ml of the NAD^+/K^+ mixture catered for 100 assays.

The enzyme solution was made on the day of the experiment. Yeast aldehyde dehydrogenase was dissolved in 0.1 M pyrophosphate buffer containing 0.1% (v/v) ß-mercaptoethanol and KCl (700 mM). An enzyme concentration of 0.4 units/ml provided a satisfactory standard curve. The enzyme mixture was kept cold throughout the assay. Approximately 18 ml of enzyme was enough for 50 assays.

2.10.3 Measurement

1 ml of acidic supernatant was diluted to 1.5 ml using ice-cold distilled water, to provide enough sample for measurement of acetaldehyde. The acetaldehyde in diluted supernatants had to be measured on the day of the experiment. They were stored in sealed autoanalyser cups at 4 °C until required for assay. The assay involved diffusion of acetaldehyde from the perchloric acid extracts into an enzyme, buffer and NAD^+ mixture. The acetaldehyde content of samples was calculated from the fluorometric measurement of NADH, using a standard curve produced with acetaldehyde solutions in a suitable concentration range.

2.10.4 **Maintenance of Apparatus**

The autoanalyser tubing was cleaned by pumping 0.1 M NaOH through the fully automated system for approximately 2 hours. This was followed by a continual wash through with deionised water.

The glass condensor, acetaldehye distillation manifold and 40 ft delay coil were cleaned by an acid wash of 50% nitric acid.

The plastic joints and tubing had to be regularly checked for deterioration and were replaced if necessary.

2.11 **ATP ASSAY**

ATP was assayed enzymatically by the method of Lamprecht and Trautschold (1974) as modified by Lund *et al.* (1975). Hexokinase phosphorylates glucose with ATP in the presence of Mg^{2+} to give glucose-6-phosphate. Glucose-6-phosphate dehydrogenase then catalyses the oxidation of glucose-6-phosphate with $NADP^{+}$. NADPH produced in the coupled reaction is measured via absorption at 340 nm.

The concentration of ATP was measured using a neutralised sample. After samples of the acidic supernatant were removed for determination of ethanol and acetaldehyde concentrations, the remaining cell extract was adjusted to pH 6.5-7.0 with 0.3 M KOH, using universal indicator solution for visual indication of pH change.

ATP content must be measured on the day of the experiment because loss of ATP occurs in the sample if kept overnight. Since 1 mole of NADPH is produced per mole of ATP, the ATP concentration can be calculated using the molar absorption coefficient of NADPH (6200 mol^{-1} l cm^{-1}).

CHAPTER 3

DEVELOPMENT OF A METHOD FOR MEASUREMENT OF SMALL CHANGES IN RATES OF ETHANOL OXIDATION

3.1 BACKGROUND TO PRESENT WORK

The modulation methodology developed by Kacser & Burns (1973) can be used to determine the flux-control coefficients of individual steps in a metabolic pathway. The modulation approach involves altering the activity of a particular enzyme by small amounts and measuring the effect this alteration has on the flux through the metabolic system. For this to be successful a method capable of detecting small changes in flux is required.

In 1986, I developed a method for measurement of small changes in rates of ethanol clearance (Page, 1986). This method involved repeated sampling from a single incubation mixture. Samples were removed every 7 minutes for 1 hour, providing enough points on a plot of concentration against time to determine the rate of ethanol clearance for that incubation mixture.

By using the repeated sampling method and applying the method of modulation, the flux-control coefficient of ADH (C^{J}_{ADH}) in hepatocytes isolated from starved rats was evaluated (Page, 1986). The activity of ADH was altered by small amounts using an ADH-specific inhibitor, tetramethylene sulphoxide (TMSO). A range of TMSO concentrations was tested (Table 3.1).

The results using this repeated sampling method (Table 3.1) emphasised the need for a new procedure in measurement of small changes in flux. Firstly, the reproducibility between experiments (as shown by the percentage inhibition values) decreased with decreasing inhibitor concentration. Secondly, at the lowest inhibitor concentration (0.05 mM) the flux was inhibited by approximately 17%. Ideally smaller changes in flux (less than 10%) need to be measured so that the initial slope (see section 1.4) on the inhibition curve may be determined more accurately and hence a more accurate value for the flux control coefficient can be calculated.

The results obtained with the repeated sampling method proved that evaluation of the C^{J}_{ADH} using the modulation approach could be accomplished. However the procedure for incubation and sampling of isolated hepatocytes was lengthy (approximately 1.5 hrs) and a maximum of only 3 inhibitor concentrations could be used per experiment. Most importantly the repeated sampling method had reached the limits of sensitivity and reproducibility.

TABLE 3.1

Percentage Inhibition of Ethanol Clearance Using Varying Concentrations of Tetramethylene Sulphoxide

[TMSO] mM	% Inhibition *	Averaged % Inhibition
0.05	12.20; 13.60; 24.10	16.60
0.15	18.70; 31.30; 32.50	27.50
0.20	27.25; 30.77; 36.00	31.24
0.40	45.10; 49.00; 52.00	49.70
0.60	61.27; 63.40; 67.60	64.10

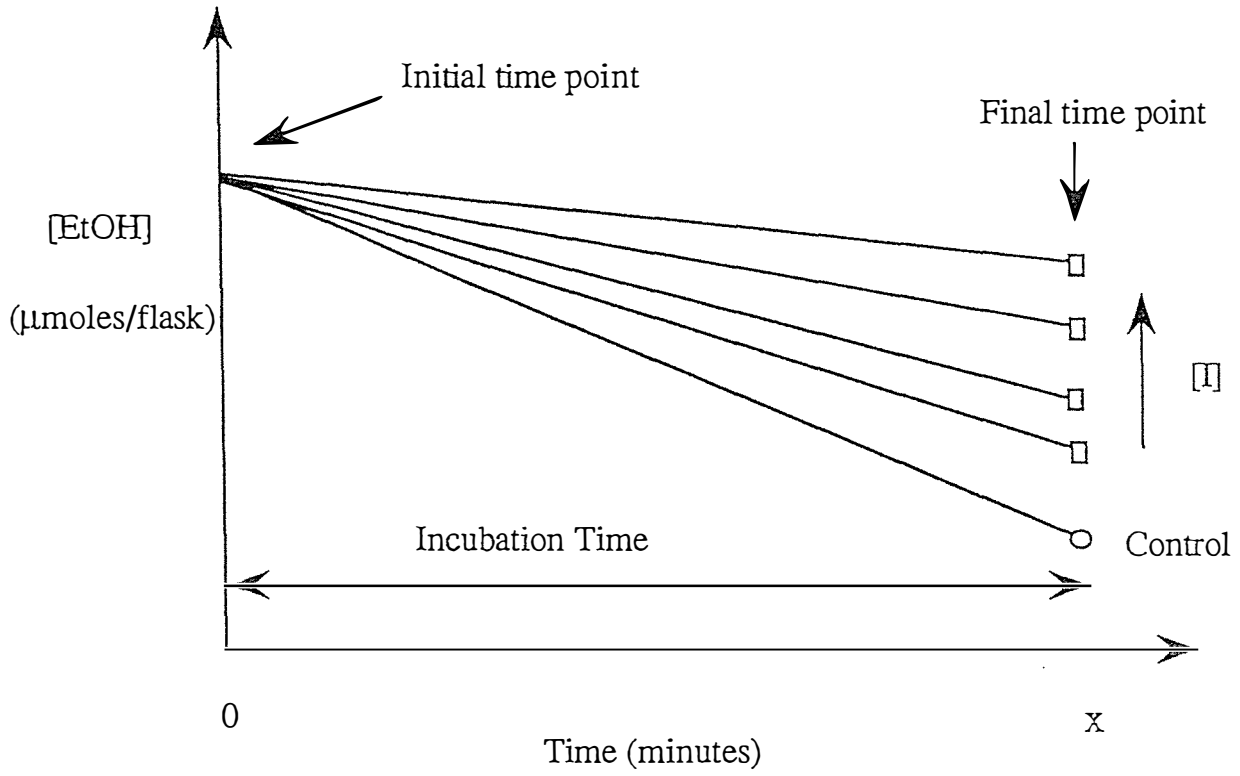
These results are from Page,1986.
* One set of experiments was performed with the higher TMSO concentrations (0.2mM to 0.6mM) and one set of experiments used the lower concentrations of TMSO (0.05mM and 0.15mM). Each set was carried out in triplicate.

A new procedure had to be developed for the sampling and incubation of isolated hepatocytes, with emphasis on improvement in the measurement of small changes in flux as well as providing a high degree of reproducibility between experiments. These two factors would increase the accuracy of the evaluated flux control coefficient (C_E^J).

The method should also be capable of examining a greater range of inhibitor concentrations in a single experiment as well as being less time-consuming. The method proposed involved using the ethanol concentration ([EtOH]) at two time points for determination of ethanol clearance rates. An initial time point represented the [EtOH] at the beginning of incubation (used for both the inhibited and noninhibited rates) and a final time point represented the concentration of ethanol at the end of incubation (where the [EtOH] is dependent on the amount of inhibitor present). I had already established, using the repeated sampling method, that ethanol clearance was linear with time under the experimental conditions to be used. Therefore the use of two time points for calculation of rates of ethanol oxidation was reasonable. The expected results using this two time point method are depicted in Figure 3.1, where with increasing inhibitor concentrations a decrease in rates of ethanol clearance is observed.

Figure 3.1

Graph of Expected Results Using the Two Time Point Method



During the developement of this new method for measurement of small changes in flux, the inhibitor TMSO was utilised. This allowed the improvement in detection of inhibition of flux to be followed, using the percentage inhibition results from the repeated sampling method (Table 3.1) as a guideline.

3.2 TWO TIME POINT METHOD

3.2.1 Procedure for Addition of Cell Suspension to Flasks and Incubation of Isolated Hepatocytes

Figure 3.2 demonstrates the procedure finally developed for the inhibitor titration studies.

The method involved the transfer of 1 ml of cell suspension (using a P_{1000} Gilson Pipette) every 45 seconds to an incubation flask (10 ml glass-stoppered glass erlenmeyer flask). All flasks contained ethanol at a starting concentration of 13 mM. The initial time samples and final time samples that did not contain inhibitor represented the control samples. All other final time flasks contained inhibitor at their respective concentrations.

Each sample was gassed with 95% O_2 : 5% CO_2 for 20 seconds and then incubated at 37 ^oC in a Citenco shaking water bath.

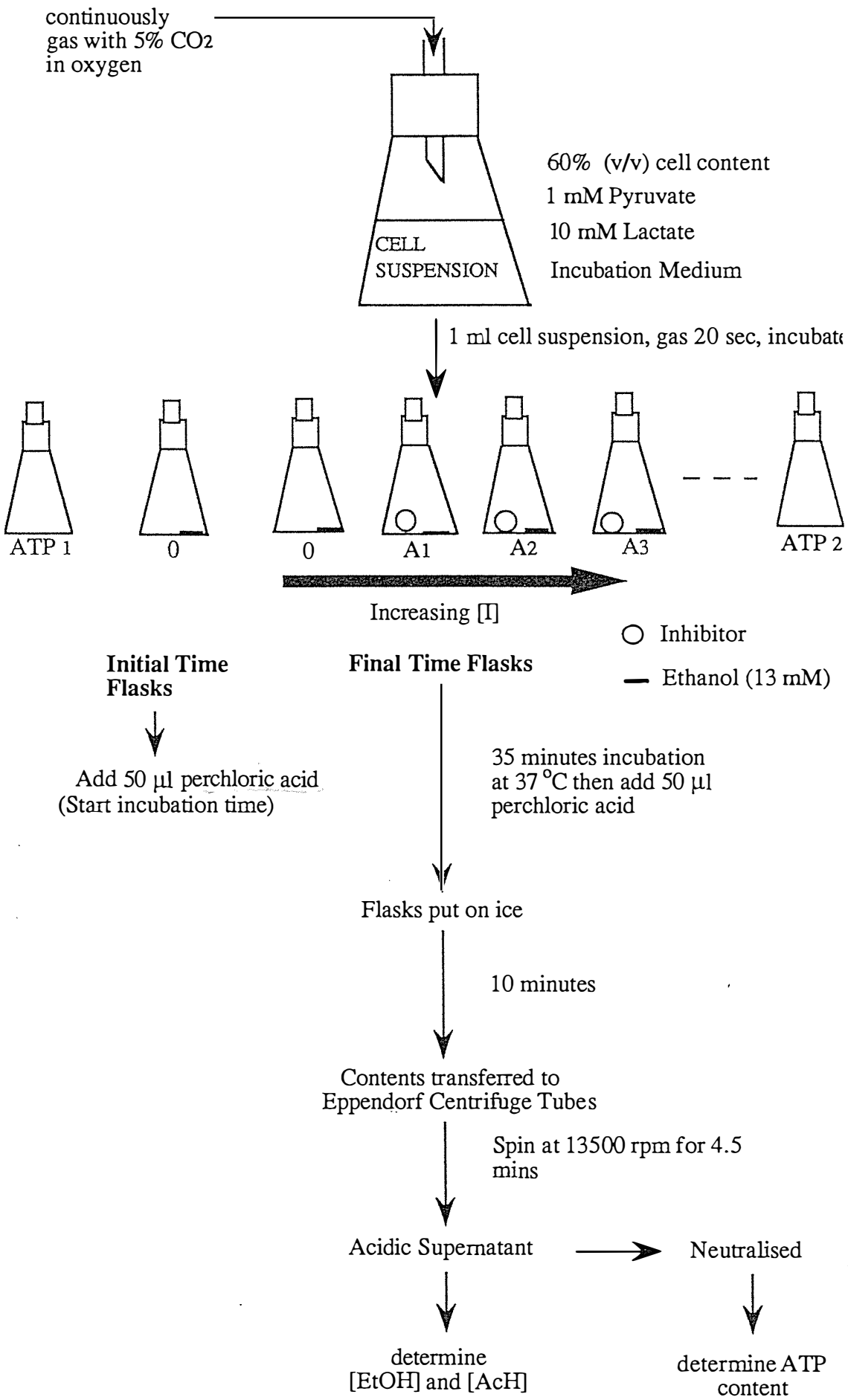
A 1 ml sample of the cell suspension was removed at the beginning (ATP_1) and end (ATP_2) of adding cell suspension to the flasks for determination of ATP content to ensure that the cells had remained viable through the sampling period.

All incubations were carried out in triplicate. This increased the accuracy of the determination of ethanol concentration for each inhibitor concentration.

Once all samples were incubating 50 µl of 60% (v/v) $HClO_4$ was added to the initial time flasks. This marked the starting time for the 35 minute incubation period for all other samples. At the end of the incubation period the reactions were stopped by addition of 50 µl 60% (v/v) $HClO_4$, with immediate mixing using a Cenco vortex. The flasks were put on ice for 10 minutes, then the contents were transferred to eppendorf centrifuge tubes and centrifuged in a microcentrifuge (model 5414) at 13500 rpm for approximately 4.5 minutes.

The acidic supernatant was then used for measurement of ethanol and acetaldehyde concentrations.

Figure 3.2: **EXPERIMENTAL PROCEDURE FOR TWO TIME POINT METHOD**



The survival of the cells in the flasks during the 35 minute incubation was determined by calculation of the ATP content (see section 2.11). ATP content is a sensitive measure for oxygen depletion of the cells and loss of ATP is in fact the first measurable sign of deterioration of metabolic performance and cell integrity (Krebs *et al.*, 1974; Cornell, 1983b). Incubations where cells had not remained viable ([ATP] < 2 μmole/g cells) were not used for determination of ethanol clearance rates.

Details of how the above method for addition of cell suspension to flasks and incubation of the isolated hepatocytes was developed are explained in section 3.3.

3.2.2 Calculation of Ethanol Clearance Rates

The ethanol concentrations ([EtOH]) measured for the control samples were important. These values were used not only for determination of the control or uninhibited rates of ethanol oxidation but also for the inhibited rates of ethanol oxidation.

For a large experiment, transfer of cell suspension to the flasks usually took 10 to 15 minutes; only after this period was perchloric acid added to the initial time flasks.

To test for ethanol disappearance during this time in inhibitor-containing incubations, hepatocyte suspensions containing ethanol plus low and high concentrations of inhibitor were incubated for the same time as hepatocyte suspensions without inhibitor (Table 3.2). The results show that the ethanol concentrations for the incubations containing TMSO and IsB were greater than for the 15 minute incubations without inhibitor. Therefore the actual value obtained for the initial time samples can not be used directly in determination of the ethanol clearance rates in the presence of inhibitors.

In view of this finding, it would have been appropriate to use an initial time sample for each inhibitor concentration. However the amount of cells collected did not allow initial time samples as well as final time samples in triplicate for the desired number of inhibitor concentrations.

TABLE 3.2

Amount of Ethanol Present in Control and Inhibitor Samples Incubated for the Same Time.

Incubations		Incubation Time (mins)	[EtOH] µmole/flask
Control	No inhibitor	0	12.22 ± 0.20
		15	11.46 ± 0.30
TMSO *	0.05 mM	15	11.54 ± 0.24
	0.60 mM	15	11.90 ± 0.30
IsB *	0.06 mM	15	11.46 ± 0.25
	0.55 mM	15	11.83 ± 0.18

*TMSO (Tetramethylene sulphoxide) and IsB (Isobutyramide) are noncompetitive inhibitors of ADH. The unincubated control samples were prepared by having $HClO_4$ present in the flask before addition of cell suspension. The rest of the samples were prepared by the method explained in the text.

It was therefore decided to use the ethanol concentrations ([EtOH]) of the uninhibited samples (see Figure 3.2) to calculate an ethanol concentration at the true zero time to use in determination of the inhibited rates. The slope of the two uninhibited time points (initial and final time samples) was extrapolated back to the true zero time (Figure 3.3). The [EtOH] at true zero time was then used in calculation of the uninhibited and inhibited ethanol clearance rates. Ethanol oxidation rates (µmole/min/g cells) were calculated by dividing the slope of the line (µmole ethanol utilised per minute in the total incubation) by the g wet wt of cells.

The ethanol concentration for the true zero time point obtained by extrapolation of the slope of the control rate and that determined experimentally by addition of $HClO_4$ to control incubations before the addition of cell suspension were compared (Table 3.3). There was no significant difference between the extrapolated and experimental ethanol concentration at true zero time.

Figure 3.3: **Extrapolation of Control Rate for Determination of theTrue Zero Time Ethanol Concentration**

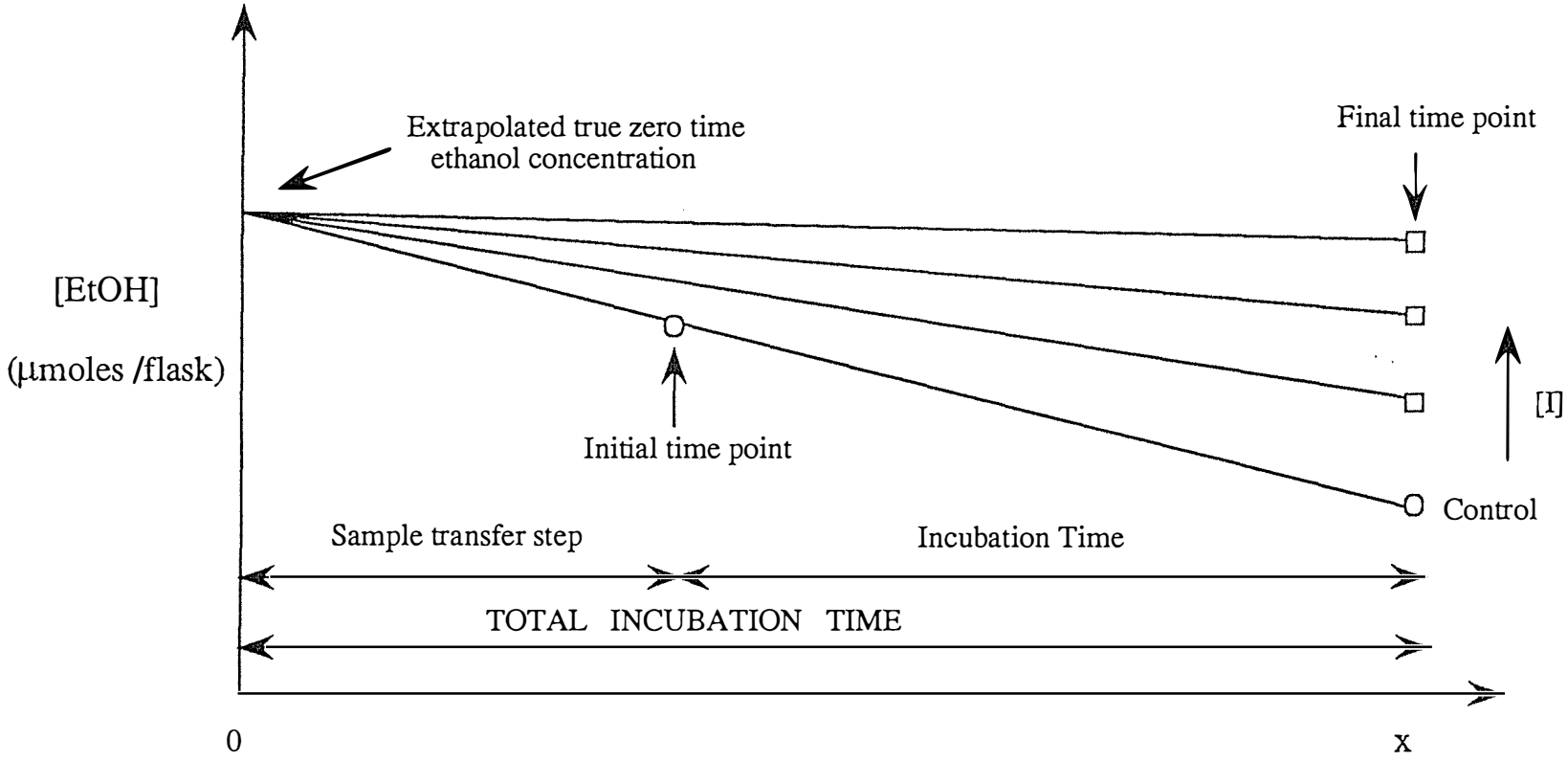


TABLE 3.3

Comparison of the Experimental and Extrapolated Ethanol Concentrations at True Zero Time.

	Name		Incubation Time (min)	[EtOH] µmoles/ml
+	Experimental True Zero Time		0	13.16 ± 0.16
*	Control Time Points	Initial	14	12.75 ± 0.08
		Final	35	12.09 ± 0.15
	Extrapolated True Zero Time			13.01

+ 1 ml samples (in triplicate) of cell suspension were transferred to 10 ml erlenmeyer flasks containing 50 µl 60% (v/v) $HClO_4$. The acidic supernatant was used to determine the the experimental [EtOH] at true zero time.

* The initial and final time samples (in triplicate)were obtained by the procedure explained in the text (see section 3.2.1). Extrapolation of the control rate to the true zero time provided the extrapolated [EtOH].

The extrapolated [EtOH] at true zero time would be the more appropriate value to use for calculation of rates of ethanol oxidation , than the experimental ethanol concentration at zero time because:

1) There is a limited volume of hepatocyte suspension.

and 2) The extrapolated [EtOH] at true zero time is determined from samples which have undergone the same conditions as those containing inhibitor.

Because the initial and final time samples were treated in triplicate it was important to assess whether the mean value for the ethanol concentration of the control samples should be used in the calculation of ethanol clearance rates. Table 3.4 compares the extrapolated [EtOH] at true zero time using the averaged ethanol concentration of the control samples and the extreme values of the triplicates. The extreme values are obtained using the upper and lower ethanol concentrations of the triplicates (see Table 3.4). The extrapolated [EtOH] and the percentage inhibition results using either the extreme or averaged extrapolated [EtOH] did not differ significantly therefore the results indicate that the averaged [EtOH] for the initial and final time points could be used for determination of the [EtOH] at true zero time.

For all samples (inhibited or control) the averaged ethanol concentration was used in the calculation of ethanol clearance rates.

TABLE 3.4

Comparison of the % Inhibition Results Obtained Using the Averaged and the Extreme Extrapolated True Zero Time Samples

	Control Flasks [EtOH] µmole / ml				Extrapolated True Zero Time [EtOH]			Inhibition Results			
			Average					[IsB]	% inhibition		
Experiment	initial time	final time	initial time	final time	A+	B++	C*	mM	A	B	C
	11.13	9.69									
1	10.96		11.01	9.63	11.90	11.59	11.70	0.05	6.09	7.10	6.80
	10.95	9.56						0.10	26.40	30.60	29.00
	10.93	8.38									
2	10.98	8.44	10.98	8.40	12.20	12.00	12.10	0.05	3.80	3.98	3.90
	11.04	8.39						0.10	26.90	28.40	27.60

IsB = Isobutyramide, an ADH inhibitor. In experiment 1 one of the three final time incubations had an ATP content < 2 µmole/ g cells; hence it was not used in the determination of the extrapolated [EtOH]

\+ (A) - extrapolated [EtOH] using the highest initial time value and lowest final time value

++ (B) - extrapolated [EtOH] using the lowest initial time value and highest final time value

* (C) - extrapolated [EtOH] using the average initial time and final time values

3.3 DEVELOPMENT OF THE TWO TIME POINT METHOD

Perseverance to improve this method to the stage finally outlined in the previous section proved worthwhile. For each experiment the weight of cells (grams) required to test a large range of inhibitor concentrations was small (eg.in total < 2 g) and enough results were obtained for the calculation of a flux control coefficient. However , development of this method was difficult and time-consuming. It involved investigation into the contents of the cell suspension , the procedure for addition of ethanol, the length of the incubation period, the method for removal of aliquots from the cell suspension with subsequent gassing of the samples, and the type of incubation vessel to be used. Details of key experiments in the development of the two time point method are given in this section.

3.3.1 Incubation Mixture and Cell Suspension

To help reduce the amount of isolated hepatocytes required for an experiment , an incubation mixture of volume 1 to 2 ml was decided upon for the two time point method. This contrasts with the 4 ml volume traditionally used in individual incubations (Cornell *et al.,* 1973; Krebs *et al.,* 1974).

The incubation mixture contained ethanol at an initial concentration of 13 mM , the substrates lactate (10 mM) and pyruvate (1 mM), isolated hepatocytes, K-H buffer containing 2.5% BSA and inhibitor when required.

For the two time point method , aliquots from a large volume of cell suspension were transferred to an incubation vessel (see Figure 3.2). Investigation into the composition of the incubation mixture (cell suspension plus contents of the incubation vessel) was required.

3.3.1.1 *Lactate and Pyruvate*

The substrates lactate and pyruvate were combined with the total cell suspension at the appropriate concentration ratio (10:1) rather than put in the individual incubation vessels as described by Cornell *et al.* (1973).

The cell suspension with substrates was continuously gassed (5%CO_2 : 95%O_2) until needed. During this waiting period, the concentration of metabolites (malate, aspartate and glutamate) lost during the preparation of the isolated hepatocytes (Cornell *et al.,* 1974, 1979; Krebs *et al.,* 1974; Crow *et al.,* 1978) would be restored. Introduction of lactate and pyruvate into the cell suspension increased the metabolic activity of the cells. Table 3.5 shows that cells suspended

either with or without substrates and continually gassed in a 250 ml erlenmeyer flask remain viable (ATP content > 2 μmole/g wet wt cells) for up to 40 minutes. Therefore addition of the substrates lactate and pyruvate to the cell suspension would not be detrimental to the survival of the cells during the period where aliquots of cell suspension are transferred to the incubation vessels.

The following points show that the substrates lactate and pyruvate are an important addition to the incubation mixture, for the study of ethanol metabolism.

1) Incubation of the isolated liver cells with lactate restores the metabolism of the freshly prepared hepatocytes to that of the intact liver (Cornell *et al.*, 1973; Krebs *et al.*, 1974; Crow *et al.*, 1978).

2) The substrate combination of lactate (10 mM) and pyruvate (1 mM) produces maximal rates of ethanol oxidation (Crow *et al.*, 1978) which are similar to those observed *in vivo* (Crow *et al.*, 1977a).

3) The results in Table 3.5 show that the survival of the cells during incubation was enhanced by the presence of lactate and pyruvate. This is important because physiological responses will be produced by cells that are metabolically viable (ATP > 2 μmole/g) at the end of the incubation period.

3.3.1.2 *Ethanol*

There were two possibilities for the introduction of ethanol into the incubation mixture and thereafter measurement of rates of ethanol clearance. The ethanol could be placed in the individual incubation vessels and the cells then added, or ethanol could be added to the cell suspension before pipetting into the incubation vessels.

The protocols for these two methods were as follows :

1) For the first method a suspension of cells, substrates, and incubation medium was prepared and continuously gassed with O_2 + CO_2 (19:1) humidified by distilled water. 13 μl of 1 M ethanol was placed in the incubation flask using a P_{20} Gilson pipette. 1 ml aliquots of the cell suspension were transferred to the incubation vessels, gassed and then incubated.

TABLE 3.5

The Viability of Cells Suspended With or Without Lactate and Pyruvate.

Samples		Viability	
		ATP Content (µmole/ g)	
	Time of Removal	No Substrates	Substrates
*	10 min	3.15	3.60
Room	20 min	3.80	3.71
Temp.	30 min	2.58	3.93
20 ° C	40 min	3.60	4.27
	Incubation Time		
**	10 min	2.44	2.81
Incubated	20 min	2.31	2.53
at	30 min	2.25	2.53
37 ° C	40 min	1.63	2.36

Two 250 ml erlenmeyer flasks were prepared . Both flasks contained 25 ml cells. The substrates lactate (10 mM) and pyruvate (1 mM) were added to one flask in a concentration ratio of 10:1. The cell suspension was made up to a total volume of 25 ml with suspension medium (see section 2.3.2). The flasks were continuously gassed with 5% CO_2 in O_2 at room temperature. The ATP content of the original cell preparation was 3.60 µmole/g cells.

* 1 ml samples (in duplicate) were transferred from each cell suspension at 10, 20, 30, and 40 minutes to 10 ml erlenmeyer flasks containing 50 µl 60% $HClO_4$, and mixed instantly (see section 2.11).

** 1 ml samples from each cell suspension (in triplicate) were transferred to 10 ml erlenmeyer flasks and incubated for 10, 20, 30, and 40 minutes. At the end of incubation , 50 µl of 60% $HClO_4$ was added. The acidic supernatant was neutralised and ATP content was determined.

2) For the second method simultaneous addition of ethanol and cells required a cell suspension containing ethanol at a starting concentration of 13 mM , incubation medium , substrates and cells. The cell suspension was continuously gassed with 95% O_2: 5% CO_2 bubbled through a 13 mM ethanol solution. This ensured that the ethanol concentration in the suspension would remain at 13 mM until it was incubated. The procedure involved removal and transfer of 1 ml aliquots of cell suspension to the incubation vessels, plus further gassing with 5% CO_2 in O_2, bubbled through 13 mM ethanol solution, before incubation.

To compare the two methods for introduction of ethanol into the incubation mixture, the procedures were examined without the presence of cells and the following factors were kept the same: the incubation vessel (glass-stoppered test tubes), sample removal from the incubation mixture (a 1 ml aliquot was transferred to each vessel every 30 seconds, gassed for 10 seconds and then incubated for 30 minutes) and addition of 50 µl 60% (v/v) $HClO_4$ for termination of the incubation period.

TABLE 3.6

Ethanol Content Present Before and After Incubation Using Method 1 and 2 for Ethanol Transfer

Method 1				Method 2			
Samples	Incubation Time	No. Samples	[EtOH] mM	Samples	Incubation Time	No. Samples	[EtOH] mM
A	0 min	4	11.09 ± 0.12	C	0 min	4	11.05 ± 0.08
				D	0 min	4	11.05 ± 0.16
B	30 min	4	11.11 ± 0.12	E	30 min	3	10.20 ± 0.22

Methods 1 and 2 are explained in the text. 50µl 60% $HClO_4$ was added immediately after 10s gassing for the unincubated samples (A,C,and D). A and C samples were removed from the solution before those to be incubated (B and E). The unincubated samples (D) were removed from the solution after the incubated samples (E) were removed.

For method 2 the concentration of ethanol in the initial incubation mixture did not alter during removal of the aliquots (compare C and D in Table 3.6) however ethanol seems to have been lost during transfer of the sample or incubation, as shown by the samples incubated for 30 minutes (E in Table 3.6). The disappearance of ethanol did not occur during incubation because the [EtOH] of the incubated samples of method 1 remained the same as the unincubated samples (compare A and B in Table 3.6). Therefore loss of ethanol must have occurred during transfer of the aliquot from the incubation mixture to the vessel. Another problem with method 2 was possible contamination of the laboratory surroundings with ethanol. The ethanol assay (see section 2.9) is very sensitive and ethanol present in the laboratory air will cause interference.

The results using methods 1 and 2 (Table 3.6) indicate that 1 ml aliquots of a cell / substrate suspension should be added to an incubation vessel containing ethanol rather than introducing ethanol combined with the hepatocyte suspension into the incubation vessel. In view of these results, method 1 was finally accepted as the best procedure for introduction of ethanol into the incubation mixture.

3.3.1.3 *Cell Content*

With past methods for measurement of ethanol clearance rates using individual incubations the incubation mixture contained 2 ml of hepatocyte suspension plus 2 ml of additions (substrates, effectors, and suspension medium). Hence the original isolated liver cell suspension made up 50% (v/v) of the final incubation mixture. There was approximately 17 to 25 mg wet wt of cells per ml of incubation mixture and the period of incubation was about 1 hour.

Initially, for the two time point method, a cell suspension containing the substrates lactate (10 mM) and pyruvate (1 mM), suspension medium and isolated hepatocytes (50% v/v) was prepared. The uninhibited rate of ethanol oxidation was around 0.020-0.030 μmole/min (see Table 3.7) compared to approximately 0.052 μmole/min observed by other research groups (Crow *et al.*, 1977a, 1978, 1983a; Cornell, 1983a). However the amount of cells present per ml of incubation mixture was only 8 to 15 mg wet wt, rather than 17-25 mg wet wt. It was decided that the low wet weight of cells could be due to insufficient removal of suspension medium during the final centrifugation step after washing the hepatocyte preparation.

Detection of small changes in flux produced by addition of inhibitor would be easier if larger differences in the rate of ethanol oxidation were produced, leading to greater changes in the concentration of ethanol. It appeared from the above results that to produce greater changes in the rate of ethanol oxidation the amount of cells per ml of incubation mixture had to be increased. To

do this, the technique for washing the isolated hepatocytes was improved and the volume of hepatocyte suspension per ml of incubation mixture was increased.

The results in Table 3.7 (60% (v/v) cell content) show that by improving the washing technique and increasing the hepatocyte content of the incubation mixture from 50% (v/v) to 60% (v/v), the wet wt of cells per ml of incubation mixture, and the rate of ethanol oxidation are increased to values comparable to those of past methods (Crow *et al.,* 1977a, 1978, 1983a; Cornell, 1983a).

The greater changes in ethanol concentration obtained with increased cell content (Table 3.7) were easier to measure and made the detection of small changes in flux less difficult.

To aid cell survival with the higher cell content per flask, the incubation period for all final time flasks (see Table 3.7) was decreased from 45 minutes to 35 minutes (i.e. total time of incubation dropped from 60 to 50 minutes). The rates of ethanol oxidation obtained for this period of incubation were still large enough for small changes in rate with the introduction of inhibitor to be detected (see Table 3.7).

3.3.2 Transfer of Cells to the Incubation Vessel

It was important that cell viability was maintained through all stages of the inhibitor titration studies.

The apparatus used for transferring cells to an incubation vessel could increase the incidence of cell damage, leading to non viable cells. Previously , detipped 2 ml glass pipettes have been used (Cornell *et al.,* 1973; Crow *et al.,* 1977a,1978) to transfer cells to the incubation vessel. The bore size (diameter 3 mm) of the detipped pipette was large enough not to damage the cell membranes, and cell viability was maintained. However this method for cell transfer was cumbersome.

An automatic pipette, such as the P_{1000} Gilson Pipetman would make the cell transfer step easier, quicker and more accurate; however the force at which the cells were drawn up and the diameter (1mm) of the C_{200} Gilson tip opening were factors that could affect the viability of the cells.

The metabolic performance and cell integrity of the hepatocytes removed by the 2 ml detipped glass pipette and the P_{1000} Gilson pipette were compared (Table 3.8). The ATP content , % viability and cell integrity all indicated that the P_{1000} Gilson Pipetman was just as good as the 2 ml detipped glass pipette and could be used for the transfer of cell suspension.

TABLE 3.7

Results of Ethanol Utilisation for Incubation Mixtures Containing 50 or 60 % of the Original Hepatocyte Suspension

	50% (v/v) Hepatocyte Content		60% (v/v) Hepatocyte Content			
	Change in [EtOH] mM	Rate µmole/min	Change in [EtOH] mM	Rate µmole/min	Change in [EtOH] mM	Rate µmole/min
Control	1.4	0.0237	1.97	0.0392	2.48	0.0498
0.1 mM TMSO	1.32	0.0224	1.92	0.0378	2.36	0.0474
0.2 mM TMSO	1.19	0.0187	1.54	0.0302	1.86	0.0374
0.4 mM TMSO	0.80	0.0102	1.17	0.023	1.54	0.0310
Total Incubation	60 minutes		50 minutes		50 minutes	
Cells (mg/ml)	10		17.8		20	

Cell suspensions containing 50 or 60% (v/v) hepatocyte suspension were prepared. Every 45 seconds 1 ml samples were removed and transferred to 10 ml erlenmeyer flasks containing EtOH (13 mM) at the appropriate concentration. The samples were gassed (5% CO_2 in O_2) for 20 seconds then incubated. Reactions were stopped by addition of 50 µl 60% (v/v) $HClO_4$. The acidic supernatant was used for determination of ethanol content (see section 2.9). µmole ethanol utilised per min was calculated as described in the text (see section 3.2.2).

For subsequent experiments, the P_{1000} automatic pipette was used for transfer of 1 ml of cell suspension and the P_{5000} (bore size of C_{6000} tip = 2mm) was used for transfer of 2 ml of cell suspension to the incubation vessels.

TABLE 3.8

Metabolic Viability and Cell Integrity For Cells Transferred by a P_{1000} Gilson Pipetman or 2 ml Detipped Glass Pipette.

		Method of Cell Transfer	
		P_{1000}	2 ml Glass Pipette
Metabolic Viability	ATP Content (μmole/g cells)	2.43	2.42
Cell Integrity	% viability viewed cells	92 membranes intact small clumps	90 membranes intact few clumps

Once the isolated hepatocytes were collected , 1 ml of cells was removed by the P_{1000} or 2 ml wide tipped pipette. 50 μl of this sample was tested for cell integrity using the Trypan blue exclusion test (see section 2.6.1). A further 1 ml of cells was removed by each pipette (in duplicate), placed in 1 ml of K-H buffer / 2.5% BSA and 100 μl 60% (v/v) $HClO_4$, mixed instantly and put on ice. The acidic supernatant was neutralised and used for determination of ATP content (see section 2.11).

3.3.3 Cell Suspension Transfer and Gassing Procedure

To start incubations, an aliquot of cell suspension was transferred to an incubation vessel which contained ethanol (13 mM) with or without inhibitor. The time taken for transfer of cell suspension was 45 seconds, which provided enough time to remove the aliquot, transfer it to the incubation vessel, gas the sample with 0_2 : CO_2 (19:1), place the vessel in the shaking water bath and then be prepared for removal of the next aliquot. Timing of sample removal was very important for calculation of ethanol elimination rates (see section 3.2.2) by this two time point method.

Once the cell suspension aliquot was transferred to the incubation vessel, it was gassed for 20 seconds to ensure that the cells remained viable during the incubation period of 35 minutes. The 20 second gassing step causes a certain amount of ethanol to be lost (Table 3.9). As long as the gas flow rate and the duration of gassing remained the same for each flask this would not cause any problems with differences being introduced between flasks. The drop in ethanol concentration was reproducible in a series of incubation mixtures as indicated by the low standard errors in Table 3.9.

TABLE 3.9

Loss of Ethanol During the 20 second Gassing Step

Name of Sample	No. of Samples	Average [EtOH] (mM)
Initial Time (nongassed)	6	12.580 ± 0.004
Initial Time (gassed)	6	11.130 ± 0.050
Final Time (gassed)	6	11.080 ± 0.120

The initial time (nongassed) samples were prepared by adding 1 ml of suspension medium to flasks containing 13 mM EtOH and 50 µl $HClO_4$. The gassed samples were prepared by adding 1 ml of suspension medium to flasks containing 13 mM EtOH , and then gassing for 20 s. The final time samples were incubated at 37 °C and at the end of 30 minutes 50 µl 60% (v/v) $HClO_4$ was added. 50 µl 60% (v/v) $HClO_4$ was added to the initial time gassed samples straight after the 20 s gassing step.

The results in Table 3.9 also show that if the control initial time samples were to be used in the calculation of inhibited and uninhibited rates as described in section 3.2.2 then those samples had to be treated the same way as all other incubated samples. Hence the 20 second gassing was performed on all samples.

3.3.4 Incubation Vessel

Because the total incubation volume was reduced from 4 ml to 1 ml, incubation vessels smaller than the 25 ml flasks previously used (Crow *et al.*, 1977a) were required.

The first alternative incubation vessel investigated was the Sorvall centrifuge tube used for the SM24 rotor (dimensions 17 x 95 mm). These plastic centrifuge tubes were thought to be suitable since other research groups (Johnson *et al.*, 1972; Pointer *et al.*, 1976; Birnbaum & Fain , 1977; Malson *et al.*, 1978; Tolbert *et al.*, 1980; Guinzberg *et al.*, 1987) had utilised plastic test tubes

(dimensions 17 x 100 mm) for the incubation of isolated hepatocytes in a total volume of 1 to 2 ml.

An appropriate stopper had to be found for the plastic tubes. Balsa corks, rubber bungs and plastic tops were examined. The rubber bungs were hard to remove for the gassing step and for the addition of perchloric acid at the end of incubation; therefore they were eliminated. The rate of ethanol disappearance was compared for cells incubated with or without TMSO (0.6 mM) in Sorvall centrifuge tubes stoppered with balsa corks or plastic tops (Table 3.10).

TABLE 3.10

Comparison of Ethanol Disappearance Rates and Percentage Inhibition Results With Plastic or Balsa Cork Stoppers

	Balsa Cork		Plastic Top	
	EtOH Oxidation Rate (µmole / min / g)	% I	EtOH Oxidation Rate (µmole / min / g)	% I
No Inhibitor	6.01		8.14	
0.6 mM TMSO	3.40	43	4.46	45

1 ml samples were removed from a cell suspension every 45 seconds and transferred to Sorvall centrifuge tubes containing 13 mM EtOH with or without TMSO (in triplicate). The samples were gassed for 20 s and then incubated for 35 minutes. Half of the samples were incubated in plastic tubes stoppered with balsa corks; the other half were incubated in plastic tubes stoppered with plastic tops. Reactions were stopped with the addition of 50 µl 60% (v/v) $HClO_4$. The acidic supernatant was used for determination of ethanol content (see section 2.9).

The percentage inhibition results were similar and compared fairly well with those of the repeated sampling method (see Table 3.1). However high apparent ethanol disappearance rates were obtained, more so for the plastic tops, indicating that ethanol was being lost by some other means in addition to oxidation by the cells. This is explained later in section 3.3.5.

The balsa cork was finally decided upon as being the most suitable stopper for the plastic incubation tubes. However, the plastic Sorvall tubes did have some drawbacks. The placement of any component into the tube and the mixing of the incubation mixture was not visible. There was a possibility that the cells and inhibitor could be adhering to the sides of the plastic tubes.

This could have been contributing to bad reproducibility between triplicates. To overcome these complications the plastic incubation tubes were replaced by 20 ml glass stoppered (Quickfit 14/20) glass test tubes (Quickfit-MF 24/1/5) of similar dimensions (17 x 105 mm).

Further inhibitor studies using these glass test tubes showed problems with survival of the cells during the incubation period. Johnson *et al.* (1972) stated that vigorous shaking was required for aeration of their cells /substrates /medium during incubation. However vigorous shaking in this instance would be too risky. Breakage of the whole cells could occur, with metabolic viability of the cells being decreased.

Survival of the cells during the incubation period is dependent on how well the oxygen diffuses from the gas space of the incubation vessel into the medium where the cells are present. In view of this, it was decided that incubation vessels giving a larger surface area of suspension in contact with the gas phase could be better, and 10 ml erlenmeyer flasks were tested . The surface area for a 1 ml incubation mixture in 20 ml glass test tubes is 2.27 cm^2 and for 10 ml erlenmeyer flasks is 5.32 cm^2. The viability of the cells (ATP content) incubated for 35 minutes was tested for both incubation vessels (Table 3.11). In all three cell preparations the ATP content of the flasks was greater than the test tubes. These results suggested that the oxygen supply was more likely to become limiting in the test tubes because of the smaller surface area ; hence the erlenmeyer flasks would be a more reliable incubation vessel to utilise in experiments using isolated hepatocytes.

The results in Table 3.11 indicated that it was necessary to determine the ATP content of every flask at the end of the 35 minutes incubation to ensure that the cells had remained metabolically viable. For determination of rates of ethanol oxidation it was important that the cells were not oxygen depleted as this could lead to breakdown of cell membranes and hence unphysiological responses. Any samples with ATP content less than 2 µmole/ g cells were not used for calculation of ethanol clearance rates (see section 3.2.2).

3.3.5 Incubation Vessel - Experimental Loss of Ethanol

To measure the disappearance of any substrate is difficult in itself. With ethanol removal the added precaution of minimising evaporative losses of ethanol was necessary so that rates of ethanol oxidation by isolated hepatocytes could be accurately measured.

Expected rates of ethanol oxidation for hepatocytes incubated in the presence of ethanol and substrates (lactate, pyruvate) are about 4.0 µmole/ min/ g cells (Crow *et al.*, 1977a). Higher rates of ethanol clearance for metabolically viable cells can be an indication of experimental loss of

TABLE 3.11

ATP Content of Hepatocytes Incubated in Flasks and Test Tubes

	Incubation Number	ATP Content (µmole/ g wet wt cells) Cell Preparation 1	Cell Preparation 2	Cell Preparation 3
	0	2.73	2.98	3.53
A	1	2.84	2.74	3.59
	2	2.61	2.60	3.19
	3	2.50	2.46	3.16
	4	2.50	2.31	2.92
	5	2.05	2.26	2.91
B	1	2.50	2.51	3.27
	2	0.95	2.20	3.19
	3	0.40	2.11	3.16
	4	0.20	2.05	2.22
	5	0.00	1.23	2.20

Hepatocytes isolated from fed rats were incubated in either 10 ml erlenmeyer flasks (A) or 20 ml glass test tubes (B). Cells (13 mg wet wt) were suspended in 1 ml of K-H buffer /2.5% BSA , 10 mM lactate and 1 mM pyruvate. Individual flasks (A_{1-5}) or test tubes (B_{1-5}) were incubated for 35 minutes, after which 0.05 ml 60% $HClO_4$ was added. Incubation number 0 indicates the ATP content of the initial cell suspensions.

ethanol. One of the reasons for not using the Sorvall centrifuge tubes as the incubation vessels was that evaporative loss of ethanol as well as metabolic loss was evident (see Table 3.10).

The 10 ml glass stoppered erlenmeyer flasks already chosen for their suitability in increasing cell survival would be excellent incubation vessels if they could also minimise loss of added ethanol. This was extremely important for the inhibitor studies because detection of small changes in rate was required.

The 10 ml flasks have Quickfit connections which should be gastight; however to test this samples containing the same concentrations of ethanol were incubated in 10 ml erlenmeyer flasks for varying times (Table 3.12).

TABLE 3.12

Ethanol Concentration Present in Flasks for Different Periods of Incubation

Incubation Time (mins)	[EtOH] µmole/ml
0	14.58 ± 0.63
15	14.61 ± 0.21
30	14.52 ± 0.36
45	14.58 ± 0.13

All flasks contained an initial ethanol concentration of 14.6 mM . 1 ml of incubation mixture (K-H Ca^{++} / 2.5 % BSA) was transferred to each flask. The samples (in triplicate)were then incubated for 0,15,30, and 45 minutes. At the end of incubation 50 µl 60% (v/v) $HClO_4$ was added to stop reactions.The perchloric acid was present in the unincubated flasks before addition of 1 ml of incubation mixture. This confirmed the initial concentration of EtOH in all flasks. The acidic supernatant was used for measurement of ethanol content (see section 2.9).

The ethanol concentration of the sample did not alter after 45 minutes incubation (Table 3.12) indicating that there was no significant loss of ethanol by evaporation. Therefore these flasks were definitely the most suitable vessels for incubation of the cells and measurement of small changes in rate of ethanol oxidation.

3.4 CONCLUSION

The technique developed for the two time point method (addition of cell suspension to the flasks and incubation of the isolated hepatocytes) can be summarised as follows :

1) The cell suspension (60% (v/v) cell content) contained the substrates lactate (10 mM) and pyruvate (1 mM). Ethanol (13 mM) and inhibitor were placed in the incubation vessel.

2) Aliquots of cell suspension were removed (using the P_{1000} or P_{5000} Gilson Pipetman) every 45 seconds and transferred to the incubation vessel where it was gassed with 5% CO_2 in O_2 for 20 seconds.

3) At the end of transferring suspension to the incubation vessels for all samples , 50 µl 60% $HClO_4$ was added to the initial time samples; this started the 35 minute incubation period for the rest of the samples.

4) The 10 ml glass stoppered erlenmeyer flask was a suitable incubation vessel as it allowed good cell survival and prevented evaporative loss of ethanol.

5) All incubations were performed in triplicate to increase reproducibility and accuracy within the experiment and between experiments.

6) To determine the uninhibited and inhibited ethanol clearance rates an extrapolated [EtOH] at true zero time was used.

7) ATP content was measured at the beginning and end of incubation to ensure that the cells were metabolically viable throughout the incubation.

An example of results obtained using this new method for detection of small changes in flux is shown in Figure 3.4.

Results of the TMSO titration studies for hepatocytes isolated from starved rats, using the two time point method were compared with the repeated sampling method previously developed (Page, 1986) (Table 3.13).

Figure 3.4

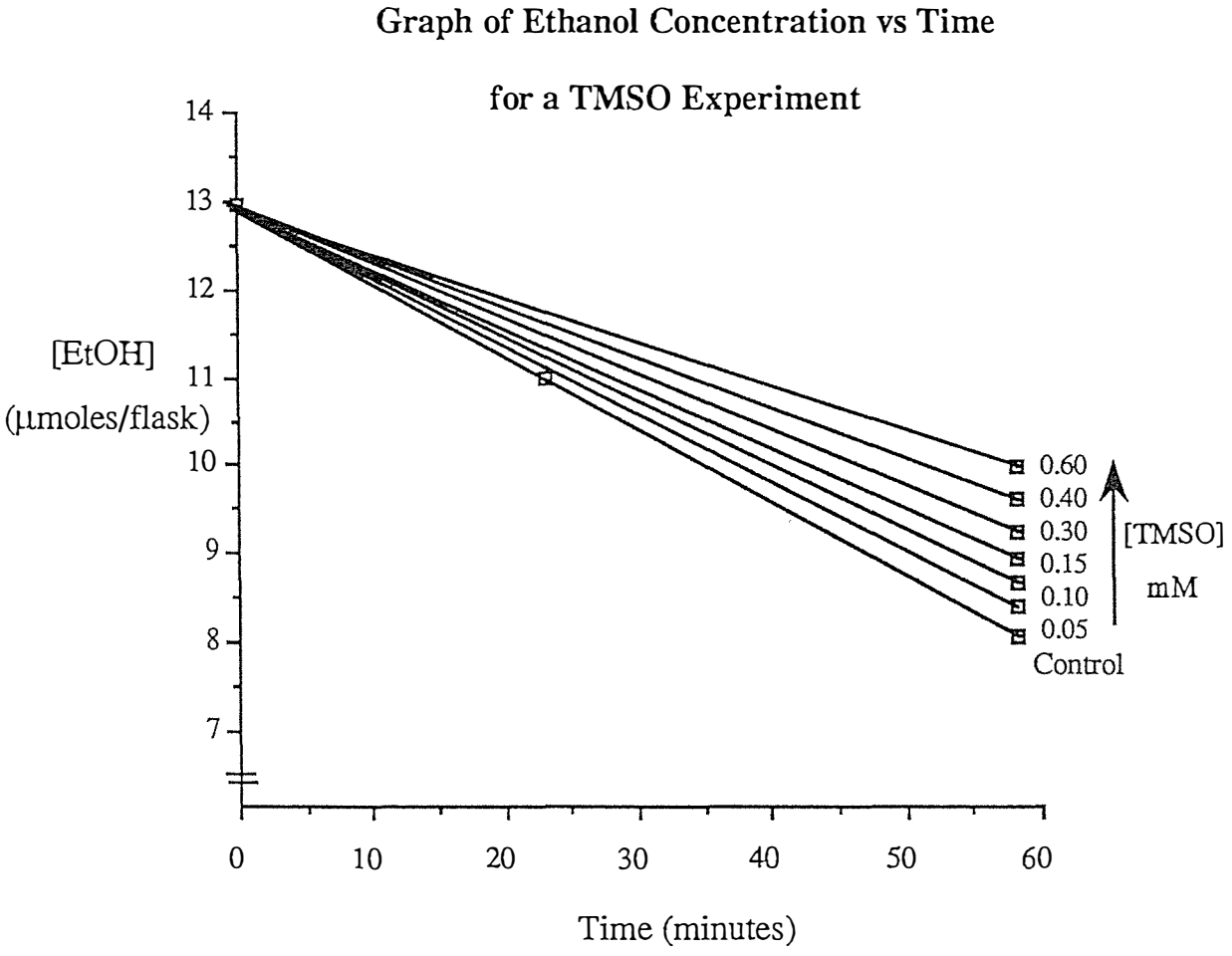

Graph of Ethanol Concentration vs Time
for a TMSO Experiment
[EtOH]
(µmoles/flask)
14
13
12
11
10
9
8
7
0
10
20
30
40
50
60
Time (minutes)
0.60
0.40
0.30
0.15
0.10
0.05
Control
[TMSO]
mM

TABLE 3.13

Percentage Inhibition of Ethanol Clearance for the Two Time Point and Repeated Sampling Methods.

Two Time Point Method		Repeated Sampling Method	
[TMSO] mM	% Inhibition	[TMSO] mM	% Inhibition
0.05	8.60 ± 2.20 (n = 4)	0.05	16.63 ± 3.75 (n = 3)
0.10	10.89 ± 1.76 (n = 10)		
0.15	17.30 ± 2.47 (n = 3)	0.15	27.50 ± 7.64 (n = 3)
0.20	21.98 ± 1.99 (n = 13)	0.20	31.34 ± 2.54 (n = 3)
0.25	23.66 ± 0.38 (n = 5)		
0.30	27.78 ± 1.30 (n = 4)		
0.40	38.20 ± 1.80 (n = 11)	0.40	49.70 ± 1.18 (n = 3)
0.60	49.90 ± 4.47 (n = 8)	0.60	64.10 ± 1.86 (n = 3)

The % inhibition results are the means ± SEM for the number of experiments indicated in brackets.

Comparing the results between the two methods, the % inhibition values obtained for the repeated sampling method are consistently higher than those of the two time point method. The most important points to note are that the two time point method is capable of detecting changes in flux of less than 10 % and that the reproducibility between experiments is increased as shown by the lower SEM , particularly at the lower [TMSO] (see Table 3.13). The reproducibility between experiments is greater using the two time point method because the value obtained for the [EtOH] at each time point is more accurate than the repeated sampling method . Each incubation is performed in triplicate and each sample is assayed in triplicate; therefore usually the [EtOH] for each time point is the mean of nine values. The higher inhibition values with the repeated sampling method probably just reflect the greater errors inherent in the method, and illustrate the difficulty in measuring small changes in rates of ethanol clearance accurately.

Overall, the new method for measurement of small changes in flux has many advantages in comparison to the repeated sampling method (see Table 3.14). The two time point method is quicker and more efficient , more sensitive and more accurate in the measurement of the C_E^J .

TABLE 3.14

Advantages of the Two Time Point Method Compared to the Repeated Sampling Method

Aspects of Method	Two Time Point Method	Repeated Sampling Method
Time	Sampling time plus incubation time is less than 55 minutes	$\frac{1}{2}$ hr preincubation plus 1 hr incubation for sample collection
Cell Content / [I] range	only 1.3 g cells required for 5 inhibitor concentrations plus control	at least 3.5 g cells required to do 4 incubations (control + 3 inhibitor concentrations)
Determination of C_E^J in one experiment	possible	not possible
Detection of small changes	< 10 % inhibition	lowest of 17 %
Reproducibility	within an experiment the use of triplicates for each time point gives low standard errors at low inhibitor concentrations, which are the critical concentrations for determining the C_E^J	only one sample per time point gives high standard errors at low inhibitor concentrations

CHAPTER 4

Alcohol Dehydrogenase

4.1 INTRODUCTION

The main reaction by which ethanol is metabolised is that catalysed by alcohol dehydrogenase (ADH).

$$\underset{\text{ethanol}}{C_2H_5OH} + NAD^+ \overset{ADH}{\rightleftharpoons} \underset{\text{acetaldehyde}}{CH_3CHO} + NADH + H^+$$

In rat tissues there are 3 different isoenzymes of ADH (Julia *et al.,* 1987), designated ADH-1, ADH-2, and ADH-3 according to their mobility on starch gel electrophoresis (Table 4.1). These isoenzymes have been purified to homogeneity and characterised (Crabb *et al.*, 1983; Julia *et al.,* 1987). All of them have a molecular weight of 80,000 with two subunits of M_r 40,000, contain 4 zinc atoms per molecule, and prefer NAD^+ as the cofactor rather than $NADP^+$.

The specific localisation and kinetic properties of rat ADH isoenzymes (Table 4.1) suggest that ADH-1 and ADH-3 may act as metabolic barriers to external alcohols and ADH-2 may have a function in the metabolism of endogenous long chain alcohols and aldehydes. The cathodic form of rat ADH (ADH-3) has a low K_m for ethanol and is abundant in the liver; therefore it plays the major role in ethanol metabolism. The greatest activity of alcohol dehydrogenase is found in the cytosolic compartment of the liver (Rognstad & Grunnet, 1979).

The steady state kinetic properties of rat liver ADH have been determined (Cornell *et al.,* 1979; Crabb *et al.,* 1983) (Table 4.2). The values of some of the kinetic parameters differ between the two studies. For example, there is a five fold difference between the K_a values (K_m for NAD^+). These values have resulted in different explanations for the fact that the rate of ethanol metabolism *in vivo* is less than the V_{max} of ADH. Cornell *et al.* (1979) stated that at physiological concentrations of NAD^+ (0.5 mM) the K_m for NAD^+ (0.15 mM) allowed ADH to operate at 0.8 V_{max}. Crabb *et al.* (1983) estimated that ADH would be over 90% saturated at physiological concentrations of NAD^+ (0.5 mM) since the $K_a(NAD^+)$ was 0.033 mM and postulated that ADH was subjected to substrate (ethanol) and product (acetaldehyde, NADH) inhibition which accounted for the enzyme operating below its maximum activity *in vivo.* The kinetic

TABLE 4.1

Characterisation of Rat Alcohol Dehydrogenase Isoenzymes

Class	Mobility	Isoelectric Point	Main Tissues Present In	K_m (ethanol) mM	K_i (pyrazole) mM	Specificity
ADH-1	Anodic	5.1	Adrenals, ocular, stomach and lung	340	0.56	broad
ADH-2	Anodic	5.95 - 6.30	in all rat organs	-	78.4	long chain alcohols and aldehydes
ADH-3	Cathodic	8.25 - 8.40	liver	1.4	0.004	broad

These results are from Julia *et al.* (1987)

characterisation of ADH in these studies was carried out under the same physiological conditions. The differences obtained for some of the kinetic parameters remain unexplained.

TABLE 4.2

Kinetic Constants of Rat Liver Alcohol Dehydrogenase

Kinetic Constant	Cornell *et al.* (1979)+ μM	Crabb *et al.* (1983)+ μM
K_a	150	33
K_{ia}	265	58
K_b	1070	480
K_{ib}	20300	810
K_p	49	37
K_{ip}	134	12
K_q	5.3	4
K_{iq}	2.3	0.9

+ Kinetic constants determined at pH 7.3, 37-38 °C, and I = 0.25
a = NAD^+ ; b = ethanol ; p = acetaldehyde ; q = NADH
K's are limiting Michaelis constants
K_i's are dissociation or inhibition constants
The Cornell *et al.* (1979) kinetic constants are slightly higher than those shown in Cornell (1983)

The reaction catalysed by rat liver ADH (Cornell *et al.*, 1979; Cornell, 1983a) follows that of an ordered bi-bi mechanism (Segel, 1975 pp 560-590); where the cofactor NAD^+ binds before ethanol (Figure 4.1). The rate-determining step in the ADH reaction is the dissociation of NADH from the enzyme (k_4) (Dalziel, 1963).

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA + B \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EAB \underset{k_{-p}}{\overset{k_p}{\rightleftharpoons}} EPQ \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EQ + P \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + Q$$

Figure 4.1: **Ordered Bi-Bi Mechanism For Rat Liver ADH.**
E = ADH ; A = NAD^+ ; B = Ethanol ; P = Acetaldehyde ; Q = NADH

The steady state rate equation that describes this mechanism (Segel, 1975 pp 563) is shown in Figure 4.2. The use of this equation with physiological concentrations of ethanol , NAD^+, NADH, and AcH predicts quite accurately the activity of the enzyme *in vivo* (Cornell, 1983a; Crabb *et al.,* 1983).

As already stated in the introduction , it is generally accepted that ADH-mediated alcohol metabolism is the main route for ethanol elimination (Hawkins & Kalant, 1972 ; Rognstad & Grunnet, 1979). However the importance of alcohol dehydrogenase in regulation of ethanol oxidation is still under debate (see section 1.2).

Pyrazole, a competitive inhibitor of ADH, and its derivatives, inhibit ethanol oxidation *in vivo* (Goldberg & Rydberg, 1969; Khanna *et al.*, 1977; Lindros *et al.*, 1977; Plapp *et al.*, 1984), in perfused liver (Thurman & McKenna, 1975) or in isolated rat hepatocytes (Crow *et al.,* 1977a; Cornell *et al.,* 1983) by 80 to 100%. Therefore qualitatively alcohol dehydrogenase activity appears to be a major factor in the control of ethanol metabolism in rats.

A theoretical framework is available for quantitatively assessing the relative importance (value of the flux control coefficient) of potential control steps in a metabolic pathway. This approach to analysis of metabolic control has been used to calculate the flux control coefficient of ADH in *Drosophila melanogaster* (Middleton & Kacser , 1983) and in horse liver (Derr & Derr, 1987).

It was concluded that in *D. melanogaster* the flux through the ethanol pathway was relatively insensitive to small variation in ADH ($C^J_{ADH} = 0.02 \pm 0.04$). The flux control coefficient of the three major ADH isoenzymes EE, ES, and SS and AlDH in horse liver were calculated using the equations derived for reactions catalysed by 3 or more isozymes (Derr & Derr 1987). The flux through the ADH isozymes was calculated from the ordered bi-bi rate equation and the elasticity coefficients were calculated using the partial derivative of the rate equation with respect to the appropriate metabolite. The flux control coefficient for the ADH and AlDH isoenzymes was not determined experimentally but from calculations. The results obtained from these studies are summarised in Table 4.3. This work by Derr and Derr (1987) was published after I started my studies on the regulation of ethanol metabolism in isolated rat hepatocytes.

The results in Table 4.3 show that under the stated cellular conditions if cytosolic AlDH is considered to be the primary enzyme in metabolising acetaldehyde, the majority of the flux control is exerted by the F_1 isoenzyme itself (see 1 & 4 in Table 4.3) . If the mitochondrial AlDH is considered the major enzyme in the metabolism of acetaldehyde, which appears to be the general case (Weiner, 1979; Harrington *et al.,* 1988), then the importance of aldehyde dehydrogenase in

$$v = \frac{\frac{V_f A B}{K_{ia} K_b} - \frac{V_r P Q}{K_p K_{iq}}}{1 + \frac{A}{K_{ia}} + \frac{K_a B}{K_{ia} K_b} + \frac{K_q P}{K_p K_{iq}} + \frac{Q}{K_{iq}} + \frac{AB}{K_{ia} K_b} + \frac{K_q AP}{K_{ia} K_p K_{iq}} + \frac{K_a BQ}{K_{ia} K_b K_{iq}} + \frac{PQ}{K_p K_{iq}} + \frac{ABP}{K_{ia} K_b K_{ip}} + \frac{BPQ}{K_{ib} K_p K_{iq}}}$$

Figure 4.2: **Steady State Rate Equation For An Ordered Bi-Bi Mechanism**

V_f and V_r are the maximal velocities in the forward and reverse directions, respectively

A = [NAD^+] ; B = [Ethanol] ; P = [Acetaldehyde] ; Q = [NADH]

$K_{a,b,q,p}$ = limiting Michaelis constants

$K_{ia,ib,iq,ip}$ are the dissociation or inhibition constants

TABLE 4.3

The Relative Importance (C_E^J) of Horse Liver ADH Isoenzymes and the Mitochondrial and Cytosolic AlDH Isozymes in the Metabolism of Ethanol

		Flux Control Coefficient (C_E^J)			
		1	2	3	4
ADH	EE	0.0873	0.316	0.366	0.100
Isoenzymes	ES	0.0362	0.131	0.152	0.041
	SS	0.0032	0.012	0.014	0.004
AlDH	F_1(cyt)	0.8733	0.000	0.687	0.940
Isoenzymes	F_2(mito)	0.000	0.541	-0.219	0.085

In this study (Derr & Derr, 1987) metabolite concentrations were ; $[NAD]_{cyt}$ = 0.4 mM ; $[NADH]_{cyt}$ = 1.5 μM; [AcH] = 200 μM; [EtOH] = 21.7 mM ; $[NAD]_{mito}$ = 4.71 mM ; total free NAD and NADH in the mitochondria is 6.33 mM

cyt = cytosolic ; mito = mitochondrial

1 - Assumed that F_1 was the primary enzyme for oxidising acetaldehyde

2 - Assumed that F_2 was the primary enzyme for oxidising acetaldehyde

3 - Assumed both F_1 and F_2 function *in vivo* and that F_2 metabolises acetaldehyde to its full capacity

4 - Assumed both F_1 and F_2 function *in vivo* and that F_1 metabolises acetaldehyde to its full capacity

control of ethanol metabolism is less. However it is still greater than the sum of the contribution of all the ADH isoenzymes to control of the ethanol pathway (see 2 & 3 in Table 4.3) . In all cases the EE isoenzyme of ADH is the most important enzyme of the three liver ADH isoenzymes in the regulation of ethanol metabolism in horse liver.

The results from these two studies are very interesting. They showed a great difference in the importance of ADH in regulation of ethanol metabolism in *D. melanogaster* and horse liver. This difference can be attributed to differences between the *D. melanogaster* ADH and mammalian alcohol dehydrogenase enzymes (Windberg and McKinley-McKee, 1988). The ADH of *Drosophila* is not a metalloenzyme, it differs significantly in size and amino acid sequence from mammalian ADH (Jornvall *et al.*, 1988), and it has AlDH activity (Heinstra *et al.*, 1989).

For rat liver , ADH-3 is the principal enzyme responsible for ethanol oxidation (Julia *et al.*, 1987).

This enzyme is analogous to the mouse A_2 isozyme (Julia *et al.*, 1987), human class I (type β) isozyme (Julia *et al.*, 1987 ; Jornvall *et al.*, 1988) and horse liver class I (type EE) isozyme (Jornvall *et al.*, 1988).

Assessment of the contribution of ADH to rates of ethanol metabolism in rats has been attempted (Rognstad and Grunnet, 1979) using the method described by Rognstad (1979). This approach involved titrating hepatocytes in the presence of ethanol with a wide range of pyrazole concentrations (2.5 μM to 2.5 mM). A linear plot of $\frac{1}{v}$ versus [I] indicates a rate-determining enzyme (Rognstad, 1979). Rognstad & Grunnet (1979) obtained a curved plot and concluded that rat liver ADH in unsupplemented cells was in excess (35% above the capacity of the overall system) and that the enzyme had to be inhibited by 35% before there was any effect on the flux through the overall pathway of ethanol utilisation. For supplemented liver cells, a linear plot of the reciprocal of rate of ethanol metabolism versus pentylpyrazole concentration was obtained (Cornell, 1983a). Using Rognstad's approach this indicated that ADH was rate-determining. The data also showed that even in supplemented cells ADH was not working at V_{max} . Therefore the assumption that if ADH operates at 35% less the V_{max} it must be inhibited by 35% before it will affect the overall rate of ethanol oxidation is incorrect. In view of this Rognstad and Grunnet's (1979) inhibition experiments do not quantitatively determine the role of ADH in regulation of ethanol metabolism.

I have determined the relative significance of rat liver ADH in the metabolism of ethanol by applying the modulation approach of Kacser and Burns (1973). The technique involved measuring the change in flux through the ethanol metabolising system by making small alterations in the activity of ADH. This was accomplished by performing inhibitor titrations i.e. changing the activity of alcohol dehydrogenase using an ADH- specific inhibitor at varying concentrations.

Several researchers (McCarthy *et al.*, 1968; Lester & Benson, 1970 ; Sigman & Winer, 1970; Goldberg & Rydberg, 1969; Chadha *et al.*, 1983, 1985; Cornell *et al.*, 1983; Plapp *et al.*, 1984) have been involved in the search for specific inhibitors of alcohol dehydrogenase and consequently ethanol metabolism.

The types of inhibitors discovered are:

1) Pyrazole and its various derivatives (Goldberg & Rydberg, 1969; Lester & Benson, 1970; Cornell *et al.*, 1983; Plapp *et al.*, 1984). These compounds are specific inhibitors of ADH *in vivo* and *in vitro* (Deis & Lester, 1979). They are competitive inhibitors with respect to ethanol since they bind tightly to the ADH-NAD^+ complex in the site normally occupied by ethanol (Eklund *et al.*, 1982).

2) Amides and Sulphoxides (Sigman & Winer, 1970 ; Porter *et al.,* 1976; Chadha *et al.,* 1983, 1985; Plapp *et al.,* 1984). For both groups of compounds, an introduction of polar groups reduced their potency as inhibitors whereas increasing the hydrophobicity resulted in greater inhibition (Chadha *et al.,* 1983). Most of the amides and sulphoxides tested were uncompetitive inhibitors of liver alcohol dehydrogenase with respect to EtOH (Chadha *et al.,* 1983). The rest exhibited noncompetitive inhibition.

3) Thyroxine and its related compounds (McCarthy *et al.,* 1968 ; Gilleland & Shore, 1969; Mezey & Potter, 1981; Mardh *et al.,* 1987). These hormones are potent inhibitors of liver alcohol dehydrogenases. Thyroxine was found to be uncompetitive against both coenzymes. Triiodothyronine inhibited ADH competitively with respect to NAD^+ and triiodothyroacetic acid was a competitive inhibitor of ADH against NADH. These thyroid hormones appear to act by interfering with the normal coenzyme binding.

For the inhibitor titration studies and modulation methodology, specific noncompetitive and irreversible inhibitors are the best to use (see section 1.4). In view of this the two ADH-specific inhibitors, tetramethylene sulphoxide (TMSO) and isobutyramide (IsB) were chosen for determining the flux control coefficient of ADH (C^J_{ADH}) in hepatocytes isolated from fed and starved rats. These inhibitors do exhibit noncompetitve inhibition against varied concentrations of ethanol (Chadha *et al.*, 1983; Plapp *et al.*, 1984)

4.2 TETRAMETHYLENE SULPHOXIDE AND ISOBUTYRAMIDE

4.2.1 Type of Inhibition

There are four types of inhibitors – competitive, uncompetitive, noncompetitive, and strict noncompetitive. Determination of the kinetic constants K_{ii} (intercept inhibition constant) and K_{is} (slope inhibition constant) establish what pattern of inhibition is observed. For competitive inhibition only K_{is} is detected; for uncompetitive inhibition only K_{ii} is found; both K_{is} as well as K_{ii} can be calculated from the double reciprocal plots for noncompetitive inhibition and strict noncompetitive inhibition is observed when the intercept inhibition constant is approximately equal to the slope inhibition constant.

The kinetic constants for inhibition of rat liver alcohol dehydrogenase by TMSO and IsB are shown in Table 4.4. For both tetramethylene sulphoxide (TMSO) and isobutytramide (IsB) the kinetic inhibition data (Chadha *et al.,* 1983 ; Plapp *et al.,* 1984) suggest at first that they are

essentially uncompetitive inhibitors against varied concentrations of ethanol. However small changes in the slopes of the double reciprocal plots are noticeable and the data fit equally well to noncompetitive inhibition. The inhibition of rat liver ADH by TMSO is more likely to be strict noncompetitive rather than uncompetititive or noncompetitive inhibition because the kinetic constants for TMSO are fairly similar ($K_{ii} \approx K_{is}$ where K_{is} has a high standard error).

TABLE 4.4

Kinetic Constants for Inhibition of Alcohol Dehydrogenase

Inhibitor	Type of Inhibition	K_{ii} (μM)	K_{is} (μM)
Isobutyramide	UC / NC	330 ± 60 [a]	1200 ± 300 [a]
Tetramethylene Sulphoxide	UC / NC	200 ± 20 [b]	230 ± 80 [b]

The inhibition constants (K_{ii} and K_{is}) were determined from in vitro experiments. Purified rat liver ADH was assayed in 83 mM potassium phosphate and 40 mM KCl buffer, pH 7.3 at 37 °C with 0.5 mM NAD^+. UC represents uncompetitive inhibition and NC represents noncompetitive inhibition.

[a] Data from Plapp *et al.*, 1984 [b] Data from Chadha *et al.*, 1983

Assuming that TMSO and IsB form the dead end complexes E-NAD^+-I and E-NADH-I (Cleland, 1967), the type of inhibition obtained by these inhibitors may be explained by applying the general rules for dead end inhibition of an ordered bi-bi mechanism, outlined in Segel (1975 pp 767 - 779). If TMSO or IsB bind only to the ADH-NADH complex then uncompetitive inhibition against ethanol or competitive inhibition against acetaldehyde should be produced. However if the inhibitor binds to both ADH-coenzyme complexes noncompetitive inhibiton against ethanol and acetaldehyde will occur. The latter pattern of inhibition has been shown for IsB (Sigman &Winer, 1970 ; Plapp *et al.*, 1984) and TMSO (Chadha *et al.*, 1983; Plapp *et al.*, 1984). From the kinetic studies (Sigman & Winer, 1970; Chadha *et al.*, 1984) it appears that TMSO and IsB bind most tightly to the ADH-NADH complex and more weakly to the ADH-NAD^+ complex. In summary, for these studies TMSO and IsB were assumed to be noncompetitive and uncompetitive inhibitors of ADH against ethanol (especially for correction of the apparent C^J_{ADH} (see section 4.5.1)) because I did not know which type of inhibition was correct for tetramethylene sulphoxide and isobutyramide.

4.2.2 Determination of Flux Control Coefficient

The flux control coefficient by inhibition of an irreversible reaction with a noncompetitive inhibitor (Groen *et al.*, 1982b ; Derr, 1986) is given by:

$$C_E^J = \frac{-K_i \, dJ}{J \, dI} \qquad (4.1)$$

where : K_i represents the inhibition constant

The inhibition constant (K_i) for TMSO and IsB had to be obtained from the literature (Chadha *et al.*, 1983; Plapp *et al.*, 1984) (see Table 4.4) since I did not carry out inhibition studies on purified rat liver ADH. For TMSO the inhibition constant used in determination of the flux control coefficient was 215 μM ± 50 μM, which is the mean value between the intercept and slope kinetic constants.

For IsB the K_{ii} value was used for determination of the flux control coefficient of ADH. The type of inhibition observed for IsB is either noncompetitive or uncompetitive. The flux control coefficient for an uncompetitive inhibitor (Derr, 1986; Page, 1986) is given by:

$$C_E^J = \frac{-K_{ii} \, dJ}{J \, dI} \left(\frac{K+S}{S}\right) \qquad (4.2)$$

where: K is equal to the K_m of the substrate, ethanol

If IsB is assumed to be an uncompetitive inhibitor, then the value obtained for the flux control coefficient will be slightly higher than if IsB is assumed to be noncompetitive (compare equations 4.1 & 4.2). The C_E^J for an uncompetitive inhibitor tends towards that for a noncompetitive inhibitor when S » K (i.e. $\frac{K+S}{S} \approx 1$). Consequently , for IsB, equation 4.1 was used for determination of the flux control coefficient of ADH with K_i represented by K_{ii}.

The $\frac{dJ}{J \, dI}$ term in equation 4.1 is calculated from the flux inhibition curve. J represents the flux at zero inhibitor and $\frac{dJ}{dI}$ is the initial slope of the inhibition curve (flux vs [I]). The flux control coefficient determined from the inhibitor studies is an apparent value and is calculated on the assumption that the ADH reaction is a single substrate/ single product reaction. The value obtained from the experimental studies needs to be corrected for possible effects of substrate (NAD^+) and/or product (NADH; acetaldehyde) concentration on the flux control coefficient . This will be addressed later on in this chapter.

4.3 THE USE OF ISOLATED HEPATOCYTES FROM FED AND STARVED RATS

The flux control coefficient of a step in a metabolic pathway does not have a fixed value, as it is dependent on the metabolic conditions. Therefore the flux control coefficient of ADH in cells from fed and starved rats was determined. This provided an overall picture of the importance of ADH in the regulation of ethanol metabolism.

Rat liver ADH is subject to product inhibition by acetaldehyde (AcH) during ethanol oxidation *in vivo* (Braggins & Crow, 1981) and in isolated rat hepatocytes (Braggins *et al.*, 1980). Acetaldehyde concentrations were determined for experiments utilising hepatocytes isolated from fed rats but not for inhibitor experiments using liver cells from starved rats. This is because the concentrations of acetaldehyde present in hepatocytes isolated from fed rats (maximum of approximately 200 μM) during ethanol elimination are capable of inhibiting rat liver ADH (Crow *et al.*, 1983a), whereas the acetaldehyde concentrations reached during ethanol elimination in hepatocytes isolated from starved rats (maximum of approximately 2 μM) will not significantly influence the activity of ADH.

The volume of the incubation mixture for experiments utilising isolated liver cells from fed rats was increased from 1 ml (for hepatocytes from starved rats) to 2 ml, to accommodate the sample volume required for measurement of acetaldehyde concentrations (see section 2.10 and chapter 3).

4.4 EXPERIMENTAL RESULTS AND DISCUSSION

For determination of the apparent flux control coefficient of alcohol dehydrogenase (C^J_{ADH}), inhibitor titration experiments were carried out using two different ADH-specific inhibitors, tetramethylene sulphoxide (TMSO) and isobutyramide (IsB). The results for each experiment were normalised (inhibited rate (v) divided by the non inhibited rate (v_o) for that particular experiment). This enabled the data from several experiments for each inhibitor to be combined.

4.4.1 Starved Rats

Inhibitor titration studies were first performed using isolated liver cells from starved rats to eliminate any complications introduced from acetaldehyde inhibition of alcohol dehydrogenase (see section 4.3). The combined results of normalised flux, inverse normalised flux, and percentage inhibition for all experiments using TMSO and IsB are shown in Tables 4.5 and 4.6 respectively.

TABLE 4.5

Combined Results for Inhibition by Tetramethylene Sulphoxide

[TMSO] mM	n	Normalised Flux (NF)	Inverse Normalised Flux	% Inhibition
0.00		1.00	1.00	0.00
0.05	4	0.914 ± 0.022	1.09 ± 0.024	8.60 ± 2.2
0.10	10	0.888 ± 0.018	1.13 ± 0.023	11.20 ± 1.83
0.15	3	0.827 ± 0.024	1.21 ± 0.035	17.30 ± 2.47
0.20	13	0.780 ± 0.020	1.28 ± 0.037	21.98 ± 1.99
0.25	5	0.763 ± 0.004	1.31 ± 0.007	23.66 ± 0.38
0.30	4	0.722 ± 0.013	1.39 ± 0.024	27.88 ± 1.30
0.40	11	0.618 ± 0.017	1.61 ± 0.045	38.24 ± 1.80
0.60	8	0.501 ± 0.045	2.16 ± 0.280	49.90 ± 4.47

All rates were normalised ($\frac{V}{V_o}$). The results are the mean ± SEM for 'n' inhibitor experiments.

% inhibition = $\frac{1\text{-NF}}{1} \times \frac{100}{1}$

TABLE 4.6

Combined Results For Inhibition by Isobutyramide

[IsB] mM	n	Normalised Flux (NF)	Inverse Normalised Flux	% Inhibition
0.00		1.00	1.00	0.00
0.06	5	0.922 ± 0.009	1.09 ± 0.012	8.63 ± 1.00
0.10	7	0.779 ± 0.028	1.29 ± 0.042	20.67 ± 2.85
0.20	3	0.780 ± 0.025	1.27 ± 0.041	22.60 ± 2.53
0.30	5	0.698 ± 0.019	1.44 ± 0.038	30.16 ± 1.88
0.40	3	0.633 ± 0.027	1.59 ± 0.064	36.70 ± 2.70
0.55	5	0.518 ± 0.035	1.89 ± 0.120	46.42 ± 3.24

All rates were normalised ($\frac{V}{V_o}$). The results are the mean ± SEM for 'n' inhibitor experiments.

% inhibition = $\frac{1\text{-NF}}{1} \times \frac{100}{1}$

The data obtained from the inhibitor titration studies may be presented as:

1) A Dixon Plot (Inverse Normalised Flux vs [I])

$$\frac{1}{v} = a + bI \qquad (4.3)$$

or 2) A Flux Inhibition Plot (Normalised Flux vs [I])

The inverse normalised flux was plotted against [I] using the combined results of the TMSO experiments (Figure 4.3) and IsB experiments (Figure 4.4). A linear regression analysis was carried out on the data of each plot, to determine the y intercept (coefficient a), slope (coefficient b), and the correlation coefficient (r). The inhibition constant (K_i) determined from the x intercept of the Dixon plot for both inhibitor studies along with the results obtained from the regression analysis are shown in Table 4.7 .

The Dixon plot was approximately linear for both inhibitor studies (TMSO: r = 0.98 ; IsB; r = 0.98). According to Rognstad (1979) this implies that rat liver ADH is a rate-limiting enzyme in the metabolism of ethanol in hepatocytes isolated from starved rats. However, the information obtained from the Dixon plot does not provide a quantitative measure of the importance of rat liver ADH in the regulation of ethanol oxidation. This may be determined by calculating the flux control coefficient (equation 4.1). The flux control coefficient of ADH was evaluated using the information obtained from the flux inhibition plot (see sections 1.4 and 4.2.2).

The normalised flux was plotted against [I] using the combined results of the TMSO experiments (Figure 4.5) and IsB experiments (Figure 4.6). For calculation of the C^J_{ADH} each inhibitor must be treated individually since it has its own characteristic flux inhibition curve (compare Figures 4.5 and 4.6).

The $\left(\frac{dJ}{JdI}\right)_{I=0}$ term of equation 4.1 is determined from the inhibition curve. J represents the flux through the ethanol pathway at zero inhibitor concentration. In this case the value of J is 1 because the rates were normalised. From the data of Figures 4.5 and 4.6, it appears that the initial slope ($\left(\frac{dJ}{dI}\right)_{I=0}$) of the plots of [I] against inhibition of ethanol clearance rates in isolated rat hepatocytes may be calculated with reasonable accuracy from the inhibition observed at 0.05 mM TMSO (Figure 4.5) and 0.06 mM IsB (Figure 4.6). The initial slope of the flux inhibition plot was derived as shown in Figures 4.5 and 4.6.

Figure 4.3: **Dixon Plot For Tetramethylene Sulphoxide**

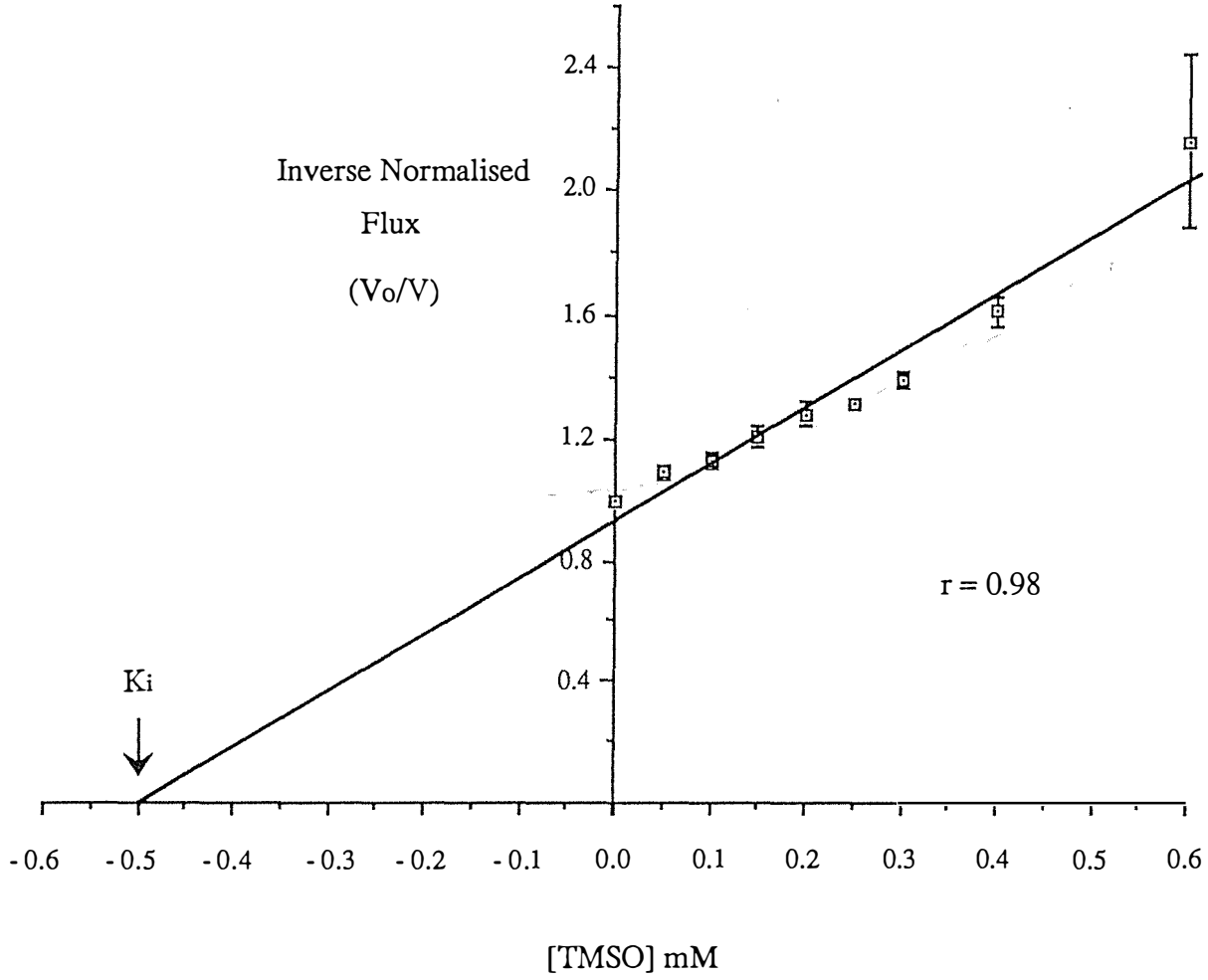


Figure 4.4: **Dixon Plot For Isobutyramide**

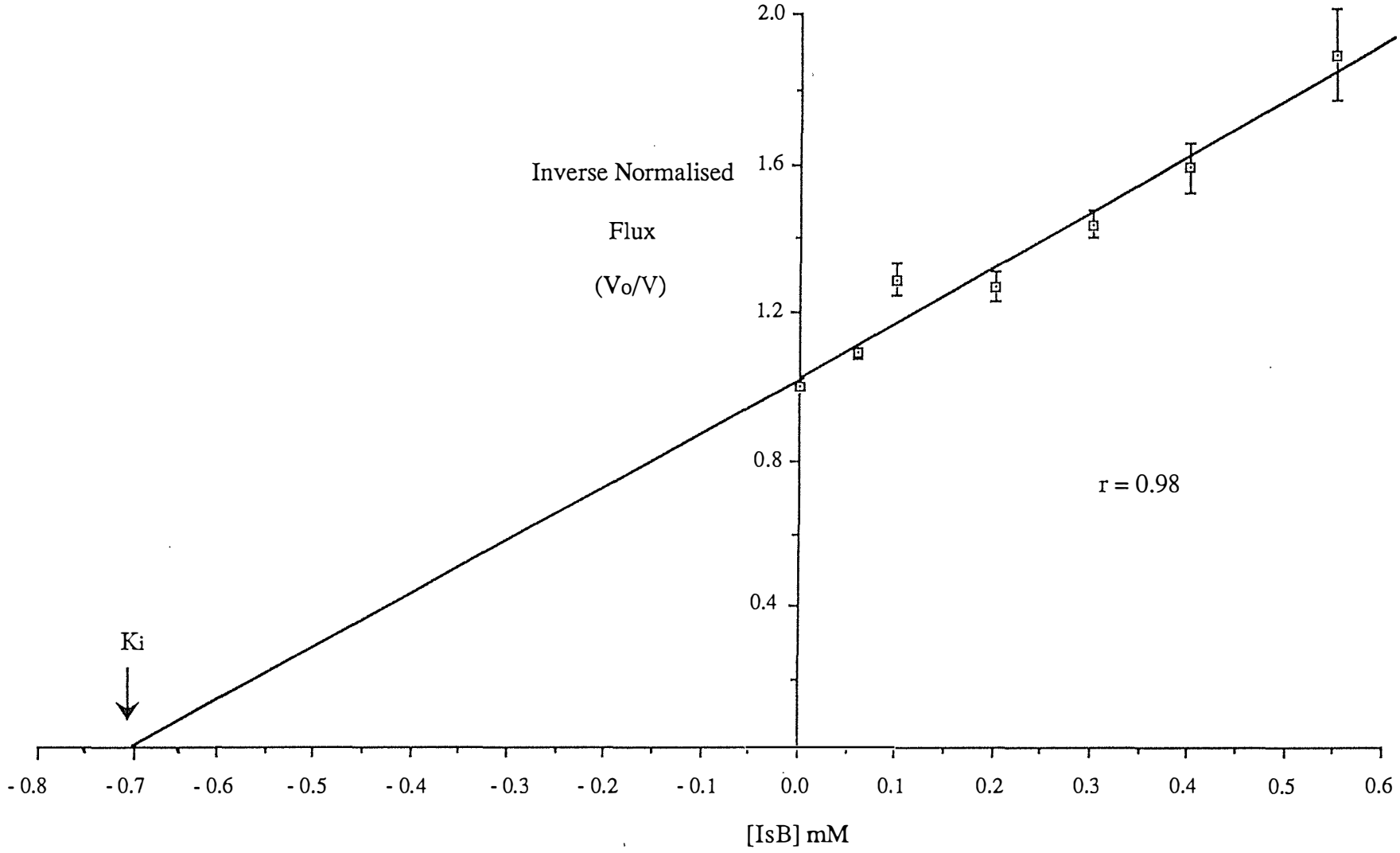
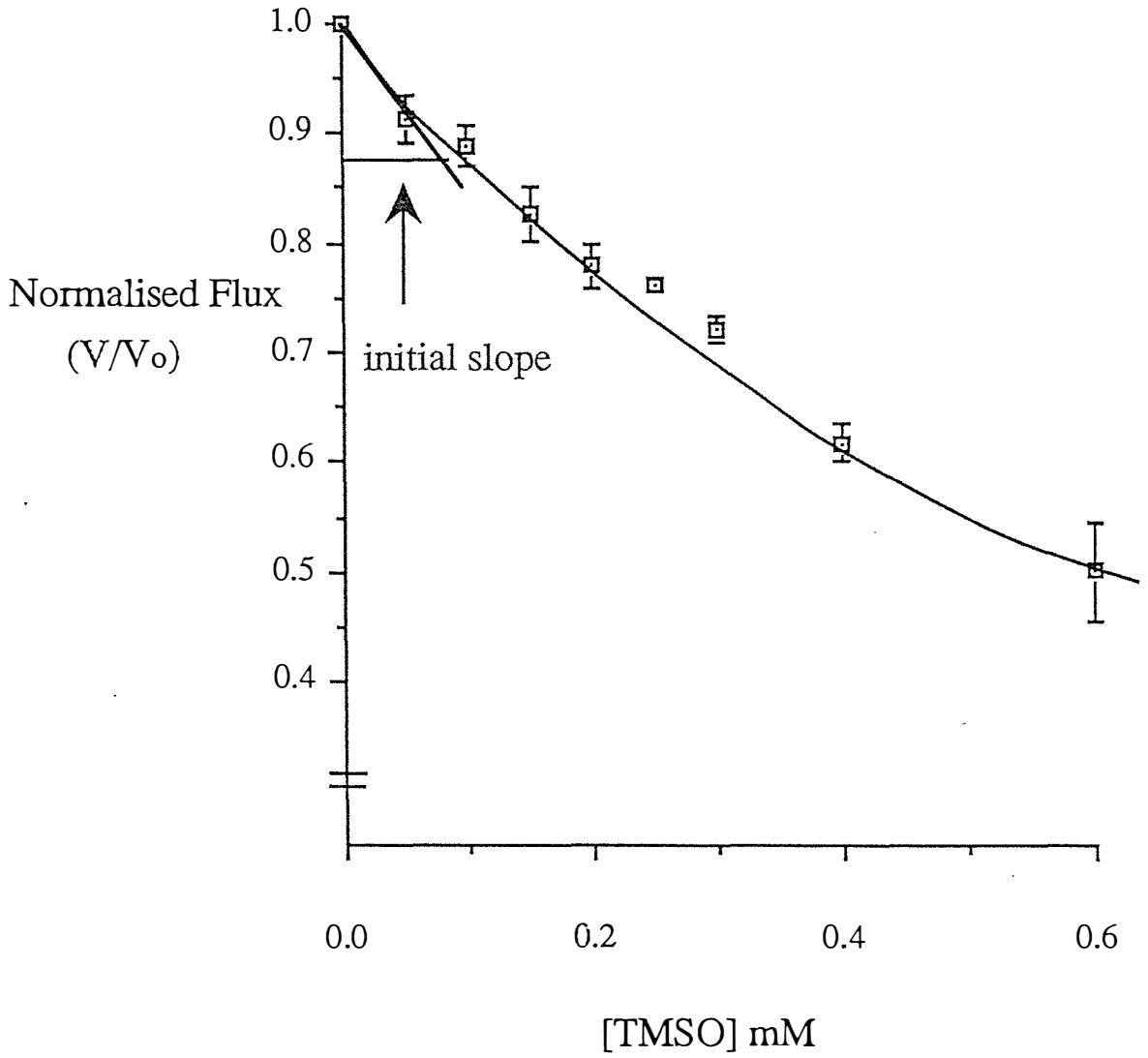


Figure 4.5

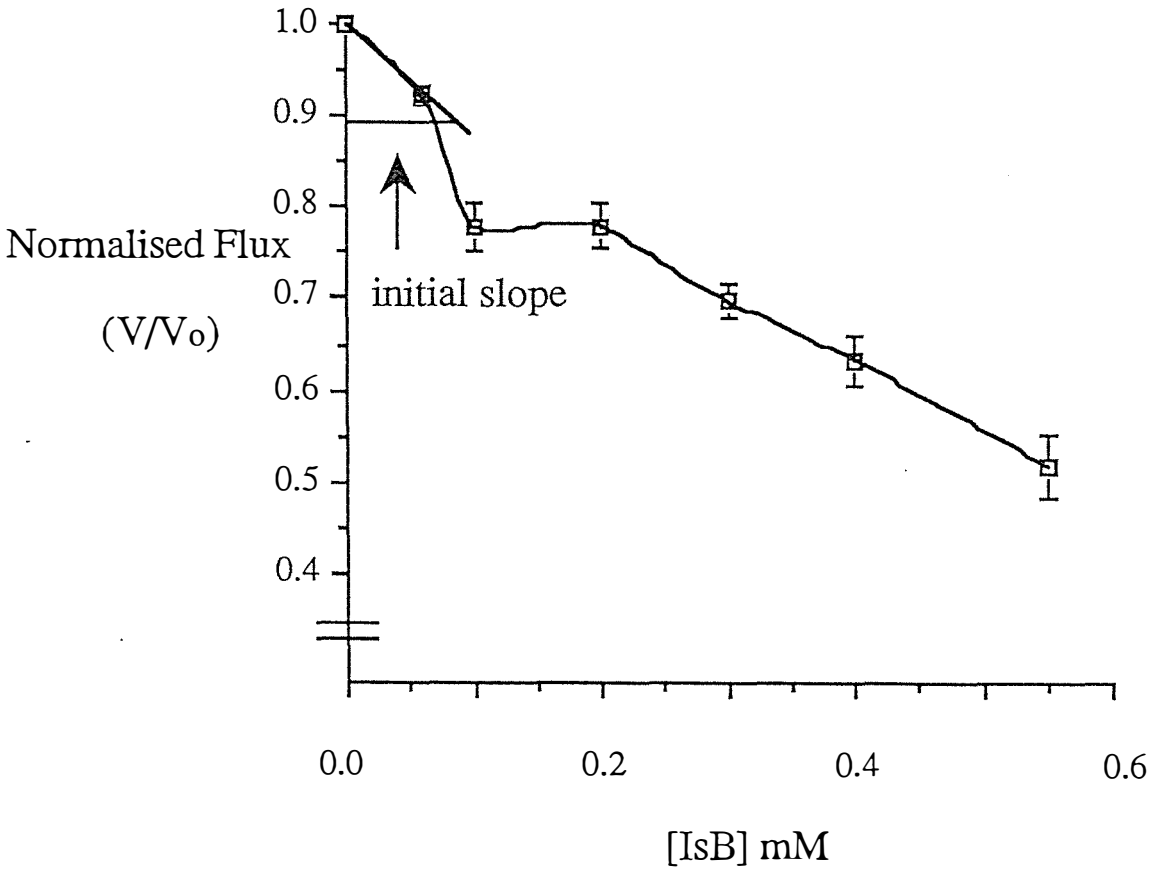
Flux Inhibition Curve for Tetramethylene Sulphoxide



The initial slope ($\left(\frac{dJ}{dI}\right)_{I=0}$) of this flux inhibition curve is shown as the chord between zero inhibitor concentration and 0.05 mM TMSO concentration.

Figure 4.6

Flux Inhibition Curve For Isobutyramide



The initial slope ($\left(\frac{dJ}{dI}\right)_{I=0}$) of this flux inhibition curve is shown as the chord between zero inhibitor concentration and 0.06 mM IsB concentration.

The flux control coefficient of ADH (C^J_{ADH}) plus the inhibition constant determined from the flux inhibition data of the combined results for TMSO and IsB are shown in Table 4.7 .

TABLE 4.7

Flux Control Coefficient of ADH and Inhibition Constant Determined Using the Combined Results for Inhibition by Tetramethylene Sulphoxide and Isobutyramide.

	TMSO		IsB	
	$\left(\frac{V}{V_0}\right)$ vs [I] ++	$\left(\frac{V}{V_0}\right)^{-1}$ vs [I] #	$\left(\frac{V}{V_0}\right)$ vs [I] ++	$\left(\frac{V}{V_0}\right)^{-1}$ vs [I] #
a	1.0	0.935 ± 0.043	1.0	1.02 ± 0.04
b $(mM)^{-1}$	1.72	1.84 ± 0.15	1.30	1.51 ± 0.14
K_i (from graph) mM		0.5		0.67
K_i (calculated) mM	0.586	0.508 ± 0.065	0.71	0.675 ± 0.089
K_i (purified)* mM	0.20 ± 0.02		0.33 ± 0.08	
C^J_{ADH}	0.369 ± 0.085		0.43 ± 0.10	

* This is the inhibition constant determined using purified rat liver ADH (from references Chadha *et al.* (1983) and Plapp *et al.* (1984).

++ This is the flux inhibition plot. # This is the Dixon plot.

For the Dixon plot , a = y int ± std. dev. and b = slope ± std. dev.

K_i (calculated) $= \frac{a}{b}$

The flux control coefficient of ADH (C^J_{ADH}) is calculated using the data from the flux inhibition plot i.e. C^J_{ADH} = initial slope (b) x inhibition constant

As can be seen the K_i determined from the Dixon plot and the data of the flux inhibition plot (Table 4.7) is reasonably close to that determined for the same inhibitor using purified rat liver ADH (Chadha *et al.*, 1983 ; Plapp *et al.*, 1984). This indicates that the inhibitor is having a similar effect in cells to that on the isolated enzyme *in vitro*. Therefore the inhibition constant determined for the isolated enzyme may be applied to results obtained using the isolated liver cells in the calculation of the C^J_{ADH} .

Tetramethylene sulphoxide was the first inhibitor used for the titration studies. Isobutyramide was used to validate the value obtained for the C^J_{ADH} in starved rats. Using the combined normalised data from all experiments (Tables 4.5 and 4.6), the apparent flux control coefficient of ADH for hepatocytes isolated from starved rats was calculated to be 0.37 ± 0.09 (using TMSO) and 0.43 ± 0.10 (using IsB) (Table 4.7), so an average value for the C^J_{ADH} is 0.4. Since, according to the summation theorem, the sum of all the flux control coefficients in a pathway is equal to one, the quantitative value of the C^J_{ADH} indicates that rat liver ADH is a major factor in the regulation of ethanol metabolism in hepatocytes isolated from starved rats.

The possible effect of substrate (NAD^+) and/or product (acetaldehyde; NADH) concentration on the apparent flux control coefficient is addressed in section 4.5.

4.4.2 **Fed Rats**

For the inhibitor titration studies using isolated hepatocytes from fed rats, only tetramethylene sulphoxide was used.

Acetaldehyde (AcH) concentrations were measured in all cases (see section 4.3), because the levels of AcH present in fed rats varies widely (Braggins & Crow, 1981) and in view of this each experiment was treated individually.

The apparent flux control coefficient of ADH was calculated as described previously in section 4.4.1 for hepatocytes isolated from starved rats. The graphs shown in Figures 4.7 and 4.8 are examples of flux inhibition curves obtained when AcH concentrations are low and high respectively.

Figure 4.7

Flux Inhibition Curve For Tetramethylene Sulphoxide when [AcH] is low (11.3 μM)

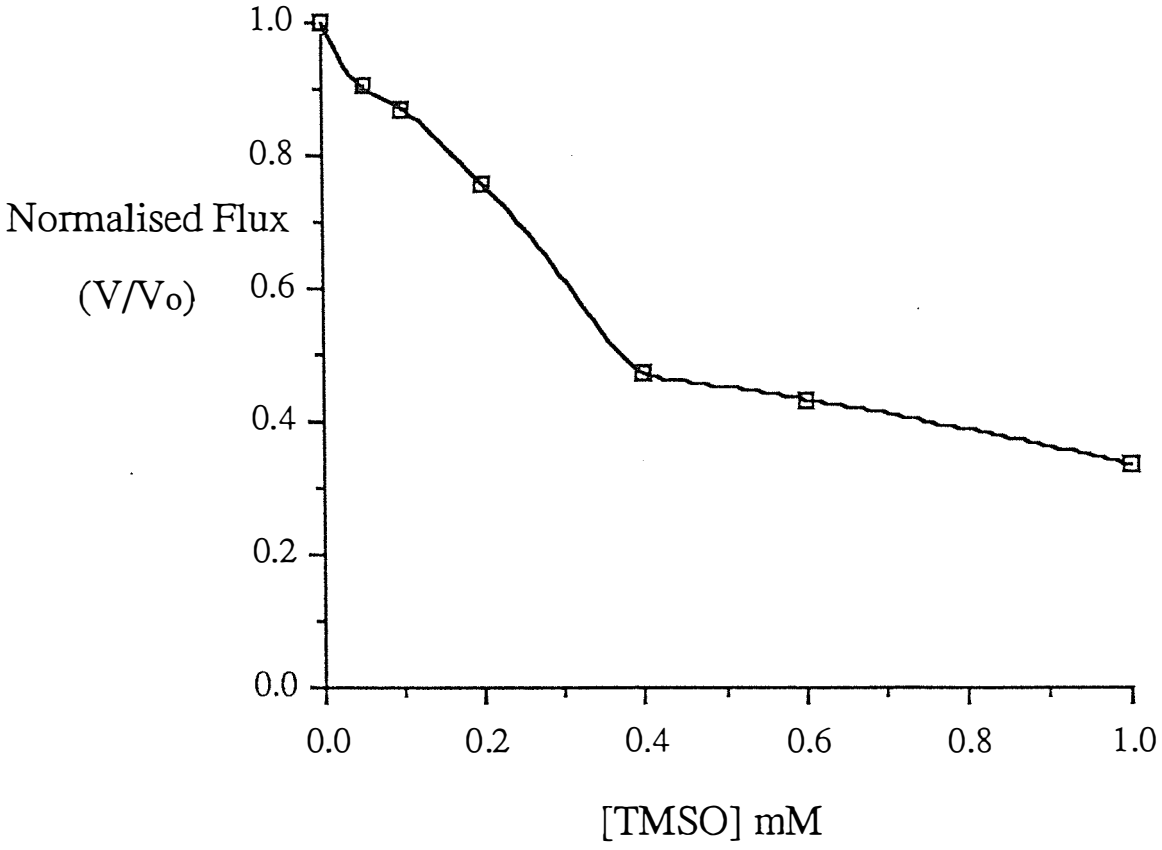
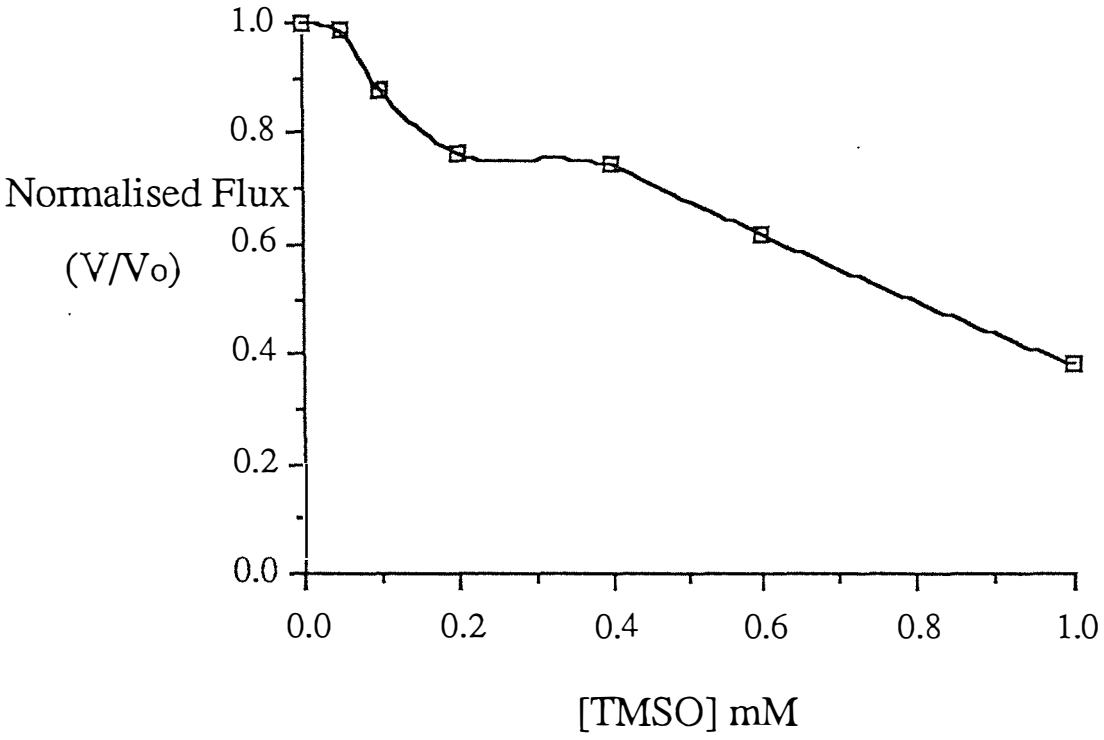


Figure 4.8

Flux Inhibition Curve For Tetramethylene Sulphoxide when [AcH] is high (80.6 μM)



The results of nine experiments with varying concentrations of acetaldehyde present in the isolated hepatocytes are shown in Table 4.8 .

TABLE 4.8

Table of Flux Control Coefficient of ADH with their Corresponding Acetaldehyde Levels

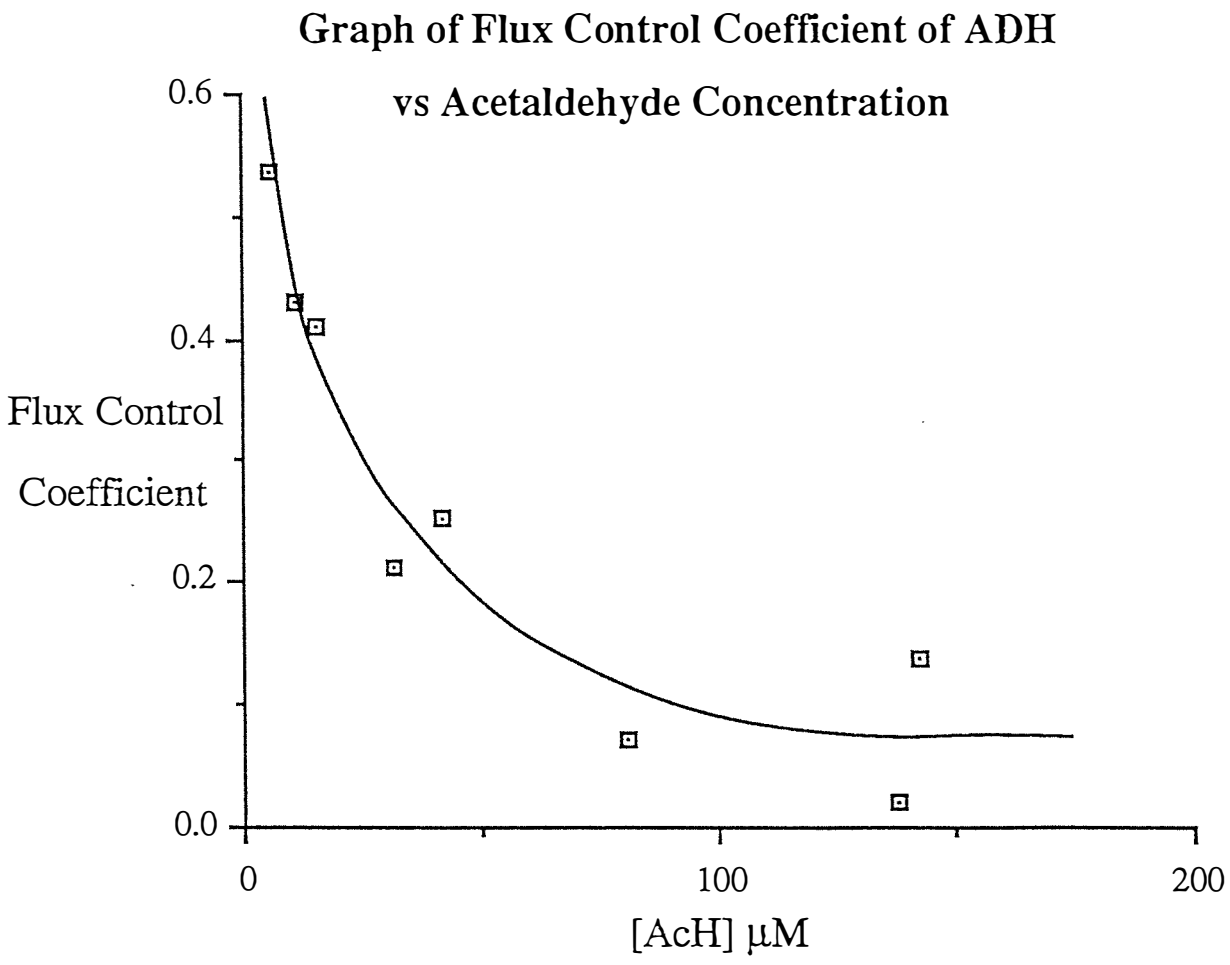
C^J_{ADH}	Maximum [AcH] μM
0.137	143.0
0.02	138.0
0.072	80.6
0.253	42.0
0.213	31.5
0.41	15.7
0.43	11.3
0.538	6.0
0.51	50.4

The acetaldehyde concentrations are from the final time control samples.

Out of the nine experiments, one appears to be an anomaly. At an acetaldehyde concentration of 50 μM a C^J_{ADH} of 0.51 was calculated. By comparison with the other results in Table 4.9, a flux control coefficient of ADH of approximately 0.3 or less should be obtained when the concentration of AcH is around 50 μM. This odd result remains unexplained. The C^J_{ADH} determined at 143 μM acetaldehyde concentration could be an anomaly, as the flux control coefficient is higher than that observed for concentrations of 138 and 80 μM. However, it still does show that a low C^J_{ADH} is obtained at a high concentration of acetaldehyde. Obviously more inhibitor experiments should be performed in hepatocytes isolated from fed rats so that the correlation observed between C^J_{ADH} and acetaldehyde concentration can be verified.

Ignoring the anomalous result determined at 50 μM acetaldehyde, Figure 4.9 graphically depicts the relationship between flux control coefficient of ADH and acetaldehyde (AcH) concentration.

Figure 4.9


Graph of Flux Control Coefficient of ADH
vs Acetaldehyde Concentration
0.6
0.4
0.2
0.0
Flux Control
Coefficient
0
100
200
[AcH] µM

Several groups (Eriksson *et al.*, 1975 ; Braggins *et al.*, 1980 ; Braggins & Crow, 1981 ; Dawson, 1981 ; Harrington *et al.*, 1988) have proposed that there is a delicate balance between the activities of alcohol dehydrogenase and aldehyde dehydrogenase (AlDH) and that the observed variability in acetaldehyde levels could be explained by small variations in enzyme activity. The results presented in this section (Table 4.8 and Figure 4.9) show that as the acetaldehyde concentration increases the C^J_{ADH} decreases. This suggests the importance of an interplay between the activities of ADH and AlDH in the overall regulation of ethanol metabolism. If acetaldehyde is produced at a faster rate than it is eliminated (i.e.the activity of ADH is greater than AlDH) its concentration will rise to levels capable of inhibiting its own production. As the product inhibition of acetaldehyde becomes more significant, the importance of ADH (as indicated by the C^J_{ADH}) in the regulation of ethanol metabolism decreases (Table 4.8 and Figure 4.9).

These results suggest that when the removal of acetaldehyde becomes limiting, the activity of AlDH should become more important in the regulation of ethanol oxidation. However this is only an assumption, and the flux control coefficient of aldehyde dehydrogenase in hepatocytes isolated from fed rats needs to be evaluated for confirmation.

What is apparent from the results using isolated hepatocytes from fed rats is that the importance of ADH in the regulation of ethanol metabolism diminishes when acetaldehyde reaches levels capable of inhibiting rat liver ADH.

4.5 SUBSTRATE AND PRODUCT CONCENTRATION EFFECT ON THE FLUX CONTROL COEFFICIENT

4.5.1 Correction of the Apparent C^J_{ADH}

The apparent flux control coefficient of ADH (C^J_{ADH}) has been evaluated from the inhibitor titration experiments on the assumption that there is a single substrate/product reaction (see section 4.2.2). In this section, the apparent C^J_{ADH} is corrected for the effect of NAD^+, NADH and acetaldehyde (AcH) concentration. This corrected C^J_{ADH} is the real value that depicts the importance of ADH in regulation of ethanol metabolism.

Equation 4.4 is the mathematical formula for the flux control coefficient, in absence of inhibitor (see section 1.4 for definition).

$$C^{J}_{Ei} = \frac{\left(\frac{dJ/J}{dI}\right)_{I=0}}{\left(\frac{\partial v_i/v_i}{\partial I}\right)_{I=0}} \qquad (4.4)$$

The denominator of this formula can be calculated from the inhibition characteristics of the enzyme.

Tetramethylene sulphoxide (TMSO) and isobutyramide (IsB), the inhibitors used in the titration experiments, form the dead end complexes EA-I (E-NAD-I) and EQ-I (E-NADH-I) (Cleland, 1967) (see section 4.2.1). The velocity equation for the uninhibited ordered bi-bi mechanism of rat liver ADH is described in Figure 4.2. Using the rules for deriving velocity equations for dead end inhibition (Segel, 1975 pp767-779), the modified rate equation for the presence of a dead end inhibitor (denominator of equation 4.4) may be determined.

The inhibition of rat liver ADH by TMSO and IsB can be either uncompetitive or noncompetitive (Chadha *et al.*, 1983; Plapp *et al.*, 1984). Because I did not know for sure whether TMSO and IsB were noncompetitve or uncompetitive inhibitors, several equations for the C^{J}_{ADH} were derived (Table 4.9) assuming that TMSO and IsB produced uncompetitive inhibition (dead end EQ-I) or noncompetitive inhibition (dead end EQ-I and EA-I) against ethanol . Expressions for the C^{J}_{ADH} were obtained for the presence of both substrates (NAD^+; EtOH) and absence of products (acetaldehyde; NADH) (Table 4.9 A, B) and derived for both substrates in the presence of NADH alone (Table 4.9 C, D) and in the presence of NADH and acetaldehyde (Table 4.9 E, F).

TABLE 4.9

Derived Equations For Substrate and Product Effect on the C^{J}_{ADH}

	Type of Dead End Inhibition	Equation
A	Dead End EQ-I Absence of products	$\frac{-K_i dJ}{J\ dI}\left(\frac{K_b(K_{ia}+A)+K_aB}{AB}+1\right)$
B	Dead End EQ-I/EA-I Absence of products	$\frac{-K_i dJ}{J\ dI}\left(\frac{K_{ia}K_b+KaB}{A\ (K_b+B)}+1\right)$
C	Dead End EQ-I Assume $P \approx 0$, $Q \neq 0$	$\frac{-K_i dJ}{J\ dI}\left(\frac{K_b(K_{ia}+A)+K_aB}{Q/K_{iq}\ (K_{ia}K_b+K_aB)+AB}+1\right)$
D	Dead End EQ-I/EA-I Assume $P \approx 0$, $Q \neq 0$	$\frac{-K_i dJ}{J\ dI}\left(\frac{K_{ia}K_b+K_aB}{Q/K_{iq}\ (K_{ia}K_b+K_a)+A(K_b+B)}+1\right)$

E Dead End EQ-I (Assume $P \neq 0$, $Q \neq 0$)

$$\frac{-K_i dJ}{J\ dI}\left(\frac{K_{iq}K_p(K_{ia}K_b+K_bA+K_aB+\frac{ABP}{K_{ip}})+K_{ia}K_b(K_qP+PQ+\frac{BPQ}{K_{ib}})+K_bK_qAP}{K_p(QK_{ia}K_b+QK_aB+ABK_{iq})}+1\right)$$

F Dead End EQ-I/EA-I (Assume $P \neq 0$, $Q \neq 0$)

$$\frac{-K_i dJ}{J\ dI}\left(\frac{K_{iq}K_p(K_{ia}K_b+K_aB+\frac{ABP}{K_{ip}})+K_{ia}K_b(K_qP+\frac{BPQ}{K_{ib}})}{K_p(K_{iq}K_bA+K_{ia}K_bQ+ABK_{iq}+K_aBQ)+K_b(K_{ia}PQ+K_qAP)}+1\right)$$

$\frac{-K_i dJ}{J\ dI}$ is the experimentally determined C^{J}_{ADH} (apparent flux control coefficient); the rest of the equation in brackets represents the correction factor (CF); the value of which can be calculated using the data of Tables 4.2 and 4.10.

Dead End EQ-I represents uncompetitive inhibition against ethanol (B) and dead end EQ-I/EA-I represents noncompetitive inhibition against ethanol.

Equations A-D were derived by Dr.M.J.Hardman and equations E and F were derived by R.Page (see Appendix for the derivations of equations A-F).

A = [NAD^+]; B = [EtOH]; Q = [NADH] ; P = [AcH]; $K_{a,b,q,p}$ are limiting Michaelis constants; $K_{ia,ib,iq,ip}$ are the dissociation constants.

The following substrate and product concentrations shown in Table 4.10, were used for correction of the apparent flux control coefficient of ADH for both starved and fed rats.

TABLE 4.10

Substrate (A,B) and Product (Q) Concentrations of ADH Reaction

		Concentration (μM)
A	free [NAD^+]	500
B	[EtOH]	10,000
Q	[NADH]	0.8 ; 1.25 ; 2.5
	free [NAD^+] / [NADH]	625 ; 400 ; 200

The actual concentration of NADH, was not measured for the liver cells from starved and fed rats in any of the inhibitor titration experiments. This is why a range of NADH concentrations (see Table 4.10) are considered. The NADH concentration range covers the free cytosolic $\frac{[NAD^+]}{[NADH]}$ ratio observed when ethanol is being metabolised. The hepatic free $\frac{[NAD^+]}{[NADH]}$ in the absence of ethanol is around 1000 (Veech *et al.*, 1972), with [NAD^+] about 0.5 mM (Bucher *et al.*, 1972). When ethanol is introduced the $\frac{NAD^+}{NADH}$ concentration ratio decreases to the range of 200 - 600 (Veech *et al.*, 1972). This reflects an increase in free [NADH] as there is no significant change in the free [NAD^+] (Crow *et al.*, 1983a). The effect of increasing NADH concentration on the apparent C^J_{ADH} could therefore be evaluated using the range of [NADH] given in Table 4.10 .

The acetaldehyde concentration was not measured in the TMSO and IsB experiments using hepatocytes isolated from starved rats (see section 4.3). However Crow *et al.* (1983a) have shown that acetaldehyde concentrations during ethanol elimination in cells from starved rats are very low (1-2 μM). Also, in inhibitor titration experiments on aldehyde dehydrogenase, using hepatocytes isolated from starved rats (see chapter 5), the concentration of acetaldehyde in control samples ranged from 0.2 - 2.0 μM. Therefore the acetaldehyde concentration range of 0.5 - 2.0 μM was chosen to evaluate the effect of increasing acetaldehyde concentration on the apparent flux control coefficient of ADH in starved rats.

For liver cells isolated from fed rats, the acetaldehyde concentration was measured (Table 4.8).

These values were used for assessing the effect of acetaldehyde on the C^J_{ADH} .

To calculate the corrected C^J_{ADH} , the apparent flux control coefficient of ADH determined from the inhibitor titration studies, must be multiplied by a correction factor (C F). This correction factor shown in the derived equations (Table 4.9) may be determined using the data in Tables 4.2, 4.8, and 4.10. Because there were differences between the kinetic parameters (Table 4.2) determined by the two research groups, the C^J_{ADH} calculated from the inhibitor experiments was re-evaluated using both sets of kinetic constants.

For cells isolated from starved rats the apparent C^J_{ADH} was 0.37 (using TMSO) and 0.43 (using IsB) (see Table 4.7). For liver cells of fed rats a range of flux control coefficients for ADH (Table 4.8) were obtained. All of these were corrected.

Correction of the apparent C^J_{ADH} for substrate and/or product concentrations is shown in Tables 4.11 - 4.13 and Tables 4.14 - 4.18 for liver cells isolated from starved and fed rats respectively. The corrected C^J_{ADH} differed considerably depending on the kinetic constants that were used (Table 4.2) ; however in all cases the apparent flux control coefficient increased when the effect of both substrates and changing product concentrations were taken into consideration.

The calculations for correction of the apparent C^J_{ADH} determined in hepatocytes isolated from starved and fed rats indicate that:

(i) For both substrates in the absence of products (Table 4.11 and 4.14), the corrected C^J_{ADH} was higher than the apparent C^J_{ADH} .

(ii) For both substrates in the presence of NADH , the corrected C^J_{ADH} decreased slightly with increasing NADH concentration (Tables 4.11, 4.15 , and 4.16).

(iii) For both substrates in the presence of NADH and acetaldehyde (Tables 4.12, 4.13, 4.17, and 4.18), the corrected C^J_{ADH} decreased slightly with increasing NADH concentration at a constant [AcH].

For liver cells isolated from starved rats, the flux control coefficient of ADH after correction increased with increasing acetaldehyde concentration at a constant NADH] (Tables 4.12 and 4.13). This increase was very small when using the kinetic parameters of Cornell *et al.* (1979). The increase was greater when the

TABLE 4.11

The Effect of Both Substrates and Changing NADH Concentration on the Apparent C^J_{ADH} Determined from Liver Cells Isolated from Starved Rats

		Kinetic Parameters of					
		Cornell *et al.* (1979)			Crabb *et al.* (1983)		
Both Substrates			C^J_{ADH}			C^J_{ADH}	
		C F	TMSO	IsB	C F	TMSO	IsB
Dead End EQ-I (a)		1.46	0.542	0.628	1.12	0.414	0.482
Dead End EQ-I/E A-I (b)		1.32	0.490	0.568	1.07	0.396	0.460
Both Substrates + [Q] Assume P ≈ 0			C^J_{ADH}			C^J_{ADH}	
	[NADH] μM	C F	TMSO	IsB	C F	TMSO	IsB
Dead End	2.5	1.33	0.494	0.572	1.10	0.407	0.473
EQ-I (c)	1.25	1.39	0.514	0.598	1.11	0.410	0.477
	0.8	1.41	0.523	0.606	1.12	0.412	0.482
Dead End	2.5	1.24	0.458	0.533	1.060	0.390	0.456
EQ-I / EA-I	1.25	1.27	0.471	0.546	1.0624	0.393	0.457
(d)	0.8	1.29	0.477	0.555	1.064	0.394	0.458

The correction factor (CF) was calculated using the data in Tables 4.2 and 4.10, and the derived equations A,B,C and D in Table 4.9

The apparent C^J_{ADH} was 0.37 (using TMSO) and 0.43 (using IsB)

TABLE 4.12

Effect of Both Substrates and Changing Product Concentrations on the C^J_{ADH} Evaluated from Liver Cells Isolated from Starved Rats Assuming TMSO and IsB are Uncompetitive Inhibitors

	Kinetic Parameters of Cornell *et al.* (1979)								
	Increasing [NADH] μM								
	0.8 μM			1.25 μM			2.5 μM		
[AcH]	C^J_{ADH}			C^J_{ADH}			C^J_{ADH}		
μM	C F	TMSO	IsB	C F	TMSO	IsB	C F	TMSO	IsB
0.5	1.42	0.525	0.611	1.40	0.518	0.602	1.34	0.496	0.526
1.0	1.43	0.529	0.615	1.40	0.518	0.602	1.34	0.496	0.526
2.0	1.44	0.533	0.619	1.42	0.525	0.611	1.35	0.500	0.581
	Kinetic Parameters of Crabb *et al.* (1983) *								
0.5	1.16	0.429	0.499	1.15	0.426	0.495	1.14	0.422	0.490
1.0	1.20	0.444	0.516	1.19	0.440	0.512	1.18	0.437	0.507
2.0	1.29	0.477	0.555	1.28	0.470	0.550	1.26	0.466	0.542

The correction factor (CF) was calculated using the data in Tables 4.2 and 4.10, and using the derived equation E in Table 4.9

The apparent C^J_{ADH} was 0.37 (using TMSO) and 0.43 (using IsB)

* The values of the corrected C^J_{ADH} are wrong because the K_{ib} and K_{ip} kinetic constants of Crabb *et al.* (1983) are incorrect

TABLE 4.13

Effect of Both Substrates and Changing Product Concentrations on the C^J_{ADH} Evaluated from Liver Cells Isolated from Starved Rats Assuming TMSO and IsB are Noncompetitive Inhibitors

[AcH] μM	Kinetic Parameters of Cornell *et al.* (1979)								
	Increasing [NADH] μM								
	0.8 μM			1.25 μM			2.5 μM		
	C^J_{ADH}			C^J_{ADH}			C^J_{ADH}		
	C F	TMSO	IsB	C F	TMSO	IsB	C F	TMSO	IsB
0.5	1.29	0.477	0.555	1.28	0.474	0.550	1.34	0.459	0.533
1.0	1.30	0.481	0.559	1.28	0.474	0.550	1.34	0.459	0.533
2.0	1.30	0.481	0.559	1.29	0.477	0.555	1.35	0.463	0.538
	Kinetic Parameters of Crabb *et al.* (1983) *								
0.5	1.10	0.407	0.473	1.10	0.407	0.478	1.09	0.403	0.469
1.0	1.14	0.422	0.490	1.14	0.422	0.490	1.13	0.418	0.486
2.0	1.22	0.451	0.525	1.21	0.448	0.520	1.20	0.444	0.516

The correction factor (CF) was calculated using the data in Tables 4.2 and 4.10, and using the derived equation F in Table 4.9

The apparent C^J_{ADH} was 0.37 (using TMSO) and 0.43 (using IsB)

* The values of the corrected C^J_{ADH} are wrong because the K_{ib} and K_{ip} kinetic constants of Crabb *et al.* (1983) are incorrect

TABLE 4.14

Effect of Both Substrates in the Absence of Products on the C^J_{ADH} Evaluated from Liver Cells Isolated from Fed Rats

Apparent C^J_{ADH}	Kinetic Parameters of: Cornell *et al.* (1979) — Dead End EQ-I (a) CF*	C^J_{ADH}	Dead End EQ-I/ EA-I (b) CF+	C^J_{ADH}	Crabb *et al.* (1983) — Dead End EQ-I (a) CF*	C^J_{ADH}	Dead End EQ-I/ EA-I (b) CF+	C^J_{ADH}
0.137	1.46	0.200	1.32	0.180	1.12	0.153	1.07	0.147
0.020	1.46	0.029	1.32	0.026	1.12	0.022	1.07	0.020
0.072	1.46	0.105	1.32	0.095	1.12	0.081	1.07	0.077
0.213	1.46	0.311	1.32	0.281	1.12	0.239	1.07	0.228
0.253	1.46	0.370	1.32	0.330	1.12	0.283	1.07	0.271
0.410	1.46	0.600	1.32	0.541	1.12	0.459	1.07	0.440
0.430	1.46	0.628	1.32	0.568	1.12	0.482	1.07	0.460
0.538	1.46	0.785	1.32	0.710	1.12	0.602	1.07	0.576

* The correction factor (CF) was calculated using the data in Tables 4.2 and 4.10, and using the derived equation A in Table 4.9

+ The correction factor (CF) was calculated using the data in Tables 4.2 and 4.10, and using the derived equation B in Table 4.9

TABLE 4.15

Effect of Both Substrates and Changing NADH Concentration on the C^J_{ADH} Evaluated from Liver Cells Isolated from Fed Rats Assuming TMSO is an Uncompetitive Inhibitor

Kinetic Parameters of

	Cornell *et al.* (1979)						Crabb *et al.* (1983)					
	Increasing [NADH] μM						Increasing [NADH] μM					
Apparent C^J_{ADH}	0.8 μM		1.25 μM		2.5 μM		0.8 μM		1.25 μM		2.5 μM	
	C F	C^J_{ADH}	C F	C^J_{ADH}	C F	C^J_{ADH}	C F	C^J_{ADH}	C F	C^J_{ADH}	C F	C^J_{ADH}
0.137	1.41	0.193	1.39	0.190	1.33	0.182	1.12	0.153	1.11	0.152	1.10	0.151
0.021	1.41	0.028	1.39	0.028	1.33	0.027	1.12	0.022	1.11	0.022	1.10	0.022
0.072	1.41	0.102	1.39	0.100	1.33	0.096	1.12	0.081	1.11	0.08	1.10	0.079
0.213	1.41	0.300	1.39	0.296	1.33	0.283	1.12	0.239	1.11	0.236	1.10	0.234
0.253	1.41	0.357	1.39	0.352	1.33	0.336	1.12	0.283	1.11	0.281	1.10	0.278
0.410	1.41	0.578	1.39	0.570	1.33	0.545	1.12	0.459	1.11	0.455	1.10	0.451
0.430	1.41	0.606	1.39	0.598	1.33	0.572	1.12	0.482	1.11	0.477	1.10	0.473
0.538	1.41	0.759	1.39	0.748	1.33	0.716	1.12	0.603	1.11	0.597	1.10	0.592

The correction factor (C F) was calculated using the data in Tables 4.2 and 4.10, and using the derived equation C in Table 4.9.

TABLE 4.16

Effect of Both Substrates and Changing NADH Concentration on the C^J_{ADH} Evaluated from Liver Cells Isolated from Fed Rats Assuming TMSO is a Noncompetitive Inhibitor

Kinetic Parameters of

	Cornell *et al.* (1979)						Crabb *et al.* (1983)					
	Increasing [NADH] μM						Increasing [NADH] μM					
Apparent C^J_{ADH}	0.8 μM		1.25 μM		2.5 μM		0.8 μM		1.25 μM		2.5 μM	
	C F	C^J_{ADH}	C F	C^J_{ADH}	C F	C^J_{ADH}	C F	C^J_{ADH}	C F	C^J_{ADH}	C F	C^J_{ADH}
0.137	1.29	0.177	1.27	0.174	1.24	0.170	1.064	0.146	1.062	0.145	1.06	0.145
0.021	1.29	0.026	1.27	0.025	1.24	0.025	1.064	0.021	1.062	0.021	1.06	0.021
0.072	1.29	0.093	1.27	0.091	1.24	0.089	1.064	0.077	1.062	0.076	1.06	0.076
0.213	1.29	0.275	1.27	0.270	1.24	0.264	1.064	0.227	1.062	0.226	1.06	0.226
0.253	1.29	0.326	1.27	0.321	1.24	0.314	1.064	0.269	1.062	0.269	1.06	0.268
0.410	1.29	0.529	1.27	0.521	1.24	0.508	1.064	0.436	1.062	0.435	1.06	0.435
0.430	1.29	0.555	1.27	0.546	1.24	0.533	1.064	0.458	1.062	0.457	1.06	0.457
0.538	1.29	0.694	1.27	0.683	1.24	0.667	1.064	0.572	1.062	0.571	1.06	0.570

The correction factor (C F) was calculated using the data in Tables 4.2 and 4.10, and using the derived equation D in Table 4.9.

TABLE 4.17

Effect of Both Substrates and Changing Product Concentrations on the C^J_{ADH} Evaluated from Liver Cells Isolated from Fed Rats Assuming TMSO is an Uncompetitive Inhibitor

Kinetic Parameters of

		Cornell *et al.* (1979)						Crabb *et al.* (1983)*					
		Increasing [NADH] μM						Increasing [NADH] μM					
[AcH]	Apparent	0.8 μM		1.25 μM		2.5 μM		0.8 μM		1.25 μM		2.5 μM	
μM	C^J_{ADH}	C F	C^J_{ADH}	C F	C^J_{ADH}	C F	C^J_{ADH}	C F	C^J_{ADH}	C F	C^J_{ADH}	C F	C^J_{ADH}
143	0.137	3.41	0.467	3.31	0.450	3.07	0.420	13.43	1.840	13.15	1.800	12.45	1.710
138	0.020	3.35	0.067	3.25	0.065	3.00	0.060	12.97	0.259	12.70	0.254	12.03	0.241
80.6	0.072	2.55	0.183	2.48	0.179	2.31	0.166	8.06	0.580	7.90	0.569	7.50	0.540
31.5	0.213	1.86	0.396	1.81	0.387	1.71	0.364	3.82	0.814	3.76	0.800	3.59	0.765
42	0.253	2.00	0.506	1.96	0.495	1.84	0.466	4.73	1.200	4.65	1.180	4.43	1.120
15.7	0.410	1.63	0.670	1.60	0.657	1.50	0.615	2.47	1.010	2.43	0.996	2.35	0.960
11.3	0.430	1.57	0.680	1.54	0.663	1.47	0.632	2.09	0.899	2.06	0.886	1.99	0.856
6	0.538	1.50	0.867	1.47	0.790	1.40	0.753	1.63	0.877	1.61	0.866	1.57	0.845

The correction factor (C F) was calculated using the data in Tables 4.2 and 4.10, and using the derived equation E in Table 4.9

* The values of the corrected C^J_{ADH} are wrong because the K_{ib} and K_{ip} kinetic constants of Crabb *et al.* (1983) are incorrect

TABLE 4.18

Effect of Both Substrates and Changing Product Concentrations on the C^J_{ADH} Evaluated from Liver Cells Isolated from Fed Rats Assuming TMSO is a Noncompetitive Inhibitor

		Kinetic Parameters of											
		Cornell *et al.* (1979)						Crabb *et al.* (1983) *					
		Increasing [NADH] μM						Increasing [NADH] μM					
[AcH] μM	Apparent C^J_{ADH}	0.8 μM		1.25 μM		2.5 μM		0.8 μM		1.25 μM		2.5 μM	
		C F	C^J_{ADH}	C F	C^J_{ADH}	C F	C^J_{ADH}	C F	C^J_{ADH}	C F	C^J_{ADH}	C F	C^J_{ADH}
143	0.137	1.97	0.270	1.92	0.263	1.82	0.249	7.30	1.000	7.24	0.990	7.04	0.960
138	0.020	1.95	0.039	1.92	0.038	1.81	0.036	7.18	0.144	7.11	0.142	6.90	0.138
80.6	0.072	1.76	0.127	1.73	0.124	1.64	0.118	5.40	0.389	5.33	0.384	5.15	0.371
31.5	0.213	1.53	0.326	1.50	0.320	1.43	0.306	3.13	0.667	3.09	0.658	2.99	0.637
42	0.253	1.59	0.402	1.56	0.394	1.49	0.376	3.70	0.936	3.65	0.923	3.53	0.893
15.7	0.410	1.43	0.587	1.41	0.577	1.35	0.553	2.18	0.894	2.15	0.882	2.09	0.857
11.3	0.430	1.40	0.600	1.38	0.592	1.32	0.569	1.88	0.808	1.87	0.804	1.82	0.783
6	0.538	1.36	0.730	1.34	0.720	1.29	0.695	1.51	0.812	1.50	0.807	1.47	0.791

The correction factor (C F) was calculated using the data in Tables 4.2 and 4.10, and using the derived equation F in Table 4.9.

* The values of the corrected C^J_{ADH} are wrong because the K_{ib} and K_{ip} kinetic constants of Crabb *et al.* (1983) are incorrect

kinetic parameters of Crabb *et al.* (1983) were used. However, the concentration of acetaldehyde present in hepatocytes of starved rats is low (maximum concentration of 2 μM) and even when using the kinetic constants of Crabb *et al.* (1983), the effect of acetaldehyde on the flux control coefficient of ADH for hepatocytes isolated from starved rats is very small.

For liver cells isolated from fed rats, at a constant [NADH] the value of the corrected C^J_{ADH} decreased with increasing [AcH] (Tables 4.17 and 4.18). When using the kinetic parameters of Cornell *et al.* (1979) the relationship observed between acetaldehyde concentration and C^J_{ADH} (see section 4.4.2) is still noticeable i.e. at low [AcH] a high C^J_{ADH} is observed and at a high [AcH] a low C^J_{ADH} is observed. However, with the kinetic parameters of Crabb *et al.* (1983), this correlation is no longer observed.

In all cases, the correction factor calculated for the equations where inhibitor binds to both EQ and EA (B, D, and F in Table 4.9) was lower than those calculated for the equations where dead end EA-I complexes were formed (A, C, and E in Table 4.9). Hence when TMSO and IsB were considered to be noncompetitive inhibitors against ethanol the corrected C^J_{ADH} was less than the corrected flux control coefficient of ADH obtained when TMSO and IsB were assumed to exhibit uncompetitive inhibition.

As already stated the corrected C^J_{ADH} obtained using the kinetic parameters of Cornell *et al.* (1979) and Crabb *et al.* (1983) (see Table 4.2) were different especially when NADH and acetaldehyde concentrations were considered (equations E and F in Table 4.9 ; Table 4.17 and 4.18). Extremely high correction factors were calculated when using Crabb *et al.* (1983) kinetic parameters (Table 4.17 and 4.18). These high correction factors (C F) were obtained because:

(1) The $\frac{ABP}{K_{ip}}$ term in the numerator of the correction factor is much higher than for Cornells' data. The K_{ip} value from Cornell *et al.* (1979) is 11 times greater than that from Crabb *et al.* (1983). With increasing acetaldehyde concentration the $\frac{ABP}{K_{ip}}$ term increases; however Cornell's K_{ip} of 134 μM in effect reduces the effect of the high AcH concentrations that can be obtained whereas Crabb's K_{ip} of 12 μM does not.

(2) Cornell's K_{ib} value (20300 μM) is 25 times greater than that for Crabb *et al.* (1983) (810 μM). Hence the $\frac{BPQ}{K_{ib}}$ term in the numerator of the correction factor is higher with the Crabb *et al.* (1983) inhibition constant for ethanol.

(3) Using Cornell *et al.* (1979) kinetic data for ADH, the denominator of the correction factor is larger than Crabb *et al.* (1983) hence smaller correction factors are obtained using Cornell's kinetic parameters.

4.5.2 Conclusion

The best set of kinetic parameters for rat liver ADH from these results (Tables 4.11- 4.18) appears to be that of Cornell *et al.* (1979). More realistic values were obtained using Cornells' kinetic data. In general, using the kinetic constants of Cornell *et al.* (1979) the apparent C^J_{ADH} in liver cells isolated from starved rats after correction for substrate and product concentration increased from 0.37 to a maximum of 0.53 (for TMSO studies) and from 0.43 to a maximum of 0.62 (for IsB studies) (see Tables 4.11- 4.13). Because the sum of all the flux control coefficients in a metabolic pathway is equal to one, the C^J_{ADH} of around 0.5 to 0.7 indicates that the rest of the pathway has a flux control coefficient of 0.3 to 0.5. Hence alcohol dehydrogenase is a major factor in the regulation of ethanol metabolism of starved rats .

The results for isolated hepatocytes from fed rats (Tables 4.14 - 4.18) using Cornells' kinetic parameters show that the C^J_{ADH} increased once it was corrected for NAD^+, NADH, and acetaldehyde concentration. The flux control coefficient of ADH determined in hepatocytes isolated from fed rats at low concentrations of acetaldehyde (Tables 4.14 - 4.18) is similar to that determined in hepatocytes isolated from starved rats (Tables 4.11 - 4.13), where acetaldehyde levels are always low (1-2 μM) (Crow *et al.*, 1983a). The observation that the importance of ADH in regulation of ethanol metabolism decreases with increasing acetaldehyde concentration in hepatocytes from fed rats is still apparent after correction of the flux control coefficent for ADH using Cornell's kinetic parameters. These results indicate that the activity of AlDH and acetaldehyde concentration may be important factors in the regulation of ethanol oxidation in fed rats. They also suggest that the relationship between the activities of ADH and AlDH is significant in regulation of ethanol metabolism in rats.

In summary, correction of the C^J_{ADH} does not affect the overall conclusion that alcohol dehydrogenase is a major flux-controlling enzyme in the metabolism of ethanol in starved rats but is less important in fed rats at high concentrations of acetaldehyde.

CHAPTER 5

ALDEHYDE DEHYDROGENASE

5.1 INTRODUCTION

Acetaldehyde is oxidised predominantly by aldehyde dehydrogenase (AlDH):

$$\underset{\text{acetaldehyde}}{CH_3CHO} + NAD^+ \xrightleftharpoons{AlDH} \underset{\text{acetate}}{CH_3COO^-} + NADH + H^+$$

There is a considerable amount of evidence that supports the importance of AlDH in the removal of acetaldehyde. For instance studies using disulfiram (Tottmar & Marchner, 1976; Crow *et al.*, 1977a; Marchner & Tottmar, 1978; Weiner, 1979) and cyanamide (Marchner & Tottmar, 1978), which are specific inhibitors of aldehyde dehydrogenase, have shown that in the presence of inhibitor, concentrations of acetaldehyde (AcH) increased during the oxidation of ethanol by rat liver. There are other enzymes that could be involved in acetaldehyde oxidation (aldehyde oxidase and xanthine oxidase); however these enzymes have K_m's for acetaldehyde in the range of 1-10 mM (Khanna & Israel, 1980; Weiner, 1980) and are unlikely to be of significance *in vivo*, where concentrations of acetaldehyde less than 20 μM are present in the liver during oxidation of ethanol (Crow *et al.*, 1983a).

In rat liver, there are several AlDH isoenzymes distributed throughout the cell (Tottmar *et al.*, 1973; Koivula & Koivusalo, 1975; Lindahl & Evces , 1984a). The isoenzymes are generally grouped into two classes based on their affinity for acetaldehyde : low-K_m (1 μM or less) and high-K_m (1 mM or greater).

The partial purification of some isoenzymes of rat liver aldehyde dehydrogenase has been reported (Tottmar *et al.*, 1973; Koivula & Koivusalo, 1975). They appear to be located in the cytosol, mitochondria, and microsomes. Further studies have shown that the AlDH activity measured in the cytosol of rat livers is inducible (e.g. phenobarbital and TCDD (2,3,7,8-tetrachlorodibenzo-*p* - dioxin) induce AlDH isoenzymes in normal liver and tumor specific isoenzymes are induced during hepatocarcinogenesis) (Lindahl & Evces, 1984a,b). Lindahl & Evces (1984b) have isolated four inducible cytosolic hepatic AlDH isoenzymes in rat which preferentially oxidise aromatic aldehydes.

There is an AlDH isozyme found exclusively in the mitochondria, that has a K_m value for acetaldehyde in the micromolar range (Tottmar *et al.* , 1973; Koivula & Koivusalo, 1975). This low-K_m hepatic AlDH has been purified from rat liver mitochondria by affinity chromatography (Poole & Halestrap, 1989).

Recently four distinct AlDH isozymes with K_m values for acetaldehyde in the millimolar range have been characterised from rat liver mitochondrial and microsomal fractions (Lindahl & Evces , 1984a; Senior & Tsai, 1988). These high-K_m isoenzymes are clearly distinguishable from each other by substrate or coenzyme specificity , response to pH and heat , sensitivity to inhibitors and apparent molecular weight (Lindahl & Evces, 1984a).

It has been shown that cyanamide is a specific inhibitor of the low-K_m mitochondrial AlDH *in vivo* (Loomis & Brien, 1983) and *in vitro* in rat liver slices, intact mitochondria (Svanas & Weiner, 1985) and subcellular fractions of rat liver (Harrington *et al.*, 1988). Through the use of this inhibitor, it has been demonstrated that the low-K_m AlDH in the mitochondria provides the major contribution to acetaldehyde oxidation at physiological concentrations of acetaldehyde (Svanas & Weiner, 1985; Harrington *et al.*, 1988). However the quantitative importance of AlDH in regulation of ethanol metabolism has not been assessed.

I have tried to determine the significance of AlDH in the regulation of ethanol oxidation in starved rats. The modulation methodology of Kacser and Burns (1973) and the procedure for measurement of small changes in flux (described in Chapter 3) were employed for evaluation of the flux control coefficient of AlDH in hepatocytes isolated from starved rats. The activity of AlDH was altered in small amounts using the AlDH- specific inhibitor disulfiram (Kitson, 1978). In rats disulfiram appears to inhibit both the mitochondrial and cytosolic forms of aldehyde dehydrogenase (Deitrich & Erwin, 1971).

Disulfiram is an irreversible inhibitor of aldehyde dehydrogenase (Kitson, 1978) and the flux control coefficient can therefore be calculated directly from the inhibition curve (flux vs [I]) using the relationship (Groen *et al.*, 1982c; Derr, 1986):

$$C_E^J = \frac{-I_{max}\, dJ}{J\ dI} \qquad (5.1)$$

where J = the flux through the pathway at zero inhibitor concentration

dJ/dI = the initial slope of the inhibition curve

I_{max} = the concentration of inhibitor required for total inhibition of the enzyme

As stated in the introduction (chapter 1) the data presented in this chapter are preliminary results since experimental work had to be stopped early as a result of an allergy to rats.

5.2 EXPERIMENTAL RESULTS AND DISCUSSION

Experiments were carried out using isolated hepatocytes from rats starved for 48 hours. Acetaldehyde concentrations were measured in all cases and the ethanol clearance rates were standardised by dividing by the noninhibited rate. Due to standardisation of the ethanol oxidation rates the normalised flux (J) through the ethanol pathway at zero inhibitor concentration is equal to one. All experiments were treated individually and the initial slope ($\left(\frac{dJ}{dI}\right)_{I=0}$) of the flux inhibition plot was determined as described in section 4.4.1.

The range of disulfiram concentrations tested in the titration experiments was 1 to 200 μM. This concentration range was chosen on the basis of previous results obtained by Crow *et al.* (1977a) in which ethanol oxidation rates in cells from starved rats were inhibited by 42% at a disulfiram concentration of 100 μM. The acetaldehyde concentrations and % inhibition results of three disulfiram titration experiments are compared to those of Crow *et al.* (1977a) in Table 5.1.

The concentrations of acetaldehyde present in the isolated hepatocytes of the three cell preparations from this study are much lower than those observed by Crow *et al.* (1977a). This difference is not unexpected as Braggins and Crow (1981) observed that the concentration of acetaldehyde formed during ethanol metabolism varied widely between rats.

For the three cell preparations (see Table 5.1) the agreement between the % inhibition values at the different disulfiram concentrations is poor. This variation was attributed to the difference in the extent that disulfiram dissolved and hence the disulfiram stock solutions of each experiment had a different concentration. This meant that the cells of all three experiments would not be receiving the same concentration of disulfiram, let alone the correct concentration. Preparation of the stock solutions of disulfiram was very difficult because disulfiram is sparingly soluble in water (Kitson, 1977). The stock solutions were prepared by trying to dissolve disulfiram in incubation medium (section 2.3.2) one to two days before the experiment. The solution was occasionally stirred and kept at a constant temperature of 37 °C.

The final dissolved concentration of the disulfiram stock solution was unpredictable and hence the exact concentration of disulfiram present in the titration experiments was not known. This is probably why the variable results shown in Table 5.1 were obtained. The results of cell preparation 3 (Table 5.1) were odd in that high inhibition was obtained when the concentrations of acetaldehyde were very low. The method for measuring the acetaldehyde concentrations (see

TABLE 5.1

% Inhibition and Acetaldehyde Concentration with Increasing Concentrations of Disulfiram in Hepatocytes Isolated from Starved Rats

Disulfiram Concentration (μM)	Crow*et al.* (1977a) +		Cell Preparation					
			1		2		3	
	[AcH] μM	%I	[AcH] μM	%I	[AcH] μM	%I	[AcH] μM	%I
0	2.10 ± 0.20		1.5		2.0		1.4	
1	4.40 ± 0.99	0.8	1.7	0.0	*	-	1.5	0.6
10	85.70 ± 13.40	25.0	13.6	3.2	*	-	2.75	30.1
100	178.40 ± 8.40	42.4	23.5	41.7	112.0	51.0	7.87	61.9
200			25.0	61.7	113.0	52.3		

+ The results of Crow *et al.* (1977a) are the means ± SEM of three experiments.

* 1 and 10 μM disulfiram concentrations were not tested in cell preparation 2; thus C^{J}_{AlDH} can not be calculated from this data.

section 2.10) was re-examined. No fault with the method was discovered so the low acetaldehyde concentrations obtained for cell preparation 3 remain unexplained. However the % inhibition values of cell preparation 3 (Table 5.1) were similar to those of Crow *et al.* (1977a).

Flux inhibition plots (normalised flux versus [I]) for the data of Crow *et al.* (1977a) and cell preparation 3 are shown in Figures 5.1 and 5.2 respectively.

The minimum amount of disulfiram required for complete inhibition of AlDH was obtained by extrapolation as indicated in Figures 5.1 and 5.2 (see also Duszynski *et al.*, 1982 ; Groen *et al.*, 1982a). The initial slope ($\left(\frac{dJ}{dI}\right)_{I=0}$) of the inhibition curve was determined from the inhibition observed at 1 μM disulfiram (i.e. the chord between zero inhibitor concentration and 1 μM disulfiram concentration). The I_{max} , initial slope and flux control coefficient of AlDH (C^J_{AlDH}) obtained from the data of Crow *et al.* (1977a) and cell preparation 3 are shown in Table 5.2 .

Table 5.2

Flux Control Coefficient of AlDH in Hepatocytes Isolated from Starved Rats

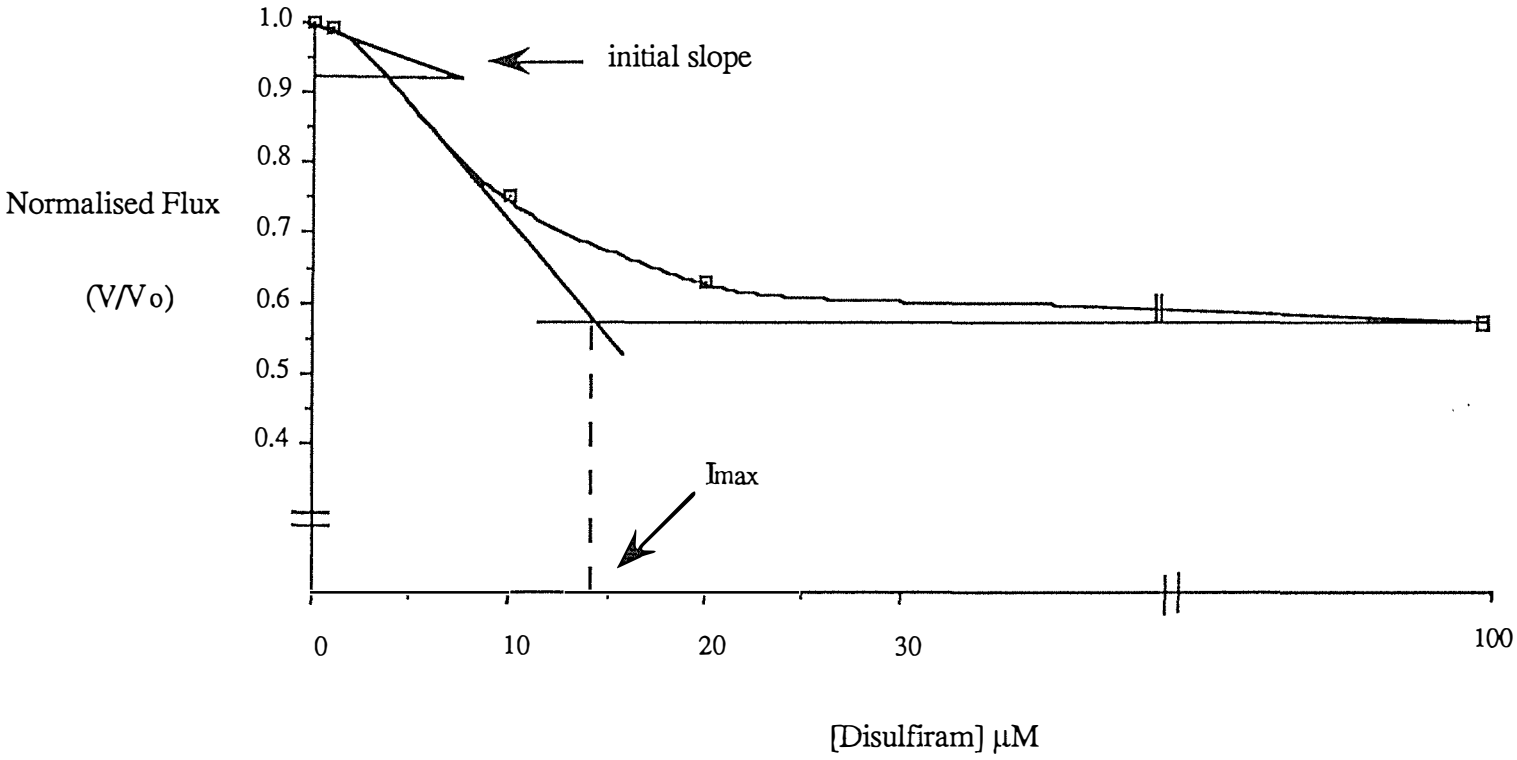
	Crow *et al.* (1977a)	Cell Preparation 3
Initial slope $(\mu M)^{-1}$	0.008	0.006
I_{max} (μM)	14	19
C^J_{AlDH}	0.11	0.11

The I_{max} value (Table 5.2) is only an estimate. More points on the flux inhibition curve in the 1-100 μM disulfiram concentration range and 100 μM onwards are required to define more closely the slopes used in determining the I_{max} value and hence provide a more accurate maximum disulfiram concentration for complete inactivation of AlDH. In view of this the C^J_{AlDH} , of about 0.1 (1 significant figure), obtained from these disulfiram experiments is only a preliminary value.

There were two possibilities for obtaining a more accurate value for the flux control coefficient of aldehyde dehydrogenase. The first was to increase the solubility of disulfiram by using an organic solvent. Ethanol is a well known solvent of disulfiram (Blake, 1943). However introducing additional ethanol into the inhibitor titration experiments would provide extra complications to consider in the final calculation of the ethanol clearance rates. Because of these possible problems,

Figure 5.1

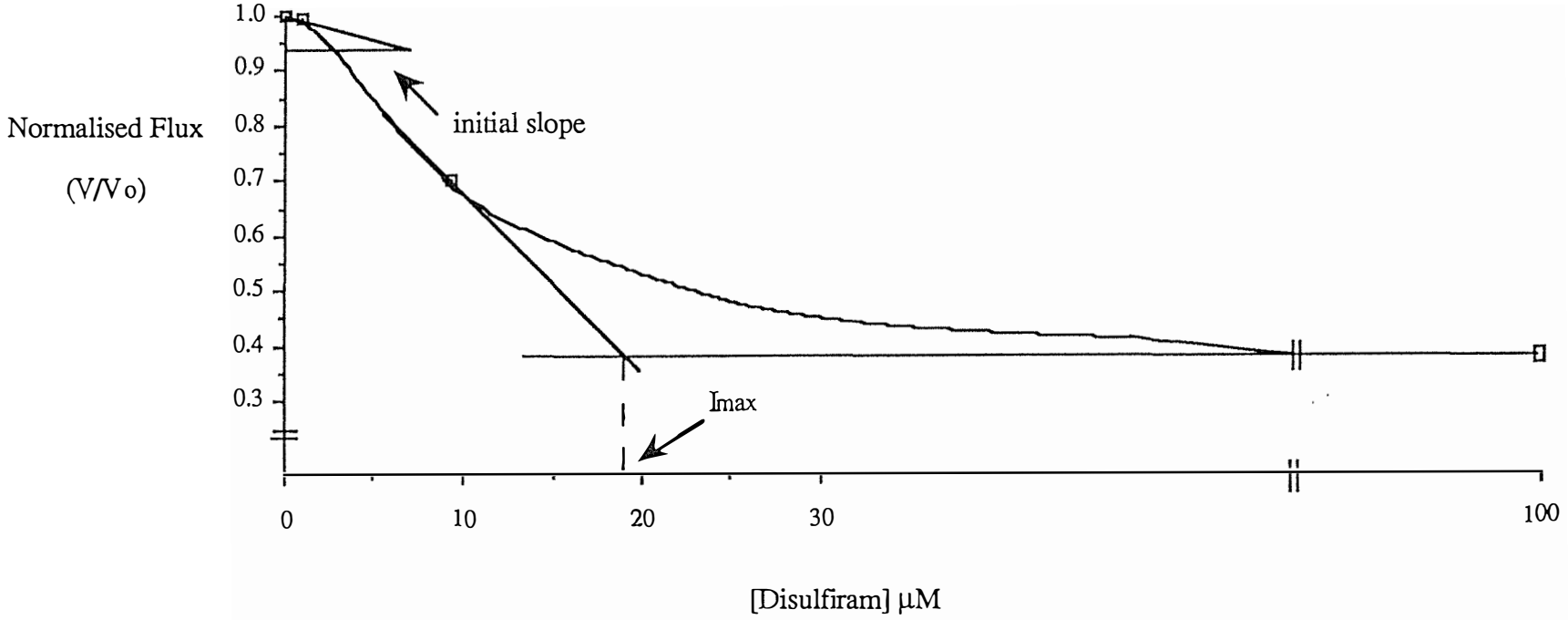
Flux Inhibition Curve for Data of Crow *et al.* (1977a)



The initial slope ($\left(\frac{dJ}{dI}\right)_{I=0}$) of this flux inhibition curve is shown as the chord between zero inhibitor concentration and 1 μM disulfiram concentration

Figure 5.2

Flux Inhibition Curve for Cell Preparation 3



The initial slope ($\left(\frac{dJ}{dI}\right)_{I=0}$) of this flux inhibition curve is shown as the chord between zero inhibitor concentration and 1 μM disulfiram concentration

this option for increasing the solubility of disulfiram was eliminated. The second possibility was to use diethyldithiocarbamate (DDC) which is a metabolite of disulfiram (Kitson, 1977, 1983). When disulfiram enters the blood stream it is rapidly reduced to DDC by reaction with glutathione (Cobby *et al.*, 1977). Dietrich and Erwin (1971) observed that DDC was as effective an inhibitor of AlDH *in vivo* as disulfiram; however DDC has little inhibitory action on AlDH *in vitro* (Kitson, 1982). In order for diethyldithiocarbamate to cause inactivation of AlDH *in vivo*, it was assumed that *in vivo* DDC is reoxidised to disulfiram in sufficient amounts for the latter to inhibit AlDH in the same way as it does *in vitro* (Kitson, 1977). There are proteins such as cyt c oxidase (DuBois *et al.*, 1977), methemoglobin (Stromme, 1963) and xanthine oxidase (Fried, 1976) that aid the conversion of DDC to disulfiram. It is also possible that DDC may be co-oxidised with some other thiol, such as glutathione, to form a mixed disulphide which is capable of inhibiting aldehyde dehydrogenase (Kitson, 1975).

Diethyldithiocarbamate seemed to be an appropiate compound to use for inactivation of AlDH. It was hoped that in the isolated hepatocytes, DDC would be oxidised to disulfiram which would then inhibit aldehyde dehydrogenase. The liver cells would be more likely to receive the correct concentrations of DDC because stock solutions of known concentrations were easy to prepare.

The results of the disulfiram experiments (Table 5.1) indicated that a greater range of inhibitor concentrations should be tested so that a more accurate value for the C^{J}_{AlDH} could be obtained. Because of the limited amount of isolated hepatocytes collected for each cell preparation I decided that the concentration of DDC required for complete inhibition of AlDH would be determined first. A range of concentrations 100 μM and upwards was tested and the results are shown in Table 5.3.

These results (Table 5.3) are very promising. They showed that in isolated hepatocytes DDC does cause inhibition of aldehyde dehydrogenase. An interesting pattern is observed for the levels of acetaldehyde from the samples containing higher DDC concentrations. For each cell preparation, similar concentrations of AcH are observed for 200 and 300 μM DDC samples and 500 and 700 μM samples. Also the concentration of AcH for 100 μM DDC samples is greater than those obtained for the samples containing higher DDC concentrations. This could indicate that at high DDC concentrations ADH is being inactivated as well as aldehyde dehydrogenase. Diethyldithiocarbamate does inhibit ADH by chelating the zinc atoms (Bethune & Vallee, 1967).

A plot of normalised flux ($\frac{100\text{-}\%I}{100}$) against the high concentrations of DDC for cell preparations 1 and 3 (Figure 5.3) shows that the inhibition of ethanol oxidation is levelling off in the 300-500 μM region and appears to drop again at 700 μM DDC. This drop noted at 700 μM could be due to experimental error or it may also suggest that another enzyme such as ADH is being inhibited. A

few more titration experiments testing the DDC concentration range of 100 μM and higher are required. However from the results (Table 5.3 and Figure 5.3) it can be assumed that complete inhibition of AlDH is obtained for diethyldithiocarbamate concentrations in the 300-500 μM range. The possibility that AlDH will not be the only enzyme being inhibited since DDC is a powerful metal ion chelator (Goldstein *et al.*, 1964) will become more significant if concentrations of DDC higher than 200 μM are used.

Table 5.3

The % Inhibition and Acetaldehyde Concentrations Obtained Using a Range of Diethyldithiocarbamate Concentrations

	Cell Preparation					
	1		2 ++		3	
[DDC] μM	[AcH] μM	%I	[AcH] μM	%I	[AcH] μM	%I
0	1.9		1.9		1.9	
100	25.1	40.0	*	-	44.6	33.5
200	21.9	61.0	7.8	-	28.5	51.2
300	20.3	64.0	6.1	-	26.9	62.9
500	*	-	4.5	-	20.4	65.7
700	*	-	4.5	-	20.4	70.6

The 0 μM DDC samples represent the final time flasks.

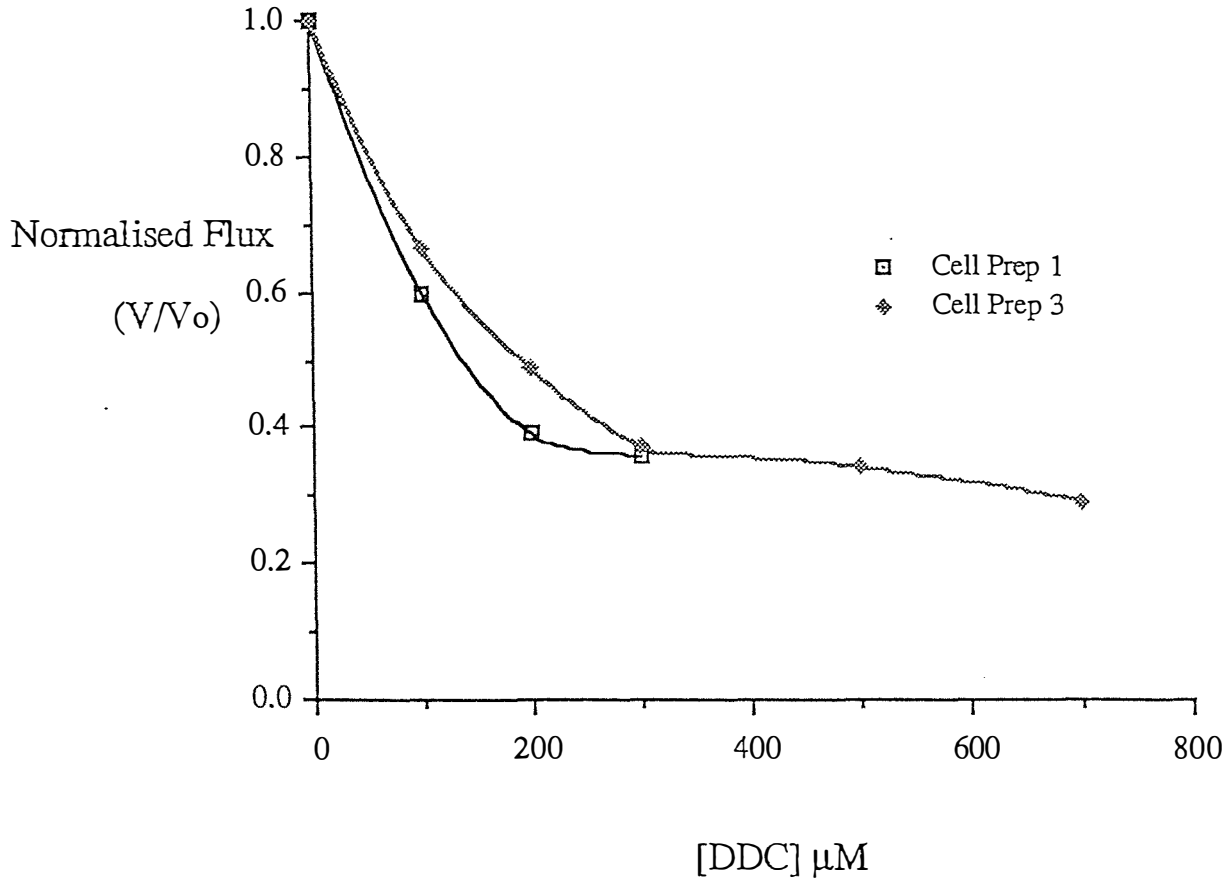
++ The % inhibition results of cell preparation 2 could not be calculated because the cells in the initial time flasks did not survive the 35 minute incubation period i.e [ATP] < 2 μmole/g wet wt cells

* There are no results for acetaldehyde conentration because the cells did not survive the 35 minute incubation period i.e [ATP]< 2μmole/ g wet wt cells

Figure 5.3

Graph of Normalised Flux vs

Diethyldithiocarbamate Concentration



At this point the titration experiments had to be stopped because of health problems developed during the course of my work. The flux control coefficient calculated for AlDH in the disulfiram studies is only a preliminary value. To obtain a satisfactory value for the C^{J}_{AlDH} it would be necessary to finish the experiments using DDC. Once the concentration of DDC for complete inactivation of AlDH is established, the flux control coefficient of AlDH could be determined using one high [DDC] (probably 300-500 μM) in the titration studies, plus a greater number of DDC concentrations tested in the 0-100 μM region than in the disulfiram experiments (Figure 5.2). This would enable the initial slope and middle section of the flux inhibition curve to be examined in more detail and hopefully provide a more accurate I_{max} value and initial slope.

Even though the inhibitor titration experiments have not been completed some interesting points have evolved from the studies carried out. I have shown that DDC does cause the inhibition of AlDH in isolated hepatocytes and the extent of inhibition at 100 and 200 μM DDC is comparable to that observed for disulfiram (compare Tables 5.1 and 5.3). This would suggest that in isolated hepatocytes DDC can be converted to disulfiram.

A major problem with these experiments was the occurence of variable concentrations of acetaldehyde with increasing concentrations of disulfiram and DDC (see Tables 5.1 and 5.3). It is possible that another enzyme such as ADH is being inhibited because DDC is capable of chelating with zinc atoms. However the variability could be attributed to formation of mixed disulphides instead of, or as well as, disulfiram. The amount of disulfiram formed would be subject to variation; hence the extent of inhibition and accumulation of AcH would change. Some mixed disulphides are capable of inhibiting AlDH (Kitson, 1975, 1989; MacKerell *et al.*, 1985). In view of these problems it may be more appropriate to use a different specific inhibitor of AlDH for determining the flux control coefficient.

A possible candidate would be cyanamide. The inhibition of aldehyde dehydrogenase by cyanamide is dependent on an enzyme catalysed conversion of cyanamide to an active metabolite (Deitrich *et al.*, 1976; DeMaster *et al.*, 1985). It has been shown that the active metabolite of cyanamide inhibits the low-K_m mitochondrial AlDH *in vivo* (Loomis & Brien, 1983) and *in vitro* (Svanas & Weiner, 1985; Harrington *et al.*, 1988). It appears that catalase is the cyanamide activating enzyme (DeMaster *et al.*, 1985; Svanas & Weiner, 1985) and that the active metabolite of cyanamide inactivates AlDH by interacting with the aldehyde binding site of the enzyme (Svanas & Weiner, 1985). Apparently the inhibition by cyanamide is essentially irreversible (Deitrich *et al.*, 1976); therefore it would be a suitable inhibitor to use in the modulation approach.

The preliminary value obtained for the C^{J}_{AlDH} of about 0.1 is dependent on the I_{max} and the value for the initial slope of the flux inhibiton curve which was calculated at 1 μM disulfiram. I have already explained how a more accurate value for the I_{max} could be obtained. At 1 μM disulfiram the extent of inhibition was 0.6% for cell preparation 3 (Table 5.1). The significance of this value and thus the inital slope is debatable. In the studies carried out for ADH the lowest extent of inhibition observed was around 8% at 0.05 mM TMSO. Comparing the standard errors of inhibition at 0.05 mM and 0.1mM TMSO in Table 4.5, it appears that the sensitivity and reproducibility of this method is reaching its limits. This indicates that the method developed for measuring the small changes in flux may not be capable of accurately detecting flux changes in the magnitude of 0.6%. Obviously more experiments involving the measurement of flux changes of less than 10% need to be carried out to see if this is true. If this is the case then perhaps a more sensitive method for measuring the small changes in flux through the ethanol pathway, imposed by increasing concentrations of inhibitor, needs to be developed, or a different approach other than the modulation approach will need to be adopted to determine the flux control coefficient of AlDH.

CHAPTER 6

SHUTTLE SYSTEMS AND ELECTRON TRANSPORT CHAIN

This chapter describes the theoretical approach for assessing the contribution of shuttle systems and the electron transport chain to the regulation of ethanol oxidation in rat liver cells.

The inhibitor titration studies were not carried out but literature research was performed on the inhibitors required for the determination of the flux control coefficient.

6.1 SHUTTLE SYSTEMS

Oxidation of ethanol to acetaldehyde by alcohol dehydrogenase results in the formation of NADH in the cytosolic compartment of the liver cell. Since intact mitochondria are impermeable to NADH (Lehninger, 1951), oxidation of NADH by the respiratory chain requires the transfer of the reducing equivalents of NADH into the mitochondria by a hydrogen shuttle mechanism. The two most important shuttle systems in the liver are the malate-aspartate shuttle (Bucher & Klingenberg, 1958) and the α-glycerophosphate (α-GP) shuttle (Klingenberg & Bucher, 1961).

In the latter shuttle (Figure 6.1), dihydroxyacetone phosphate (DHAP) is reduced to glycerol-3-phosphate in the cytosol by glycerol-3-phosphate dehydrogenase and NADH. A flavin linked glycerophosphate dehydrogenase is bound to the outer surface of the inner mitochondrial membrane and catalyses the regeneration of DHAP with a concomitant reduction of a membrane flavoprotein. The reducing equivalents are transferred directly to ubiquinone in the electron transport chain. As a result 1 mole of cytosolic NADH oxidised by the cycle produces 2 moles of ATP during oxidative phosphorylation.

The malate-aspartate shuttle (Figure 6.2) is more complex than the α-GP shuttle. NADH regeneration in the mitochondria is made possible by the presence of a malate dehydrogenase (MDH) and glutamate-aspartate aminotransferase (AAT) in both the cytosol and mitochondria. For the malate-aspartate shuttle to function, glutamate and malate must enter the mitochondria while aspartate and α-ketoglutarate (α-KG) leave. The mitochondria contain carrier systems for all these anions (Chappell, 1968) . As shown in Figure 6.2, the cytosolic NADH reduces oxaloacetate (OAA) to malate, which exchanges for α-KG across the membrane and is then oxidised by the mitochondrial malate dehydrogenase. Because of the restricted permeability of the mitochondrial membrane to OAA (Haslam & Krebs, 1968), oxaloacetate can not itself return

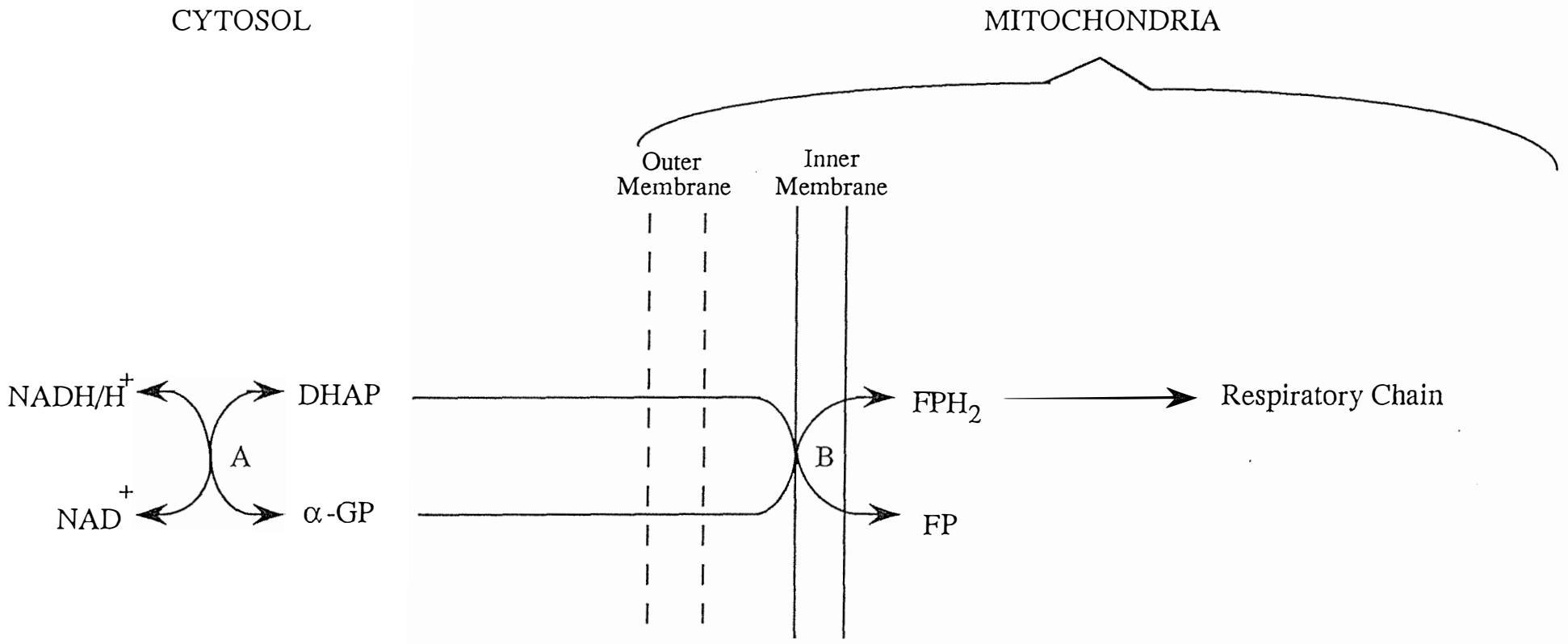


Figure 6.1: **Schematic Diagram of the α-Glycerophosphate Shuttle.**

DHAP is dihydroxyacetone phosphate; α- GP is α-glycerol-3-phosphate; FPH_2 is reduced flavoprotein; FP is oxidised flavoprotein; A is glycerol -3-phosphate dehydrogenase; B is a flavin linked glycerophosphate dehydrogenase.

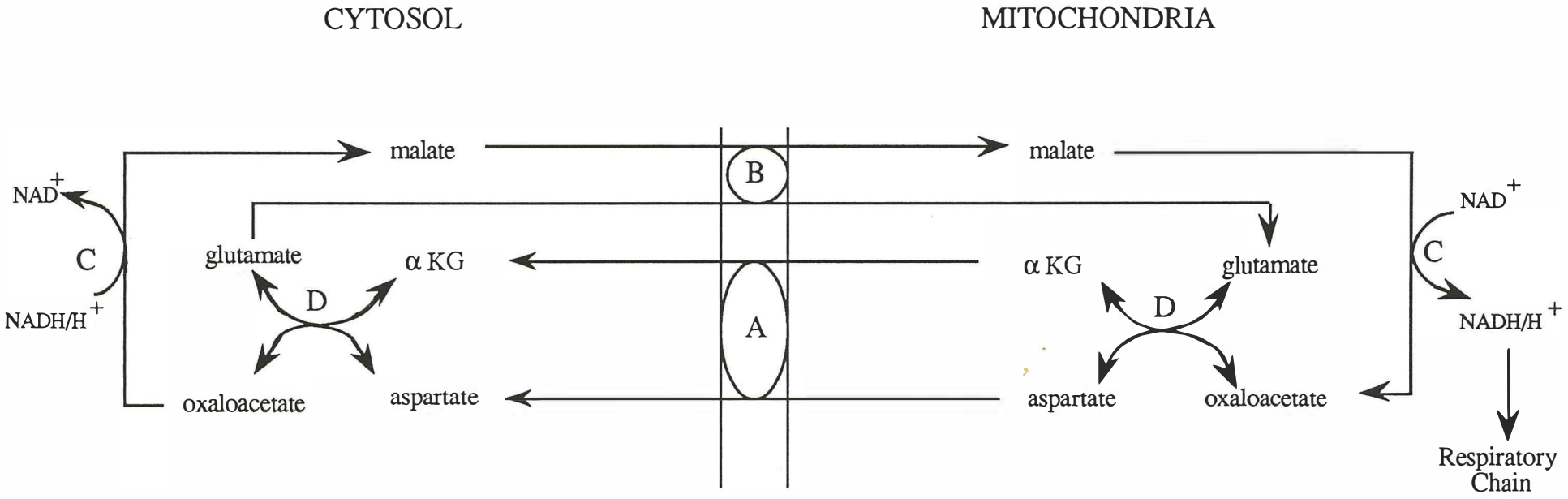


Figure 6.2: **Schematic Diagram of the Malate-Aspartate Shuttle.**
A and B are antiporters; C is malate dehydrogenase; D is glutamate aspartate aminotransferase.

across the membrane. This return is achieved by transamination from glutamate to α-KG to form aspartate which is exchanged for glutamate across the membrane; transamination in the cytosol regenerates OAA to initiate the next cycle. For every mole of NADH regenerated in the mitochondrial matrix 3 moles of ATP are synthesised.

The participation of the malate-aspartate shuttle in ethanol metabolism has been demonstrated by the use of inhibitors of aspartate aminotransferases (Williamson *et al.,* 1974; Krebs & Stubbs, 1975; Cederbaum *et al.,* 1977; Williamson & Tischler, 1979), an inhibitor of glutamate/aspartate exchange (Cederbaum *et al.,* 1973, 1977), and an inhibitor of malate transport (Cederbaum *et al.,* 1973, 1977). Ethanol oxidation was inhibited by 40-60% by these agents. Furthermore rotenone, which blocks the oxidation of mitochondrial NADH by inhibiting the electron transport chain at complex I, also inhibits ethanol oxidation to the same extent as the inhibitors of the malate-aspartate shuttle (Cederbaum *et al.,* 1977). These results suggested that :

1) The malate-aspartate shuttle is very important in oxidation of ethanol.

and

2) Approximately half the reducing equivalents generated by alcohol dehydrogenase are transferred to the mitochondria by this NAD^+-dependent shuttle system while the remainder is transferred by a rotenone-insensitive process such as the α-GP shuttle.

Another NAD^+-dependent shuttle that is operational in the rat liver is the fatty acid shuttle (Whereat *et al.,* 1969; Cederbaum *et al.,* 1973) which involves the elongation and ß-oxidation of fatty acids. The involvement of this shuttle in ethanol oxidation has been ruled out. Studies have shown that rotenone, which should inhibit the fatty acid shuttle as well as the malate-aspartate shuttle has no greater effect on the oxidation of ethanol than do specific inhibitors of the malate-aspartate shuttle (Dawson, 1979).

The importance of the α-GP shuttle in the metabolism of ethanol was further supported by the observation that antimycin, which inhibits the mitochondrial oxidation of flavoproteins as well as NADH, decreased ethanol oxidation by 75% (Cederbaum *et al.,* 1977). This inhibition was greater than that caused by rotenone or by the inhibitors of the malate-aspartate shuttle, indicating the presence of another shuttle system, such as the α-GP shuttle, which is insensitive to rotenone. Also ethanol oxidation was stimulated by the addition of DHAP which increases the intracellular concentration of glycerol-3-phosphate, a metabolite of the glycerophosphate shuttle (Williamson *et al.,* 1974).

Other attempts to evaluate the importance of the shuttle systems in the metabolism of ethanol have involved incubating isolated rat hepatocytes with [1 R-^{3}H] ethanol and [1 S-^{3}H] ethanol to

estimate the rate of transfer of reducing equivalents by FAD- or NAD^+-dependent shuttles (Grunnet *et al.*, 1977). If R-ethanol-1-^3H is the substrate for liver cells, NAD^3H is formed exclusively in the cytosol. If S-ethanol-1-^3H is the substrate, NAD^3H will be formed almost exclusively in the mitochondria. The total transfer of cytosolic NAD^3H to the mitochondria may be estimated from the tritium incorporation into water from R and S-ethanol-1-^3H and the transfer of cytosolic NAD^3H into the mitochondria via NAD^+-dependent shuttles may be calculated from the incorporation of tritium into ß-hydroxybutyrate from R- and S-ethanol-1-^3H. The difference between these two values provides an estimation for the transfer of reducing equivalents via FAD-linked shuttles. The results showed that of the total reducing equivalents from [1-^3H] ethanol, 55-60% were transferred by the NAD^+-dependent shuttle, 20-25% by the FAD-dependent shuttle, and less than 20% of the acetaldehyde oxidation occurred extramitochondrially. The figure calculated for the involvement of NAD^+-dependent shuttles in transfer of reducing equivalents is similar to results from experiments where malate-aspartate shuttle inhibitors and rotenone were used. This indicates that 50-60% of NADH from the oxidation of ethanol to acetaldehyde is transferred to the mitochondria via the malate-aspartate shuttle.

All of the above studies have shown that the malate-aspartate shuttle and the α-GP shuttle are essential for the metabolism of ethanol. However, the quantitative importance of these shuttle systems in the regulation of ethanol oxidation has not been assessed.

To apply the modulation methodology of Kacser and Burns (1973), enzyme-specific inhibitors are required. For the malate-aspartate shuttle there are a number of inhibitors that have been used to quantitatively assess the role of this shuttle in removal of reducing equivalents (Williamson *et al.*, 1974; Krebs & Stubbs, 1975; Cederbaum *et al.*, 1973, 1977; Williamson & Tischler, 1979). The most widely used inhibitors are the transaminase-specific inhibitors, aminooxyacetate (John *et al.*, 1978) and cycloserine (Wong *et al.*, 1973). However neither of these inhibitors are useful for the modulation approach (Kacser & Burns, 1973) because:

1) Aminooxyacetate (AOA) inhibition of aspartate aminotansferase (AAT) can be blocked by acetaldehyde (Smith *et al.*, 1977), which forms a complex with the inhibitor (Jones *et al.*, 1970). It has been shown that AOA inhibition of AAT may be partially overcome by substrate combinations that increase the cellular content glutamate or aspartate (Cornell *et al.*, 1981). Also aminooxyacetate is an effective inhibitor at low concentrations (Williamson *et al.*, 1974) but at high concentrations (greater than 1 mM) AOA affects ethanol oxidation in a manner not explained by inhibition of transaminases (Krebs & Stubbs, 1975; Harris *et al.*, 1982).

2) Cycloserine inhibition of AAT in rat liver extracts may be partially blocked or reversed by glutamate (Janski & Cornell , 1981).

3) Both of these inhibitors react with transaminases other than aspartate aminotransferase (Smith *et al.*, 1977; John *et al.*, 1978).

The above points indicate there are too many nonspecific effects and complications that need to be considered when using aminooxyacetate or cycloserine; therefore they should not be used in inhibitor titration experiments for assessing the importance of the malate-aspartate shuttle in regulation of ethanol oxidation.

An inhibitor that is totally specific for the aspartate aminotransferase is the bacterial toxin, L-2-amino-4-methoxy-*trans* -3-butenoate (AMB) (Rando *et al.*, 1976). This inhibitor should be suitable for evaluating the importance of the malate-aspartate shuttle in the metabolism of ethanol because it affects only AAT (Rando *et al.*, 1976) and is not subject to the nonspecific effects and complications noted for aminooxyacetate and cycloserine (Smith *et al.*, 1977; John *et al.*, 1978; Janski & Cornell, 1981). Furthermore the inhibition of aspartate aminotransferase is irreversible (Rando *et al.*, 1976) in contrast to that by AOA (Smith & Freedland, 1981) and cycloserine (Janski & Cornell, 1981). Because AMB is an irreversibe inhibitor the end point of the titration may be used to calculate the amount of enzyme present in the system. Therefore AMB is an excellent inhibitor to use in the inhibitor titration experiments.

AMB inhibits both the cytosolic and mitochondrial aspartate aminotransferases (Smith & Freedland, 1981) hence the flux control coefficient calculated would represent the contribution of both enzymes to the regulation of ethanol metabolism.

Experimental determination of the flux control coefficient for each individual reaction of the malate-aspartate shuttle system would be difficult as it would involve determining the contribution of the mitochondrial and cytosolic malate-aspartate shuttle components to the overall regulation of ethanol metabolism. Specific inhibitors for the mitochondrial and cytosolic enzymes of the shuttle would be required. Meijer *et al.* (1978) stated that cycloserine did not penetrate the mitochondrial membrane and therefore inhibited only the cytosolic AAT. This would allow the determination of the importance of the mitochondrial and cytosolic AAT in regulation of ethanol oxidation by using cycloserine and AMB. However, Janski and Cornell (1981) improved the cell fractionation procedure of Meijer *et al.* (1978) and found that only 16% of the cellular aspartate aminotransferase is located in the cytosol, not 50%. The inhibition studies indicated that cycloserine reacts equally with the mitochondrial and cytosolic aspartate aminotransferases (Janski & Cornell, 1981). This evidence, plus the fact that too many non-specific effects and

complications are introduced when using cycloserine, emphasises the difficulty in finding specific inhibitors for the modulation approach in this study. In view of the difficulty in determining control factors for individual reactions, with such a complex system that incorporates both cytosolic and mitochondrial reactions, an overall flux control coefficient may need to be determined.

The irreversible inhibitor AMB would appear to be ideal for the inhibitor titration experiments necessary to study the regulation of ethanol metabolism. The contribution of aspartate aminotransferase as a combined factor (i.e. mitochondrial plus cytosolic involvement) in regulation of ethanol oxidation, would be determined using the inhibitor L-2-amino-4-methoxy-*trans* -3-butenoate. This would give some indication on the importance of the malate-aspartate shuttle in regulation of ethanol oxidation in isolated rat hepatocytes.

The importance of the α-glycerophosphate shuttle in regulation of ethanol metabolism can not be determined using the methods outlined in this study because no direct inhibitors of this shuttle are known. The flux control coefficient of the α-GP shuttle would have to be determined in another way.

The contribution of the α-GP shuttle in regulation of ethanol oxidation could be evaluated using the flux control summation theorem (see section 1.4), assuming that all the flux-controlling steps in the ethanol pathway are incorporated in the calculations. If some steps have been omitted then the value obtained for the flux control coefficient would be an upper estimate.

6.2 ELECTRON TRANSPORT CHAIN

The relationship of ethanol metabolism to the electron transport chain has been investigated by using uncouplers to increase the rate of electron flow by the removal of respiratory control. Uncouplers such as carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) and 2,4-dinitrophenol (DNP) and have been shown to stimulate the rate of ethanol oxidation in rat liver slices (Videla & Israel, 1970), isolated rat hepatocytes (Meijer *et al.*, 1975; Cederbaum *et al.*, 1977) and in rats *in vivo* (Israel *et al.*, 1970). Cederbaum *et al.* (1977) stated that under conditions where the shuttle system metabolites are at a sufficient level, ethanol oxidation is regulated by the capacity of the mitochondrial respiratory chain to reoxidise reducing equivalents generated by the alcohol dehydrogenase reaction.

The quantitative importance of the respiratory chain in regulation of ethanol oxidation has not been determined; however quantitative analysis of regulation of the electron transport chain itself has

been carried out (Duszynski *et al.*, 1982; Groen *et al.*, 1982b; Tager *et al.*, 1983; Westerhoff *et al.*, 1983; Wander *et al.*, 1984).

The studies of Duszynski *et al.* (1982) and Groen *et al.* (1982b) involved the use of specific inhibitors for the reactions in the electron transport chain. The inhibitors that could be useful in studying the chain in relation to regulation of ethanol metabolism are the noncompetitive inhibitors azide and hydroxyquinoline-N-oxide and the irreversible inhibitor carboxyatractyloside (CAT). Azide inhibits cytochrome c oxidase, hydroxyquinoline-N-oxide inhibits the cytochrome bc_1 complex and CAT inhibits the adenine nucleotide translocator (AT).

For the above noncompetitive inhibitors and irreversible inhibitor, substrate and product concentrations do not have to be measured and the following equations for calculating the flux control coefficients would be used.

1) For a noncompetitive inhibitor in an irreversible reaction.

$$C_E^J = \left(\frac{dJ}{J\,dI}\right)_{I=0} - K_i \qquad (6.1)$$

2) For an irreversible inhibitor in an irreversible reaction.

$$C_E^J = \left(\frac{dJ}{J\,dI}\right)_{I=0} - I_{max} \qquad (6.2)$$

Equations (6.1) and (6.2) were used for determining the flux control coefficient of ADH (chapter 4) and AlDH (chapter 5) respectively. The experimental approach using these electron transport chain inhibitors would be the same as that used for the alcohol dehydrogenase and aldehyde dehydrogenase experiments.

6.3 CONCLUSION

In summary, the significance of the aspartate aminotransferases of the malate-aspartate shuttle and the cyt c oxidase, cyt bc_1 complex and the adenine nucleotide translocator (AT) of the electron transport chain in regulation of ethanol oxidation could be determined by using specific inhibitors. The values obtained for the flux control coefficient of each of these steps in combination with the flux control coefficients calculated for ADH and AlDH under the same conditions may be used to determine an upper estimate for the flux control coefficient of the

α-glycerophosphate shuttle ($C^J_{\alpha GP}$) by applying the flux control summation theorem (see section 1.4).

$$C^J_{\alpha GP} = 1 - (C^J_{ADH} + C^J_{AlDH} + C^J_{AAT} + C^J_{cyt\ c} + C^J_{cyt\ bc_1} + C^J_{AT}) \qquad (6.3)$$

The flux control coefficient of α-GP using equation (6.3) would be an upper estimate or a maximum value because not all the steps for reoxidation of NADH are included. For instance, there are other enzymes in the electron transport chain that have to be considered as well as the malate dehydrogenase enzymes in the malate-aspartate shuttle. Also studies by Grunnet *et al.* (1977) have shown that approximately 20% of NADH reoxidation occurs in the cytosol. The flux control coefficient of enzymes such as lactate dehydrogenase and glyceraldehyde dehydrogenase would need to be calculated to determine the significance of cytosolic NADH reoxidation in regulation of ethanol oxidation.

Theoretically, by adding the flux control coefficients of ATT, cyt c, cyt bc_1, and AT, the importance of mitochondrial NADH reoxidation in regulation of ethanol metabolism could be calculated. This would only be a maximum estimate because at present several steps will have been omitted.

CHAPTER 7

DISCUSSION

Analysis of metabolic control in a quantitative manner has become more prominent in biochemistry over the last two decades. The quantitative approaches that have been developed (Biochemical Systems Theory, Metabolic Control Theory and Flux Oriented Theory) have tended to be regarded as theoretical applications rather than of practical value. However, over the past few years these theoretical frameworks have begun to be experimentally applied to biochemical systems (Duszynski *et al.,* 1982; Groen *et al.,* 1982b; Crabtree & Newsholme, 1985a; Irvine & Savageau, 1985a,b; Salter *et al.,* 1986; Sorribas & Savageau, 1989a,b).

I have used the theoretical approach of Kacser and Burns (1973) and Heinrich and Rapoport (1974) to study the regulation of ethanol metabolism in isolated rat hepatocytes. This approach required the determination of a flux control coefficient for each potential control step to assess its contribution to the overall regulation of ethanol oxidation in isolated rat hepatocytes.

To determine the flux control coefficient, the modulation approach described by Kacser and Burns (1973) was used. This involved altering the activity of the enzyme in question by small amounts and measuring the effect of this alteration on flux through the ethanol pathway. Small changes in the ethanol clearance rates were imposed by using enzyme-specific inhibitors.

The control analysis was carried out for both fed and starved rats as the values of the flux control coefficients change with different metabolic conditions. The flux control coefficient of ADH was determined for both fed and starved rats. This provided an overall assessment of the importance of alcohol dehydrogenase in regulation of ethanol metabolism in isolated rat hepatocytes.

7.1 SUMMARY OF RESULTS

The results obtained from this study are outlined in Table 7.1

The flux control coefficient describes in quantitative terms the relative role which each enzyme plays in regulation of the pathway. In isolated hepatocytes from starved rats the results (see Table 7.1) indicate that ADH is more important than AlDH in regulation of ethanol metabolism ($C^J_{ADH} > C^J_{AlDH}$).

TABLE 7.1

Acetaldehyde Concentrations and Flux Control Coefficients of Alcohol Dehydrogenase and Aldehyde Dehydrogenase for Hepatocytes Isolated from Fed and Starved Rats

Enzyme	Isolated Hepatocytes From			
	Starved Rats [AcH] μM	C_E^J	Fed Rats [AcH] μM	C_E^J
Alcohol Dehydrogenase+ (ADH)	0 – 2.0	0.5 – 0.6	0 – 6.0	0.70
			30 – 40	0.3 – 0.4
			138	0.04
Aldehyde Dehydrogenase* (AlDH)	0 – 2.0	0.1	not determined	

+ This is the maximum value of the C_{ADH}^J after correction for substrate/product concentrations using the kinetic parameters of Cornell *et al.* (1979), for inhibition by TMSO and IsB in starved rats and inhibition by TMSO in fed rats. The results for the fed rats cover a range of acetaldehyde (AcH) concentrations present in the isolated hepatocytes.

* This a preliminary value for the C_{AlDH}^J.

The summation of all flux control coefficients in any pathway of any complexity equals unity (Kacser & Burns, 1973; Heinrich & Rapoport, 1974). Therefore according to the summation property:

$$C_{ADH}^J + C_{REST}^J = 1 \qquad (7.1)$$

where: C_{ADH}^J = flux control coefficient of ADH.

C_{REST}^J = sum of the flux control coefficients for the remaining enzymes in the ethanol system.

Since the flux control coefficient of ADH is 0.5 – 0.6, the flux control coefficient for the remainder of the enzymes in the metabolism of ethanol will be between 0.4 and 0.5. This indicates that

alcohol dehydrogenase is a major flux controlling enzyme in the oxidation of ethanol in hepatocytes isolated from starved rats.

The flux control coefficient for AlDH in isolated hepatocytes from starved rats is a preliminary value. Initially disulfiram, an irreversible inhibitor of AlDH was used in the titration experiments. However I found that because disulfiram was sparingly soluble in water, it was a difficult compound to use in the titration experiments. An alternative inactivator of AlDH was sought. I used a metabolite of disulfiram called diethyldithiocarbamate (DDC). Diethyldithiocarbamate causes inactivation of AlDH *in vivo* (Deitrich & Erwin, 1971); however DDC has no effect on the AlDH *in vitro* (Kitson, 1982). How DDC causes the inactivation of AlDH in rats is not known. It has been suggested that DDC is converted to disulfiram, which subsequently inhibits aldehyde dehydrogenase (Kitson,1977). Despite all the problems encountered with the inhibitor studies of AlDH, I have shown that DDC does cause the inactivation of AlDH in isolated rat hepatocytes and will probably be useful in further studies for determining the flux control coefficient of AlDH.

It has been suggested that oxidation of ethanol in liver cells from fasted rats is limited by the rate of removal of reducing equivalents from the cytosol, which in turn is regulated by the intracellular concentrations of the intermediates of the shuttle systems (Meijer *et al.,* 1975; Cederbaum *et al.,* 1977). However this conclusion is not valid because isolated liver cells that were depleted of substrates and metabolising ethanol at low rates were used in these experiments. The concentrations of the shuttle intermediates, in freshly prepared hepatocytes from starved rats, are much lower than those in freeze-clamped livers from starved rats (Krebs *et al.,* 1974; Cornell *et al.,* 1979). After addition of the appropriate substrates, the shuttle intermediates are restored (Crow *et al.,* 1978 ; Cornell *et al.,* 1979) and the rate of ethanol oxidation in the isolated hepatocytes is similar to those observed *in vivo* . Thus the activity of the shuttle systems is probably not rate limiting for hepatocytes from starved rats *in vivo* , where depletion of the shuttle metabolites does not normally occur.

In this study the cells were supplemented with lactate and pyruvate (see section 3.3.1.1), so that the metabolites of the shuttle systems were restored and the rate of ethanol oxidation was similar to that *in vivo* . The results of this study show that reoxidation of NADH is not as important as alcohol dehydrogenase in the regulation of ethanol metabolism in hepatocytes isolated from starved rats ($C^J_{ADH} > C^J_{REST}$). The overall process of NADH reoxidation involves the cytosolic reoxidation of NADH by dehydrogenase enzymes, transfer of reducing equivalents from the cytosol to the mitochondria by the shuttle systems, and the reoxidation of NADH in the mitochondria by the respiratory chain. Thus the suggestion that the transfer of reducing equivalents is 'rate-limiting' in starved rats is not correct.

The results for the isolated hepatocytes from fed rats (see Table 7.1) are very interesting. They show that the importance of alcohol dehydrogenase in regulation of ethanol metabolism can be as great as in starved rats, but this is only the case when the concentration of acetaldehyde is low. Generally, as the acetaldehyde concentration present in the isolated hepatocytes increased the flux control coefficient and hence the significance of ADH in regulation of ethanol metabolism decreased. As the importance of alcohol dehydrogenase in regulation of ethanol oxidation decreases with increasing AcH, the control on the flux through the ethanol pathway would be expected to be shifted to other enzymes. The main enzyme expected to become important with increasing acetaldehyde concentration is aldehyde dehydrogenase. This suggests that the activity of AlDH may at times play a significant regulatory role in the pathway of ethanol oxidation, and that the balance of activities of ADH and AlDH is a critical control feature as has been suggested previously (Eriksson *et al.*, 1975; Braggins *et al.*, 1980; Braggins & Crow, 1981; Dawson, 1981; Harrington *et al.*, 1988) on the basis of non-quantitative studies. It is important to note that the enzymes involved in the reoxidation of NADH (especially the electron transport chain) may also play a greater part in regulation of ethanol metabolism in hepatocytes isolated from fed rats.

7.2 PROBLEMS

Considerable difficulties were encountered in the practical application of the modulation approach to analysis of metabolic control. The major problem with this study on regulation of ethanol metabolism in isolated rat hepatocytes was the development of a method for detecting small changes in flux through the ethanol metabolising pathway. The difficulty lies in measuring a small decrease in a high substrate concentration rather than measuring an increase in a product from zero concentration.

The method finally developed, designated the two time point method (see chapter 3) can be used to determine flux control coefficients of enzymes that do produce observable changes in flux with increasing inhibitor concentrations. This is validated by the fact that there is good agreement in the value obtained for C^{J}_{ADH} using the two time point method with two different ADH-specific inhibitors. The apparent flux control coefficient of ADH was calculated to be 0.37 ± 0.09, using TMSO, and 0.43 ± 0.10, using IsB. After correction for substrate and product concentrations, using the kinetic parameters of Cornell *et al.* (1979), the flux control coefficient for ADH increased to a value as high as 0.5 - 0.6 (see Table 7.1)

However in the experiments using disulfiram, an AlDH inhibitor, at 1 μM the inhibition was found to be about 0.6%. The significance of this value is debatable. Firstly it was only measured in one experiment and secondly the experimental results of ADH (see Tables 4.5 and 4.6) show that at the

lowest inhibitor concentration tested, a % inhibition of around 8.0 was obtained and the method seemed to be reaching the limit of its sensitivity at this point as shown by the standard errors at the lower inhibitor concentrations. This indicates that the two time point method may not be capable of detecting the small changes in flux required for measurement of flux control coefficients in the range of 0.1 or less.

The most probable value of the flux control coefficient for any enzyme in the pathway is approximately $\frac{1}{n}$, where n represents the number of elements in the metabolic pathway. Thus the larger the number of enzymes, the smaller the mean flux control coefficient for all enzymes. Therefore it is not surprising that many experimentally estimated flux control coefficients are small (Groen *et al.*, 1982b; Salter *et al.*, 1986; Torres *et al.*, 1986). In this study it has been established that the overall flux control coefficient for the remainder of the ethanol pathway in isolated hepatocytes from starved rats is between 0.4 and 0.5, since C^J_{ADH} is 0.5 – 0.6 (see Table 7.1). There are many individual steps involved in the oxidation of ethanol other than ADH (see Figures 1.1 and 1.2). It is expected that some of the enzymes will have zero flux control coefficients or very small flux control coefficients; therefore to measure accurately the flux control coefficients for other enzymes in the metabolism of ethanol in hepatocytes isolated from starved rats, it may be necessary to use a method other than the modulation approach or develop a better method for measuring ethanol removal. For example a procedure using a continuous ethanol assay for measuring the disappearance of ethanol could be developed. This method might be sensitive enough to detect changes in flux of less than 8.0% , which is the smallest extent of inhibition registered for the two time point method.

Another problem with the inhibitor titration studies, apart from the titration of enzymes that do not result in conveniently measurable changes in flux, is the inaccuracy in determination of the initial slope of the flux inhibition curve. In other studies where inhibitors have been used to determine the flux control coefficient of a particular enzyme (Duszynski *et al.*, 1982; Groen *et al.*, 1982a,b; Salter *et al.*, 1986), the only information provided on how the initial slope has been calculated is a reference to a diagram of the inhibition curve showing the tangent ($\left(\frac{dJ}{dI}\right)_{I=0}$). In this study the slope of the chord from zero inhibitor concentration to the smallest inhibitor concentration was taken as the initial slope on the flux inhibition curve. It is assumed that the slope remains unchanged between the two points so that the chord will have the same slope as the tangent. The good agreement in the value obtained for C^J_{ADH} using the two time point method with two different ADH-specific inhibitors, indicates that the method for calculating the initial slope produces reasonably accurate and reproducible results. However a more accurate assay which allowed reliable measurement of small changes in flux would better define the initial slope.

In summary, the results using the two time point method have shown that this method is capable of detecting changes in flux through the ethanol pathway, as low as 8.0% and the method provides reproducible and accurate results (see chapter 4). However, the two time point method and even the concept of the modulation methodology may not be useful in calculating very small flux control coefficients.

7.3 FUTURE WORK

To determine the importance of the shuttle systems and electron transport chain in the regulation of ethanol metabolism, the flux control coefficients of the individual enzymes participating in these systems would need to be calculated. This would be difficult using the modulation approach (Kacser & Burns, 1973) because specific inhibitors for all enzymes that operate in the malate-aspartate shuttle (mitochondrial and cytosolic) and respiratory chain are required as well as for the α-glycerophosphate shuttle, for which no direct inhibitors are currently known.

As described in chapter 6, with increasing concentrations of L-2-amino-4-methoxy-*trans* -3-butenoate (AMB), a combined flux control coefficient for the mitochondrial and cytosolic aspartate aminotransferase (AAT) could be calculated. This would provide some insight into the importance of the transfer of reducing equivalents from the cytosol to the mitochondria by the malate-aspartate shuttle during ethanol oxidation. Also the flux control coefficients of the cytochrome c oxidase enzyme, cytochrome bc_1 complex and adenine nucleotide translocator could be calculated using the inhibitors azide, hydroxyquinoline-N-oxide and carboxyatractyloside respectively. This would help to determine the significance of these steps in the electron transport chain during ethanol oxidation. However other steps can not be quantitated at present.

Other methods that are available in the metabolic control theory for calculating the flux control coefficients are the connectivity theorem (Kacser & Burns, 1973; Westerhoff *et al.*, 1984) and flux control summation theorem (Kacser & Burns, 1973; Heinrich & Rapoport, 1974).

The connectivity theorem would be very useful in determining flux control coefficients that are too small to be measured by the modulation approach. For example, the preliminary work on AlDH (see chapter 5) indicates that C^J_{AlDH} for starved rats is going to be small and it may be more appropriate to use the connectivity theorem for determining the flux control coefficient of AlDH.

The connectivity theorem states that the ratio of the flux control coefficients of adjacent steps is equal to the inverse ratio of the two elasticity coefficients (see section 1.3.2 for definition) with

respect to the shared substrate. In the case of ADH and AlDH acetaldehyde is the shared substrate and it follows that:

$$\frac{C^{J}_{ADH}}{C^{J}_{AlDH}} = \left| \frac{\varepsilon^{AlDH}_{AcH}}{\varepsilon^{ADH}_{AcH}} \right| \qquad (7.2)$$

The C^{J}_{ADH} has been calculated in this study for both fed and starved rats (see Table 7.1) thus only the elasticities need to be evaluated to allow calculation of C^{J}_{AlDH}. The elasticity coefficient (see 1.3.2) may be calculated, in the case of ADH, because the kinetics of the enzyme and the metabolite concentrations of the enzyme reaction are known (Groen *et al.,* 1982a; Westerhoff *et al.,* 1984 ; Derr, 1986). In the absence of enzyme kinetic data, as for AlDH, direct experimental determination of the elasticity coefficient according to the definition is possible (Kacser & Burns, 1973; Groen *et al.,* 1982a; Derr, 1986; Salter *et al.,* 1986). In starved rats the C^{J}_{AlDH} may have to be determined using the connectivity theorem. However, using hepatocytes from fed rats where it is expected that the control coefficient of AlDH will be high at high acetaldehyde concentrations, the modulation approach may be implemented.

The summation theorem can be used for determining flux control coefficients if used properly . However some people have employed this theorem to validate the completeness of the studied metabolic pathway (Groen *et al.,* 1982b, 1983; Salter *et al.,* 1986; Torres *et al.,* 1986). For example if the flux control coefficients of a pathway do not add up to one then some steps have been omitted from the pathway or if the sum of the flux control coefficients in the pathway is one then it is complete and all steps in the pathway have been considered. These assumptions can be very risky because even if experimentally determined flux control coefficients sum to unity, several elements may have been missed from the metabolic pathway. These elements could have a zero flux control coefficient under the conditions tested or there may be positive and negative contributions from the neglected components that fortuitously cancel. Since the flux control coefficients can be positive, negative or greater than one , the ability to test for completeness of a metabolising system is lost. Nevertheless it can be said that the values determined using the summation theorem are maximum values representing the importance of the element under consideration, in control of the pathway. Because the metabolism of ethanol is a **simple, well defined** pathway, the summation theorem can probably be used for determining flux control coefficients. However, it is necessary for at least one flux control coefficient in the pathway to be experimentally determined (e.g. inhibitor studies) otherwise the validity of using the summation theorem is lost. Because small errors in experimental data is possible, it is important that the control coefficients are ascertained by at least two independent methods. In this study C^{J}_{ADH} was determined using two different inhibitors.

7.4 CONCLUSION

From measurement of the control coefficients of an enzyme, it is possible to obtain a quantitative assessment of the importance of that enzyme in control of a pathway under one given set of conditions. Inhibitors can be used to quantify the degree of control that an enzyme exerts on a steady state flux through a metabolic pathway. This study shows that ADH is the major enzyme in regulation of ethanol metabolism in hepatocytes isolated from starved rats. This conclusion agrees with those of previous qualitative studies (Crow *et al.*, 1977a,b; Cornell *et al.*, 1979). The significance of alcohol dehydrogenase in hepatocytes from fed rats decreases as the concentration of acetaldehyde increases implying that other steps of the ethanol pathway are also important in its regulation. The major candidate for regulation of ethanol oxidation in fed rats is aldehyde dehydrogenase.

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Appendix

DERIVATION OF EQUATIONS A to F in TABLE 4.9

Dr M.J.Hardman derived equations 4.9 A-D and I derived equations 4.9 E and F.

The following points are essential for the derivation of equations A to F in Table 4.9

1) The enzyme distribution terms for an ordered bi-bi mehanism are described in Segel (1975 pp 560-561).

2) For dead end EQ-I , the $\frac{[EQ]}{[E_t]}$ enzyme distribution term is affected. This contains the terms [Q], [B][Q], and [A][B].

3) For dead end EA-I, the $\frac{[EA]}{[E_t]}$ enzyme distribution term is affected. This contains the terms [A], [A][P], and [Q][P].

4) In the absence of products the rate equation for an ordered bi-bi mechanism (Segel, 1975 pp 564) is given by:

$$v = \frac{V_{max}[A][B]}{K_{ia}K_b+K_b[A]+K_a[B]+[A][B]} \qquad \text{(A-1)}$$

5) In the presence of products the rate equation for an ordered bi-bi mechanism (Segel,1975 pp 560) is that shown in Figure 4.2

6) The mathematical formula for the flux control coefficient in the absence of inhibitor (Groen *et al.*, 1982b; Derr *et al.*, 1987) is given by:

$$C^J_{Ei} = \frac{\left(\frac{dJ/J}{d[I]}\right)_{I=0}}{\left(\frac{\partial v_i/v_i}{\partial[I]}\right)_{I=0}} \qquad \text{(A-2)}$$

Derivation of Equation A and B in Table 4.9

For absence of products and dead end EQ-I, the coefficients of [A][B] in equation (A-1) are multiplied by $\left(1+\frac{[I]}{K_i'}\right)$, and the modified rate equation is:

$$v = \frac{V_{max}[A][B]}{K_{ia}K_b+K_b[A]+K_a[B]+[A][B]\left(1+\frac{[I]}{K_i'}\right)} \qquad \text{(A-3)}$$

Differentiation of (A-3) with respect to I leads to (A-4):

$$\frac{dv}{d[I]} = \frac{-V_{max}[A][B]}{\left(K_{ia}K_b+K_b[A]+K_a[B]+[A][B]\left(1+\frac{[I]}{K_i'}\right)\right)^2} \times \left(\frac{[A][B]}{K_i'}\right) \qquad \text{(A-4)}$$

$$\therefore \quad \frac{dv}{d[I]} = \frac{-v}{K_{ia}K_b+K_b[A]+K_a[B]+[A][B]\left(1+\frac{[I]}{K_i'}\right)} \times \left(\frac{[A][B]}{K_i'}\right) \qquad \text{(A-5)}$$

$$\therefore \quad \frac{dv}{vd[I]} = \frac{-[A][B]}{K_i'\left(K_{ia}K_b+K_b[A]+K_a[B]+[A][B]\left(1+\frac{[I]}{K_i'}\right)\right)} \qquad \text{(A-6)}$$

when [I] is zero, equation (A-6) reduces to:

$$\left(\frac{dv}{vd[I]}\right)_{I=0} = \frac{-[A][B]}{K_i'(K_{ia}K_b+K_b[A]+K_a[B]+[A][B])} \qquad \text{(A-7)}$$

Substitution of (A-7) into (A-2) leads to:

$$C_{Ei}^{J} = \frac{dJ}{Jd[I]} \times \frac{-K_i'(K_{ia}K_b+K_b[A]+K_a[B]+[A][B])}{[A][B]}$$

$$= \frac{-K_i dJ}{J\, d[I]} \left(\frac{K_b(K_{ia} + [A]) + K_a[B]}{[A][B]} + 1 \right) \qquad \text{(A-8)}$$

Equation (A-8) is equivalent to equation A in Table 4.9. Equation B in Table 4.9 is derived in the same fashion as for equation A. For dead end EQ-I and dead end EA-I the coefficients of [A] are multiplied by $\left(1+\frac{[I]}{K_i}\right)$ and [A][B] multiplied by $\left(1+\frac{[I]}{K_i'}\right)$. The modified rate equation is:

$$v = \frac{V_{max}[A][B]}{K_{ia}K_b+K_b[A]\left(1+\frac{[I]}{K_i}\right)+K_a[B]+[A][B]\left(1+\frac{[I]}{K_i'}\right)} \qquad \text{(A-9)}$$

On the assumption that K_i is equal to K_i', equation (A-9) reduces to:

$$v = \frac{V_{max}[A][B]}{K_{ia}K_b+K_a[B]+(K_b[A]+[A][B])\left(1+\frac{[I]}{K_i}\right)} \qquad \text{(A-10)}$$

Differentiation of (A-10) and substitution of $\left(\frac{dv}{vd[I]}\right)_{I=0}$ into equation (A-2) leads to equation B of Table 4.9.

Derivation of Equation C and D in Table 4.9

Assuming that $P \approx 0$, then all terms in the rate equation containing[P] will be deleted. For dead end EQ-I the coefficients of [Q] and [B][Q] are multiplied by $\left(1+\frac{[I]}{K_i}\right)$ and the coefficient of [A][B] is multiplied by $\left(1+\frac{[I]}{K_i'}\right)$, where K_i does not equal K_i'.

The modified rate equation is given by:

$$v = \frac{\frac{V_f[A][B]}{K_{ia}K_b}}{1+\frac{[A]}{K_{ia}}+\frac{K_a[B]}{K_{ia}K_b}+\frac{[Q]}{K_{iq}}\left(1+\frac{[I]}{K_i}\right)+\frac{[A][B]}{K_{ia}K_b}\left(1+\frac{[I]}{K_{i'}}\right)+\frac{K_a[B][Q]}{K_{ia}K_bK_{iq}}\left(1+\frac{[I]}{K_i}\right)} \quad \text{(A-11)}$$

$$= \frac{V_f AB}{K_{ia}K_b+K_bA+K_aB+\frac{K_{ia}K_bQ}{K_{iq}}\left(1+\frac{I}{K_i}\right)+AB\left(1+\frac{I}{K_{i'}}\right)+\frac{K_aBQ}{K_{iq}}\left(1+\frac{I}{K_i}\right)} \quad \text{(A-12)}$$

where [A] = A; [B] = B; [Q] = Q; [I] = I

Differentiation of (A-12) with respect to I, leads to:

$$\frac{dv}{dI} = \frac{-V_f AB}{(\text{ denominator })^2}\left(\frac{AB}{K_{i'}}+\frac{K_{ia}K_bQ}{K_{iq}K_i}+\frac{K_aBQ}{K_{iq}K_i}\right) \quad \text{(A-13)}$$

$$\frac{dv}{dI} = \frac{-v}{\text{denominator}}\left(\frac{Q}{K_{iq}K_i}(K_{ia}K_b+K_aB)+\frac{AB}{K_{i'}}\right)$$

$$\therefore \quad \frac{dv}{vdI} = \frac{-\left(\frac{Q}{K_{iq}K_i}(K_{ia}K_b+K_aB)+\frac{AB}{K_{i'}}\right)}{K_{ia}K_b+K_bA+K_aB+\frac{K_{ia}K_bQ}{K_{iq}}\left(1+\frac{I}{K_i}\right)+AB\left(1+\frac{I}{K_{i'}}\right)+\frac{K_aBQ}{K_{iq}}\left(1+\frac{I}{K_i}\right)} \quad \text{(A-14)}$$

When I = 0, equation (A-14) reduces to:

$$\left(\frac{dv}{vdI}\right)_{I=0} = \frac{-\left(\frac{Q}{K_{iq}K_i}(K_{ia}K_b+K_aB)+\frac{AB}{K_{i'}}\right)}{K_{ia}K_b+K_bA+K_aB+\frac{K_{ia}K_bQ}{K_{iq}}+AB+\frac{K_aBQ}{K_{iq}}} \quad \text{(A-15)}$$

Substitution of (A-15) into (A-2) gives:

$$C_{Ei}^{J} = \frac{-K_i dJ}{J\,dI}\left(\frac{K_{ia}K_b+K_bA+K_aB+\frac{K_{ia}K_bQ}{K_{iq}}+AB+\frac{K_aBQ}{K_{iq}}}{\left(\frac{Q}{K_{iq}K_i}(K_{ia}K_b+K_aB)+\frac{AB}{K_{i'}}\right)}\right) \quad \text{(A-16)}$$

Now K_i' must be $\geq K_i$ because:

$$K_i' = \left(\frac{k_3+k_4}{k_3}\right) K_i \quad \text{for mechanism on pp561 in Segel (1975)}$$

Since $k_3 \gg k_4$ for ADH, $K_i' \approx K_i$. When $K_i' \approx K_i$, equation (A-16) becomes:

$$C_{Ei}^{J} = \frac{-K_i\left(K_{ia}K_b+K_bA+K_aB+\frac{K_{ia}K_bQ}{K_{iq}}+AB+\frac{K_aBQ}{K_{iq}}\right)}{\left(\frac{Q}{K_{iq}}(K_{ia}K_b+K_aB)+AB\right)}$$

$$C_{Ei}^{J} = \frac{-K_i dJ}{J\,dI}\left(\frac{K_b(K_{ia}+A)+K_aB}{Q/K_{iq}\,(K_{ia}K_b+K_aB)+AB}+1\right) \qquad \text{(A-17)}$$

Equation (A-17) is equivalent to equation C in Table 4.9. Equation D in Table 4.9 is derived in the same fashion as for equation C. All K_i's are assumed to be the same. Thus the modified rate equation for dead end EQ-I / EA-I is determined by multiplying the coefficients of [A], [A][B],[Q], and [Q][B] by $\left(1+\frac{[I]}{K_i}\right)$.

Derivation of Equation E and F in Table 4.9

Assuming that all K_i's are the same, for dead end EQ-I the coefficients of [Q],[B][Q], and [A][B] are multiplied by $\left(1+\frac{I}{K_i}\right)$ to give:

$$v = \frac{\frac{V_f AB}{K_{ia}K_b} - \frac{V_rPQ}{K_pK_{iq}}}{1+\frac{A}{K_{ia}}+\frac{K_aB}{K_{ia}K_b}+\frac{K_qP}{K_pK_{iq}}+\frac{Q}{K_{iq}}\left(1+\frac{I}{K_i}\right)+\frac{AB}{K_{ia}K_b}\left(1+\frac{I}{K_i'}\right)+}$$

$$\frac{K_qAP}{K_{ia}K_pK_{iq}}+\frac{K_aBQ}{K_{ia}K_bK_{iq}}\left(1+\frac{I}{K_i}\right)+\frac{PQ}{K_pK_{iq}}+\frac{ABP}{K_{ia}K_bK_{ip}}+\frac{BPQ}{K_{ib}K_pK_{iq}} \qquad \text{(A-18)}$$

$$v = \frac{V_f ABK_pK_{iq} - V_rPQK_{ia}K_b}{K_{ia}K_bK_{iq}K_p+K_{iq}K_pK_bA+K_{iq}K_pK_aB+K_{ia}K_bK_qP+K_{ia}K_bK_pQ\left(1+\frac{I}{K_i}\right)+ K_{iq}K_pAB\left(1+\frac{I}{K_i}\right)}$$

$$+ K_bK_qAP + K_pK_aBQ\left(1+\frac{I}{K_i}\right)+ K_{ia}K_bPQ +\frac{K_pK_{iq}ABP}{K_{ip}}+\frac{K_{ia}K_bBPQ}{K_{iq}} \qquad \text{(A-19)}$$

Differentiation of (A-19) with respect to I leads to:

$$\frac{dv}{dI} = \frac{-V_f ABK_pK_{iq} - V_rPQK_{ia}K_b}{(\text{ denominator })^2} \times \left(\frac{K_pK_aBQ}{K_i}+\frac{K_{iq}K_pAB}{K_i}+\frac{K_{ia}K_bK_pQ}{K_i}\right)$$

$$\therefore \qquad \frac{dv}{vdI} = \frac{-K_p(K_aBQ+K_{iq}AB+K_{ia}K_bQ)}{K_i(\text{ denominator })} \qquad \text{(A-20)}$$

When I = 0, equation (A-20) reduces to:

$$\left(\frac{dv}{vdI}\right)_{I=0} = \frac{-K_p/K_i(K_aBQ+K_{iq}AB+K_{ia}K_bQ)}{K_{ia}K_bK_{iq}K_p+K_{iq}K_pK_bA+K_{iq}K_pK_aB+K_{ia}K_bK_qP+K_{ia}K_bK_pQ+ K_{iq}K_pAB}$$

$$+ K_bK_qAP + K_pK_aBQ+ K_{ia}K_bPQ +\frac{K_pK_{iq}ABP}{K_{ip}}+\frac{K_{ia}K_bBPQ}{K_{iq}} \qquad \text{(A-21)}$$

Substitution of (A-21) into (A-2) leads to:

$$C_{Ei}^{J} = \frac{-K_i dJ}{J\ dI}\left(\frac{K_{iq}K_p(K_{ia}K_b+K_bA+K_aB+\frac{ABP}{K_{ip}})+K_{ia}K_b(K_qP+PQ+\frac{BPQ}{K_{ib}})+K_bK_qAP}{K_p(QK_{ia}K_b+QK_aB+ABK_{iq})} + 1\right) \qquad \text{(A-22)}$$

Equation (A-22) is equivalent to equation E in table 4.9. Equation F in Table 4.9 is derived in the same fashion as for equation E. All K_i's are assumed to be the same. Thus the modified rate equation for dead end EQ-I/EA-I is determined by multiplying the coefficients [A],[Q],[A][B],[B][Q],[A][P], and [P]Q] by $\left(1+\frac{I}{K_i}\right)$.