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The importance of alcohol dehydrogenase in regulation of ethanol metabolism in rat liver cells

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We used titration with the inhibitors tetramethylene sulphoxide and isobutyramide to assess quantitatively the importance of alcohol dehydrogenase in regulation of ethanol oxidation in rat hepatocytes. In hepatocytes isolated from starved rats the apparent Flux Control Coefficient (calculated assuming a single-substrate irreversible reaction with non-competitive inhibition) of alcohol dehydrogenase is 0.3–0.5. Adjustment of this coefficient to allow for alcohol dehydrogenase being a two-substrate reversible enzyme increases the value by 1.3–1.4-fold. The final value of the Flux Control Coefficient of 0.5–0.7 indicates that alcohol dehydrogenase is a major rate-determining enzyme, but that other factors also have a regulatory role. In hepatocytes from fed rats the Flux Control Coefficient for alcohol dehydrogenase decreases with increasing acetaldehyde concentration. This suggests that, as acetaldehyde concentrations rise, control of the pathway shifts from alcohol dehydrogenase to other enzymes, particularly aldehyde dehydrogenase. There is not a single rate-determining step for the ethanol metabolism pathway and control is shared among several steps.

INTRODUCTION

The factors that control the rate of alcohol metabolism in mammals have been the subject of much debate, with two main hypotheses proposed for the rate-limiting step of the major pathway (for reviews see Crow, 1985; Crow & Hardman, 1989). The first hypothesis states that the rate of ethanol oxidation is limited by the rate at which NADH can be reoxidized to NAD^+ (Hawkins & Kalant, 1972; Khanna & Israel, 1980), and the second hypothesis proposes that the rate of ethanol metabolism is regulated by the amount of alcohol dehydrogenase in the liver (Crow *et al.*, 1977; Cornell *et al.*, 1979). Qualitative analysis of the control of the pathway has not disproved either of these hypotheses. Current approaches to quantitative analysis of metabolic regulation suggest that control is usually shared among several steps in a pathway and that the degree of control exerted by a step depends on the metabolic state of the system. Therefore there is probably not a single rate-determining step for the pathway of ethanol metabolism in the liver. The enzymes that may be important in regulating the rate of ethanol oxidation are (1) alcohol dehydrogenase, which catalyses the oxidation of ethanol to acetaldehyde, (2) aldehyde dehydrogenase, which catalyses the oxidation of acetaldehyde to acetate, and (3) the enzymes of the hydrogen shuttles and the electron transport chain, which are involved in the reoxidation of the NADH that is formed in the alcohol dehydrogenase and aldehyde dehydrogenase reactions.

A theoretical framework developed by Kacser & Burns (1973) and Heinrich & Rapoport (1974) allows the determination of a Flux Control Coefficient (C_E^J; nomenclature of Westerhoff *et al.*, 1984), which is a quantitative measure of the importance of an enzyme in the control of flux through a pathway. In the present paper the principles developed by Kacser & Burns (1973) were used to determine the Flux Control Coefficient of alcohol dehydrogenase during ethanol metabolism in hepatocytes isolated from starved rats and fed rats. We used the method of modulation, which involves altering the activity of one enzyme by small amounts (for example with an enzyme-specific inhibitor) and measuring the small changes in pathway flux that this produces.

EXPERIMENTAL

Rats

Male Sprague–Dawley rats, weighing 160–300 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 h-light/12 h-dark cycle. The rats were fed on a standard pellet diet, supplied *ad libitum*, or starved for 48 h before the experiment. In both cases there was free access to tap-water.

Preparation of isolated liver cells

Isolated hepatocytes were prepared by the method of Berry & Friend (1969) as modified by Cornell *et al.* (1973). The viability of the cell preparation was initially assessed by the Trypan Blue exclusion test (Phillips, 1973). Only cell preparations with a viability count of 85% or greater were used in the metabolic experiments. The extent of survival of the cells during preparation and incubation was determined by measurement of the ATP content, which is a sensitive indicator of O_2 depletion of the cells and of deterioration of metabolic performance and cell integrity (Krebs *et al.*, 1974; Cornell, 1983). ATP was assayed enzymically (Lamprecht & Trautschold, 1974), after neutralization of the acidic supernatant with 3 M-KOH. Cell preparations or individual incubations where the ATP content was less than 2 μmol/g of cells (Cornell, 1983) were not used for determination of ethanol clearance rates. In this paper metabolite contents (μmol/g) and metabolic rates (μmol/min per g) are expressed on the basis of cell wet weight, which was determined as described by Krebs *et al.* (1974).

Inhibitor titration experiments

Two procedures were used to measure the small decreases (required by the modulation approach) in rates of ethanol

Abbreviations: TMSO, tetramethylene sulphoxide; ADH (subscript or superscript), alcohol dehydrogenase; ALDH (subscript or superscript), aldehyde dehydrogenase.

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oxidation produced by increasing concentrations of inhibitor. Procedure A allowed the determination of several time points for a small number of inhibitor concentrations, whereas procedure B allowed a larger number of inhibitor concentrations to be used with one batch of hepatocytes, but with only a small number of time points for each rate measurement.

Procedure A. Hepatocytes (7.5 ml of suspension) were incubated with ethanol (13 mM), lactate (10 mM), pyruvate (1 mM) and inhibitor, plus Krebs–Henseleit (1932) bicarbonate buffer, which contained 2.5% (w/v) BSA, in a total volume of 15 ml. The incubation mixture was held in a 150 ml conical flask with a side arm fitted with a Suba-Seal; an 18-gauge needle entered through the seal. The mixture was gassed with O_2/CO_2 (19:1) for 30 s immediately after addition of the cells and then for four more 30 s periods during a 30 min preincubation in a shaking water bath at 37 °C. Samples (0.4–0.9 ml) for determination of ethanol concentration were removed through the needle into a 1 ml syringe at the end of preincubation and then at 7 min intervals for 63 min. Each sample was injected immediately into a centrifuge tube containing 25 μl of 60% (v/v) $HClO_4$ and mixed with the use of a vortex mixer, before centrifugation in an Eppendorf microcentrifuge (model 5414) at 13500 rev./min for approx. 4.5 min. The supernatants were used for determination of ethanol concentration by the method of Cornell & Veech (1983). Rates of ethanol removal were calculated by linear regression of plots of ethanol concentration against time. For all experiments the ethanol oxidation rates were normalized (inhibited rate, J, divided by non-inhibited rate, J_0). This enabled the data from several experiments for each inhibitor to be combined and used in evaluation of the Flux Control Coefficient.

Samples (about 3 ml) for determination of ATP were removed after 20 min and 50 min, by vacuum suction with the use of a Becton–Dickinson stopcock, into a 10 ml syringe containing 150 μl of 60% (v/v) $HClO_4$. Samples were then centrifuged in a Sorvall RC2B centrifuge at 15000 rev./min for 10 min at 4 °C.

Procedure B. A bulk incubation mixture was prepared by mixing cell suspension (0.6 ml per 1.0 ml total volume), lactate (10 mM), pyruvate (1 mM) and Krebs–Henseleit (1932) bicarbonate buffer containing 2.5% (w/v) BSA.

Individual incubation mixtures were prepared by transfer of 1 ml (hepatocytes from starved rats) and 2 ml (hepatocytes from fed rats) of cell suspension every 45 s to an incubation flask (10 ml glass-stoppered glass Erlenmeyer flask). The sample was gassed with O_2/CO_2 (19:1) for 20 s and then incubated at 37 °C in a shaking water bath. Before the addition of cell suspension, all flasks contained ethanol (final conc. 13 mM) and, except for control incubation samples, inhibitor at the chosen concentration. All incubations were carried out in triplicate.

Once all incubations were prepared (about 10 min), 50 μl of 60% (v/v) $HClO_4$ was added to the first three control incubations to give the initial time samples. This marked the starting time for the 35 min incubation period for all remaining incubations (final-time samples). At the end of the incubation period the reactions were stopped by addition of 50 μl of 60% (v/v) $HClO_4$. The flasks were put on ice for 10 min, then transferred to Eppendorf centrifuge tubes and centrifuged as for procedure A. The supernatant was used for measurement of the concentrations of ethanol (Cornell & Veech, 1983) and, for hepatocytes from fed rats, acetaldehyde (Stowell *et al.*, 1978).

The ethanol concentration at true zero time (determined by extrapolation of the control rate back to the true zero time) was used in the calculation of the uninhibited and inhibited rates of ethanol oxidation. For all experiments the ethanol oxidation rates were normalized as described for procedure A.

The concentration of acetaldehyde present in the liver cells of fed rats was measured because acetaldehyde concentrations during ethanol metabolism vary widely between individual rats (Braggins & Crow, 1981), and can be high enough to inhibit alcohol dehydrogenase (Braggins *et al.*, 1980; Crow *et al.*, 1983*b*). Because of this, Flux Control Coefficients for hepatocytes isolated from fed rats were determined from individual experiments.

RESULTS

The inhibitors tetramethylene sulphoxide (TMSO) and isobutyramide, which are non-competitive inhibitors (nomenclature of Cleland, 1970) with respect to ethanol (Chadha *et al.*, 1983; Plapp *et al.*, 1984), were chosen for determining the Flux Control Coefficient of alcohol dehydrogenase. We have found that these inhibitors did not have any effect on the activity of either low-K_m or high-K_m aldehyde dehydrogenase in rat liver homogenates.

For pure non-competitive inhibition ($K_{is} = K_{ii}$), an apparent Flux Control Coefficient may be calculated, assuming that the alcohol dehydrogenase reaction can be treated as a single-substrate irreversible reaction, by using eqn. (1) (Groen *et al.*, 1982; Derr, 1986):

$$C_E^J = \frac{-K_i \cdot dJ}{J \cdot dI} \quad (1)$$

We discuss in another section below additional factors that apply for other types of inhibition, for reversible reactions and for reactions with two substrates.

The $dJ/J \cdot dI$ term can be determined from a plot of normalized flux (J/J_0) against inhibitor concentration (see below). The inhibition constants (K_i) for TMSO and isobutyramide were obtained from the literature (Chadha *et al.*, 1983; Plapp *et al.*, 1984).

Because of the limited volume of hepatocyte suspension that could be isolated from a single rat liver, we were unable to measure ethanol clearance rates using repeated sampling (procedure A) for more than two inhibitor concentrations in a single experiment. This made it very time-consuming to accumulate sufficient data for calculation of a Flux Control Coefficient. We therefore developed a simpler and more rapid procedure (B) where the ethanol concentration at a single, final, time point was used to calculate the clearance rate, so that a wider range of inhibitor concentrations could be used in each experiment. Although each individual clearance rate was less accurate, more replicate determinations could be carried out to compensate for this. For hepatocytes from starved rats the results obtained with procedure B were similar to those obtained with procedure A (see below). For hepatocytes from fed rats procedure B had to be used because the variation in acetaldehyde concentration required Flux Control Coefficients to be determined separately for each liver.

Hepatocytes from starved rats

We have previously found that acetaldehyde concentrations are very low during ethanol metabolism by incubations of hepatocytes from starved rats. We therefore used starved rats for our initial experiments to avoid the complicating factor of variability in acetaldehyde concentrations (see below).

Procedure A. The dependence of the rate of ethanol removal on TMSO concentration in experiments using procedure A is shown in Fig. 1. A plot of the reciprocal of the normalized flux against [TMSO] appears to be linear (inset to Fig. 1). This does not necessarily imply that the flux is linearly dependent on enzyme activity; a linear plot would also arise, for example, if the

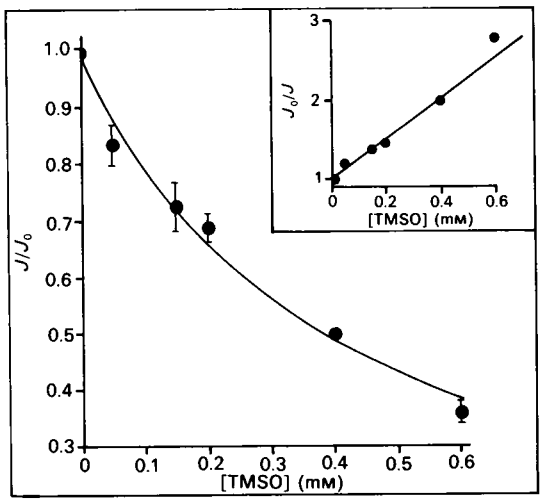

Fig. 1. Dependence on TMSO concentration of the rate of ethanol metabolism in hepatocytes from starved rats

The flux through the pathway was determined by using procedure A, as described in the Experimental section. Error bars represent the S.E.M.; where error bars are not shown, the error is less than the radius of the circle. The line represents the computer-calculated fit to eqn. (3), with $a = 1.02 \pm 0.03$ and $b = 2.56 \pm 0.22$.

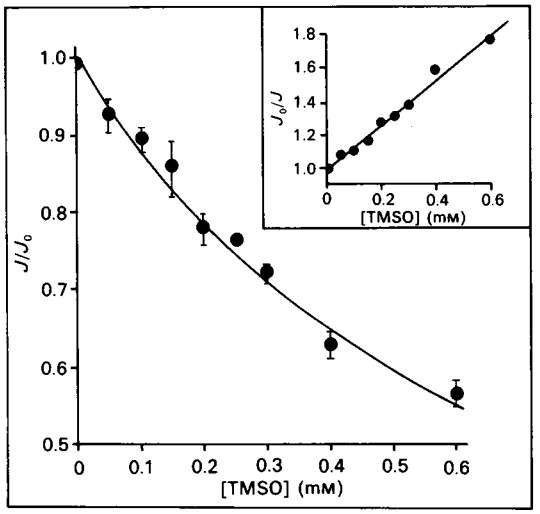

Fig. 2. Dependence on TMSO concentration of the rate of ethanol metabolism in hepatocytes from starved rats

The flux through the pathway was determined by using procedure B, as described in the Experimental section. Error bars represent S.E.M.; where error bars are not shown, the error is less than the radius of the circle. The line represents the computer-calculated fit to eqn. (3), with $a = 0.996 \pm 0.011$ and $b = 1.36 \pm 0.07$.

dependence of flux on enzyme activity was hyperbolic. The value of $dJ/J \cdot dI$ can be shown to be given by eqn. (2):

$$\frac{dJ}{J \cdot dI} = \frac{-b}{a} \quad (2)$$

where a is the intercept of the reciprocal plot (close to 1 for normalized flux) and b is the slope of this plot. In fact it is preferable to fit the untransformed flux to eqn. (3):

$$J = 1/(a + b \cdot I) \quad (3)$$

Using non-linear fitting of the data in Fig. 1, with the program ENZFITTER (Leatherbarrow, 1987), we calculated the value of $dJ/J \cdot dI$ to be -2.52 ± 0.22. Since K_i for TMSO is 200 ± 20 μM (Chadha *et al.*, 1983), eqn. (1) gives an apparent C^J_{ADH} of 0.50 ± 0.09. Over half of the error in this result arises from the error in the value of K_i.

Procedure B. Figs. 2 and 3 represent plots of flux against inhibitor concentration for TMSO and isobutyramide respectively, obtained by using the combined results for all experiments for each inhibitor using procedure B. The plots of 1/flux against [inhibitor] are again linear (insets of Figs. 2 and 3). The values of $dJ/J \cdot dI$ calculated from fitting the data in Figs. 2 and 3 to eqn. (3) are -1.37 ± 0.08 for TMSO and -1.42 ± 0.06 for isobutyramide. The inhibition constants (K_i) for TMSO (as above) and isobutyramide (330 ± 60 μM; Plapp *et al.*, 1984) were used to calculate apparent Flux Control Coefficients for alcohol dehydrogenase 0.27 ± 0.04 (with TMSO) and 0.47 ± 0.10 (with isobutyramide).

The value of the apparent Flux Control Coefficient with TMSO as inhibitor is lower than that obtained with procedure A (0.50). Two possible reasons for this difference are (1) a barrier to diffusion of TMSO into liver cells and (2) a difference between the animals used for the two sets of experiments. In the former case, the shorter preincubation in procedure B would lead to a smaller inhibitory effect. However, a value of C^J_{ADH} of 0.44 was obtained with procedure B and TMSO for a hepatocyte preparation from a fed rat (see below); this shows that the shorter incubation time does not necessarily lead to a low C^J_{ADH}. We

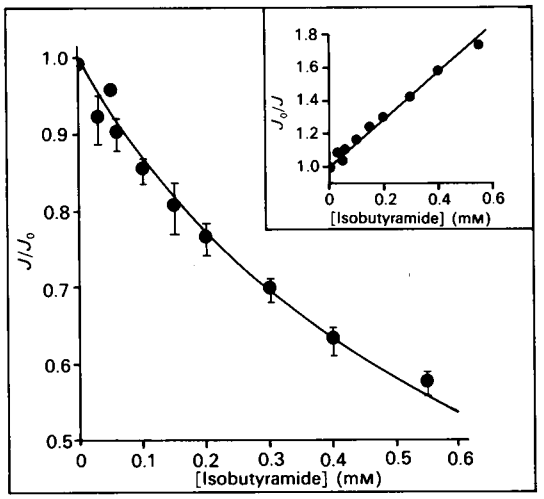

Fig. 3. Dependence on isobutyramide concentration of the rate of ethanol metabolism in hepatocytes from starved rats

The flux through the pathway was determined by using procedure B, as described in the Experimental section. Error bars represent S.E.M.; where error bars are not shown, the error is less than the radius of the circle. The line represents the computer-calculated fit to eqn. (3), with $a = 1.004 \pm 0.007$ and $b = 1.43 \pm 0.05$.

therefore believe that the variation is more likely to be due to a difference in liver alcohol dehydrogenase activity between the animals used in the two sets of experiments. The value of the Flux Control Coefficient is not a constant and it will vary if the activities of some of the enzymes in a pathway change. For example, rats with high liver alcohol dehydrogenase activity would be expected to show low values of C^J_{ADH}. We have previously observed a variation in liver alcohol dehydrogenase activity of almost 2-fold between different groups of rats of the same strain as those used in this study (Braggins & Crow, 1981; Gillion *et al.*, 1985).

The value of C^J_{ADH} obtained in the experiments using iso-

butyramide (0.47) is intermediate between the two values obtained from experiments using TMSO (0.27 and 0.50). We therefore conclude that for starved rats the apparent value of C^J_{ADH} is 0.3–0.5.

Adjustment of apparent Flux Control Coefficient. The values of C^J_{ADH} given above were calculated by using eqn. (1), which was derived for pure non-competitive inhibition of a single-substrate irreversible reaction (Groen *et al.*, 1982). For this type of inhibition the slope and intercept inhibition constants, K_{is} and K_{ii}, are equal, as for TMSO inhibition of alcohol dehydrogenase (Chadha *et al.*, 1983), and K_i in eqn. (1) represents both K_{is} and K_{ii}. For uncompetitive inhibition (Derr, 1986), or for non-competitive inhibition with K_{is} not equal to K_{ii} (also called mixed inhibition; Cornish-Bowden, 1979, pp. 76–78), the expression for the Flux Control Coefficient should also include a substrate concentration term (see the Appendix); inhibition by isobutyramide is of one of these types (Plapp *et al.*, 1984). Derr (1986) has also shown that the presence of product in a reversible reaction leads to an additional term in the equation for the Flux Control Coefficient, which increases the calculated value of C^J_E. We have extended this analysis to include the presence of two substrates and two products (see the Appendix). Since all four species are present during ethanol metabolism, their concentrations should be taken into account. The correction factor to be applied to the results calculated by using eqn. (1) depends on the type of inhibition and the concentrations of substrates and products. We have assumed the following concentrations: free [NAD^+], 500 μM (Bücher *et al.*, 1972); free [NADH], 0.8–2.0 μM (Veech *et al.*, 1972; Crow *et al.*, 1983*a*); [ethanol], 10 mM; [acetaldehyde], 1–2 μM (Crow *et al.*, 1983*b*). Calculations using the kinetic parameters given by Cornell *et al.* (1979) show that inclusion of these factors would increase the values of C^J_{ADH} quoted above by 1.3–1.4-fold.

Because of some uncertainty as to the type of inhibition displayed by TMSO and isobutyramide and the assumptions that must be made about metabolite concentrations, such as [NAD^+] and [NADH], that cannot be measured directly, it would be unrealistic to be any more precise in correcting the apparent Flux Control Coefficients. For the purposes of this study, it is sufficient to say that the apparent values are minimum ones; the additional factors necessary to allow for a two-substrate–two-product reversible reaction and for types of inhibition other that the simplest case of pure non-competitive will all tend to increase the final value of C^J_{ADH}.

Hepatocytes from fed rats

The concentrations of acetaldehyde measured in incubations of hepatocytes isolated from fed rats varied widely (from 6 μM to 143 μM) among different animals. We have previously found similar variation in acetaldehyde concentrations in hepatocytes from fed rats (Crow *et al.*, 1983*b*), as well as in perfused liver (Braggins *et al.*, 1980) and in rats *in vivo* (Braggins & Crow, 1981).

With procedure B, an apparent Flux Control Coefficient for alcohol dehydrogenase was determined individually for each of five fed rats. Plots of flux against [TMSO] for experiments with 16 μM-acetaldehyde and 138 μM-acetaldehyde are shown in Fig. 4. The effect of [TMSO] on flux is less for the higher acetaldehyde concentration (curve *B*). The values of the apparent Flux Control Coefficient, calculated by fitting to eqn. (3), are 0.44 ± 0.10 for 16 μM-acetaldehyde and 0.14 ± 0.03 for 138 μM-acetaldehyde. There is some indication in Fig. 4 that curve *B* is sigmoid (compare Groen *et al.*, 1982). If this is so, the value of C^J_{ADH} from fitting to eqn. (3) will be an overestimate; a linear fit to the first four points of curve *B* gives an apparent C^J_{ADH} of about 0.06. When the acetaldehyde concentration was between 16 and 138 μM, the value of the apparent C^J_{ADH} was intermediate between 0.14 and 0.44 (Table 1). The values of C^J_{ADH} in hepatocytes from fed rats are therefore similar to those in hepatocytes from starved rats, although C^J_{ADH} appears to decrease as the acetaldehyde concentration increases. Inclusion of additional factors for the presence of products and the second substrate (see the Appendix) would increase the value of the Flux Control Coefficient by 1.4-fold (at [acetaldehyde] = 16 μM) to 1.9-fold (at [acetaldehyde] = 138 μM) but not alter the overall trend.

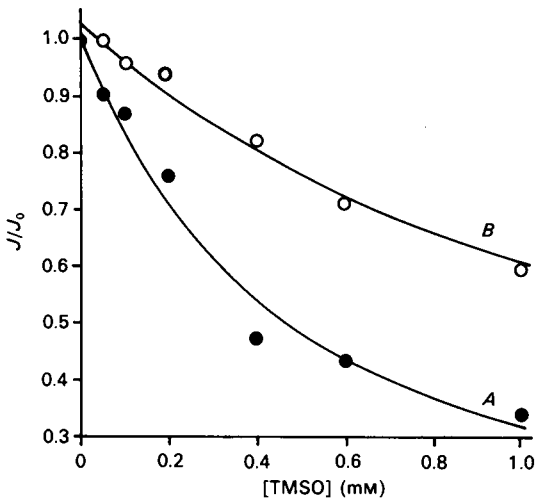

Fig. 4. Dependence on TMSO concentration of the rate of ethanol metabolism in hepatocytes from fed rats

The flux through the pathway was determined in hepatocytes from one rat per experiment by using procedure B, as described in the Experimental section. The acetaldehyde concentrations, measured in the absence of inhibitor, were 16 μM (●, curve *A*) and 138 μM (○, curve *B*). The lines represent the computer-calculated fits to eqn. (3), with $a = 0.982\pm0.032$ and $b = 2.16\pm0.22$ for curve *A* and $a = 0.972\pm0.015$ and $b = 0.67\pm0.05$ for curve *B*.

Table 1. Relationship between acetaldehyde concentration and apparent Flux Control Coefficient for alcohol dehydrogenase in hepatocytes from fed rats

All data were obtained as described in the text.

[Acetaldehyde] (μM)	$-\frac{dJ}{J\cdot dI}$ (mM^{-1})	C^J_{ADH}
16	2.19 ± 0.30	0.44 ± 0.10
29	1.43 ± 0.23	0.29 ± 0.08
50	1.52 ± 0.27	0.30 ± 0.08
81	1.26 ± 0.22	0.25 ± 0.07
138	0.69 ± 0.06	0.14 ± 0.03

DISCUSSION

The apparent Flux Control Coefficients for alcohol dehydrogenase determined in hepatocytes from starved rats, or in hepatocytes from fed rats when acetaldehyde concentrations are low (less than about 50 μM), range from 0.3 to 0.5. Correction of these values, as described above, would give Flux Control Coefficients in the range 0.4–0.7. The Summation Theorem (Kacser & Burns, 1973) states that the sum of all the Flux Control Coefficients in a metabolic pathway should equal 1. It is clear therefore that, when acetaldehyde concentrations are low,

alcohol dehydrogenase plays an important role in the regulation of flux through the pathway of ethanol metabolism. It is probably the most important individual enzyme, since the remaining contribution to the total of 1 would be made by a combination of the Flux Control Coefficients for aldehyde dehydrogenase and for the processes involved in NADH reoxidation, including the reactions of the malate–aspartate shuttle and the electron-transport chain. This would involve a total of at least ten enzymes and transport proteins, and therefore the contribution from each individual step is likely to be small.

When the acetaldehyde concentration is high (above 100 μM) the apparent C^J_{ADH} is decreased (Table 1), and other enzymes may play a more important role in regulation of ethanol metabolism. The most likely candidate for this role is aldehyde dehydrogenase. It has been suggested previously that the concentration of acetaldehyde is governed by a balance between the activities of alcohol dehydrogenase and aldehyde dehydrogenase (Eriksson *et al.*, 1975; Braggins *et al.*, 1980; Braggins & Crow, 1981; Dawson, 1981; Crow *et al.*, 1982; Harrington *et al.*, 1988). The steady-state concentration of acetaldehyde may vary widely as a result of small changes in the relative activities of these two enzymes. Dawson (1981) suggested, on the basis of qualitative experiments, that when the acetaldehyde concentration is elevated aldehyde dehydrogenase becomes an important rate-determining step. The decrease in the Flux Control Coefficient for alcohol dehydrogenase with increasing acetaldehyde concentration provides quantitative support for this idea.

Using the connectivity property (Kacser & Burns, 1973; Kacser, 1983*a*) and the values of C^J_{ADH} that we have determined experimentally, we have calculated approximate values for the Flux Control Coefficient of aldehyde dehydrogenase. This involved use of the kinetic parameters for alcohol dehydrogenase (Cornell *et al.*, 1979) and aldehyde dehydrogenase to calculate Elasticity Coefficients ($\epsilon^E_{acetaldehyde}$; Westerhoff *et al.*, 1984) towards acetaldehyde for these two enzymes. Rat liver has low-K_m and high-K_m forms of aldehyde dehydrogenase (Siew *et al.*, 1976), with K_m values about 1 μM and 1 mM respectively. These values were not determined at physiological pH, and we have used them only as reasonable approximations.

For hepatocytes from starved rats, where the acetaldehyde concentration is about 1 μM, only the low-K_m form of aldehyde dehydrogenase is relevant. The value of C^J_{ADH} is then given by:

$$C^J_{ALDH} = -C^J_{ADH} \cdot \left(\frac{\epsilon^{ADH}_{acetaldehyde}}{\epsilon^{ALDH}_{acetaldehyde}} \right) \quad (4)$$

We calculated an Elasticity Coefficient for aldehyde dehydrogenase ($\epsilon^{ALDH}_{acetaldehyde}$) of 0.50. For alcohol dehydrogenase the Elasticity Coefficient is -8.4×10^{-3} (assuming [NADH] = 2 μM; see above). Since C^J_{ADH} is about 0.5, C^J_{ALDH} will be less than 0.01.

In hepatocytes from fed rats the acetaldehyde concentration can be as high as 138 μM; we found a value of C^J_{ADH} of 0.14 at this concentration. The Elasticity Coefficients at 138 μM-acetaldehyde are -0.88 for alcohol dehydrogenase and 0.0070 (for K_m = 1 μM) for the low-K_m aldehyde dehydrogenase. Assuming that only the low-K_m aldehyde dehydrogenase is involved, C^J_{ALDH}/C^J_{ADH} is therefore about 125 and the calculated C^J_{ALDH} is much greater than 1; this is not realistic (Kacser, 1983*a*). The calculated value of C^J_{ALDH} is lower if the high-K_m aldehyde dehydrogenase, which will have a much higher Elasticity Coefficient towards acetaldehyde at 138 μM (0.88 for a K_m of 1 mM), contributes significantly to removal of acetaldehyde. Calculation of the relative Flux Control Coefficients of the three enzymes by the method of Kacser (1983*a*) shows that, if flux through the high-K_m enzyme is 10% of the total flux, C^J_{ALDH} of the low-K_m enzyme will be only about 8 times C^J_{ADH}. These calculations show that C^J_{ALDH} increases as C^J_{ADH} decreases with increasing acetaldehyde concentration, and that the importance of aldehyde dehydrogenase in control of ethanol metabolism depends on the proportions of the high-K_m and low-K_m forms of the enzyme in the liver.

In humans, acetaldehyde concentrations present in peripheral blood during ethanol metabolism are usually very low (< 1 μM), but liver concentrations may be considerably higher (Lindros, 1989). Therefore aldehyde dehydrogenase may play a significant regulatory role in human ethanol metabolism.

The quantitative analysis of this pathway has provided support for the hypothesis that alcohol dehydrogenase is, under metabolic conditions where the acetaldehyde concentration is low, an important regulatory enzyme in the pathway. Assumptions that this enzyme is 'present in excess' and 'not rate-limiting' (Hawkins & Kalant, 1972; Kalant *et al.*, 1975) are clearly not correct. It is also clear, however, that alcohol dehydrogenase is not the only rate-determining enzyme; if it were, a Flux Control Coefficient close to 1.0 would be observed. This explains why, in the past, some experiments have lent support to the ideas that aldehyde dehydrogenase or the processes of NADH reoxidation are rate-limiting. The relative contributions of these factors under various metabolic conditions have yet to be quantitatively determined. This study illustrates very clearly that, as suggested by Kacser (1983*b*), we should not be looking for a single rate-determining step for this pathway. There are a number of rate-determining steps, and the relative significance of each of these varies with the metabolic state of the liver.

It has been assumed for many years that, once the rate-determining step for the pathway of ethanol metabolism was identified, it might be possible to develop drugs that would provide accelerated ethanol clearance (Alkana & Noble, 1979). Since this pathway has several rate-determining steps, it will probably not be possible to develop a single rapid-detoxification drug, and it is probably not surprising that attempts to increase the rate of ethanol oxidation in human subjects have not been particularly successful (Crow & Hardman, 1989).

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APPENDIX

Calculation of Flux Control Coefficients from inhibition data

The Flux Control Coefficient for an enzyme can be determined from inhibition studies by using eqn. (A1) (Groen *et al.*, 1982):

$$C^J_{E_f} = \frac{\left(\frac{dJ/J}{dI}\right)_{I=0}}{\left(\frac{\partial V_f/V_f}{\partial I}\right)_{I=0}} \quad (A1)$$

The numerator of eqn. (A1) represents the effect of the inhibitor on flux through the pathway and is determined from a plot of flux against [inhibitor]. The denominator of eqn. (A1) represents the effect of the inhibitor on the enzyme isolated from the pathway flux but under the same conditions as those which apply at flux J. This term can be calculated from the inhibition characteristics of the enzyme.

For a general non-competitive inhibitor (nomenclature of Cleland, 1970), also known as a mixed inhibitor (Cornish-Bowden, 1979, pp. 76–78), the rate in the absence of products, v, is given by eqn. (A2), where K_m represents the Michaelis constant for the substrate and K_{is} and K_{ii} represent the slope and intercept inhibition constants for the inhibitor (Plapp, 1970):

$$v = \frac{V \cdot S}{K_m\left(1+\frac{I}{K_{is}}\right)+S\left(1+\frac{I}{K_{ii}}\right)} \quad (A2)$$

Differentiation with respect to I, followed by taking the limit as I approaches zero, and substitution into eqn. (A1), gives:

$$C^J_E = \frac{-dJ}{J \cdot dI} \cdot \left(\frac{K_m+S}{K_m/K_{is}+S/K_{ii}}\right) \quad (A3)$$

For the following special cases eqn. (A3) reduces to previously published equations: pure non-competitive (Groen *et al.*, 1982) when $K_{is} = K_{ii}$ (eqn. 1 of the main paper), uncompetitive (Derr, 1986) when K_{is} is infinite, and competitive (Derr, 1986) when K_{ii} is infinite. We wish to emphasize that many of the examples of non-competitive inhibition given in the literature are in fact cases of general non-competitive inhibition (mixed inhibition) and the general equation, eqn. (A3), which involves a substrate concentration term, should be used. For example, for inhibition of rat liver alcohol dehydrogenase by isobutyramide values of both K_{is} and K_{ii} were determined (Plapp *et al.*, 1984); therefore eqn. (A3) applies.

Although Derr (1986) has derived expressions for the Flux Control Coefficient of a one-substrate enzyme in the presence of a product, enzymes with two substrates and two products, such as alcohol dehydrogenase, have not previously been considered. Rat liver alcohol dehydrogenase has an Ordered Bi Bi mechanism (Cornell *et al.*, 1979). TMSO is a pure non-competitive inhibitor with respect to ethanol, with $K_{is} = K_{ii}$; therefore TMSO binds to both E–NADH and E–NAD^+, with equal dissociation constants (Chadha *et al.*, 1983; Plapp *et al.*, 1984). Isobutyramide is an uncompetitive or general non-competitive (mixed) inhibitor and binds to E–NADH more tightly than to E–NAD^+ (Plapp *et al.*, 1984). We have derived expressions for the Flux Control Coefficient for this system on the basis that the inhibitor binds to both E–NADH and E–NAD^+ or to E–NADH alone.

The denominator of eqn. (A1) can be derived from the rate equation for an Ordered Bi Bi system in the presence of a dead-end inhibitor (Segel, 1975, pp. 767–779). For an inhibitor that binds to E–NADH (EQ), the full rate equation for an Ordered Bi Bi reaction (Cornish-Bowden, 1979, p. 105) is modified by multiplying the terms contributed by [EQ] (Segel, 1975, pp. 560–563) by $(1+I/K_i)$. In pure non-competitive inhibition, the inhibitor also binds equally well to E–NAD^+ (EA) and the terms contributed by EA are also multiplied by $(1+I/K_i)$. For the former case, which represents uncompetitive inhibition with respect to ethanol, the modified rate equation is given by eqn. (A4):

$$v = \frac{\dfrac{V_f[A][B]}{K_{ia}K_b} - \dfrac{V_r[P][Q]}{K_pK_{iq}}}{1+\dfrac{[A]}{K_{ia}}+\dfrac{K_a[B]}{K_{ia}K_b}+\dfrac{K_q[P]}{K_pK_{iq}}+\dfrac{[Q]}{K_{iq}}\left(1+\dfrac{I}{K_i}\right)+\dfrac{[A][B]}{K_{ia}K_b}\left(1+\dfrac{I}{K_i}\right)+\dfrac{K_q[A][P]}{K_{ia}K_pK_{iq}}+\dfrac{K_a[B][Q]}{K_{ia}K_bK_{iq}}\left(1+\dfrac{I}{K_i}\right)+\dfrac{[P][Q]}{K_pK_{iq}}+\dfrac{[A][B][P]}{K_{ia}K_bK_{ip}}+\dfrac{[B][P][Q]}{K_{ib}K_pK_{iq}}} \quad \text{(A4)}$$

Re-arrangement of eqn. (A4), followed by differentiation with respect to I and taking the limit when $I = 0$, gives:

$$\left(\frac{dv}{v\cdot dI}\right)_{I=0} = \frac{-K_p(K_a[B][Q]+K_{iq}[A][B]+K_{ia}K_b[Q])/K_i}{K_{ia}K_bK_{iq}K_p+K_{iq}K_pK_b[A]+K_{iq}K_pK_a[B]+K_{ia}K_bK_q[P]+K_{ia}K_bK_p[Q]+K_{iq}K_p[A][B]+K_bK_q[A][P]+K_pK_a[B][Q]} + K_{ia}K_b[P][Q]+\frac{K_pK_{iq}[A][B][P]}{K_{ip}}+\frac{K_{ia}K_b[B][P][Q]}{K_{ib}} \quad \text{(A5)}$$

Substitution of eqn. (A5) into eqn. (A1) leads to:

$$C_E^J = \left(\frac{-K_i\cdot dJ}{J\cdot dI}\right)\left(\frac{K_{iq}K_p\left(K_{ia}K_b+K_b[A]+K_a[B]+\dfrac{[A][B][P]}{K_{ip}}\right)+K_{ia}K_b\left(K_q[P]+[P][Q]+\dfrac{[B][P][Q]}{K_{ib}}\right)+K_bK_q[A][P]}{K_p([Q]K_{ia}K_b+[Q]K_a[B]+[A][B]K_{iq})}+1\right) \quad \text{(A6)}$$

Comparison with eqn. (1) of the main paper shows that the apparent Flux Control Coefficient calculated by using that equation should be modified by multiplication by the term in large parentheses in eqn. (A6) to allow for the presence of the second substrate and the products.

For pure non-competitive inhibition, the same procedure gives eqn. (A7):

$$C_E^J = \left(\frac{-K_i\cdot dJ}{J\cdot dI}\right)\left(\frac{K_{iq}K_p\left(K_{ia}K_b+K_a[B]+\dfrac{[A][B][P]}{K_{ip}}\right)+K_{ia}K_b\left(K_q[P]+\dfrac{[B][P][Q]}{K_{ib}}\right)}{K_p(K_{iq}K_b[A]+K_{ia}K_b[Q]+[A][B]K_{iq}+K_a[B][Q])+K_b(K_{ia}[P][Q]+K_q[A][P])}+1\right) \quad \text{(A7)}$$

The corresponding equation for a general non-competitive (mixed) inhibitor would be more complex, involving two different inhibition constants. We found that the value of C^J_{ADH} obtained by using eqn. (A3), which assumes general non-competitive inhibition of a single-substrate irreversible reaction, was intermediate between the values from equations for pure non-competitive and uncompetitive inhibition for a single-substrate reaction. We have therefore used eqns. (A6) and (A7) to calculate a range of values of the modified Flux Control Coefficient for alcohol dehydrogenase, which we expect to include the values for general non-competitive inhibition.

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