Quantitative analysis of intermediary metabolism in rat hepatocytes incubated in the presence and absence of ethanol with a substrate mixture including ketoleucine

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Hepatocytes isolated from livers of fed rats were incubated with a mixture of glucose (10 mM), ribose (1.0 mM), acetate (1.25 mM), alanine (3.5 mM), glutamate (2.0 mM), aspartate (2.0 mM), 4-methyl-2-oxovaleric acid (ketoleucine) (3.0 mM), and, in paired flasks, 10 mM-ethanol. One substrate was ^{14}C -radiolabelled in any given incubation. Incorporation of ^{14}C into glucose, glycogen, CO_2 , lactate, alanine, aspartate, glutamate, acetate, urea, lipid glycerol, fatty acids and the 1- and 2,3,4-positions of ketone bodies was measured after 20 and 40 min of incubation under quasi-steady-state conditions. Data were analysed with the aid of a realistic structural metabolic model. In each of the four conditions examined, there were approx. 77 label incorporation measurements and several measurements of changes in metabolite concentrations. The considerable excess of measurements over the 37 independent flux parameters allowed for a stringent test of the model. A satisfactory fit to these data was obtained for each condition. There were large bidirectional fluxes along the gluconeogenic/glycolytic pathways, with net gluconeogenesis. Rates of ureagenesis, oxygen consumption and ketogenesis were high under all four conditions studied. Oxygen utilization was accurately predicted by three of the four models. There was complete equilibration between mitochondrial and cytosolic pools of acetate and of CO_2 , but for several of the metabolic conditions, two incompletely equilibrated pools of mitochondrial acetyl-CoA and oxaloacetate were required. Ketoleucine was utilized at a rate comparable to that reported by others in perfused liver and entered the mitochondrial pool of acetyl-CoA directly associated with ketone body formation. Ethanol, which was metabolized at rates comparable to those *in vivo*, caused relatively few changes in overall flux patterns. Several effects related to the increased NADH/NAD $^{+}$ ratio were observed. Pyruvate dehydrogenase was completely inhibited and the ratio of acetoacetate to 3-hydroxybutyrate was decreased; flux through glutamate dehydrogenase, the citric acid cycle, and ketoleucine dehydrogenase were, however, only slightly inhibited. Net production of ATP occurred in all conditions studied and was increased by ethanol. Futile cycling was quantified at the glucose/glucose 6-phosphate, glycogen/glucose 6-phosphate, fructose 6-phosphate/fructose 1,6-bisphosphate, and phosphoenolpyruvate/pyruvate/oxaloacetate substrate cycles. Cycling at these four loci consumed about 22% of cellular ATP production in control hepatocytes and 14% in ethanol-treated cells.

INTRODUCTION

In a preceding study of liver cell metabolism (Crawford & Blum, 1983), hepatocytes isolated from fed rats were incubated with a mixture of glucose (10 mM), ribose (1 mM), mannose (4 mM), glycerol (3 mM), acetate (1.25 mM), and ethanol (5 mM). Label incorporation into metabolic products was analysed with the aid of a full model of the 'upper' pathways (gluconeogenesis, glycolysis, and the pentose phosphate shunt), and a minimal model of mitochondrial reactions such as the citric acid cycle. Futile cycling at several major metabolic crossroads was quantified and the classical formulation of the pentose phosphate cycle was confirmed. Rabkin & Blum (1985) expanded the previous model by using a more 'physiological' substrate mixture, which permitted more comprehensive modelling of the 'lower' mitochondrial reactions including the citric acid and urea cycles.

In the present study, hepatocytes from fed rats were incubated, in the presence and absence of ethanol, with a mixture containing glucose, ribose, alanine, glutamate, aspartate, acetate and 4-methyl-2-oxovaleric acid (ketoleucine). Radiolabel incorporation data were supplemented with measurements of the concentrations of glucose, glycogen, alanine, lactate, urea, aspartate, glutamate, acetate, 3-hydroxybutyrate and acetoacetate, and of the rate of oxygen consumption. Agreement between the model's predictions and these measurements, as well as the fit to the isotopic flow data, indicate the validity of the model.

Acute ethanol administration alters both the cytosolic and mitochondrial oxidation/reduction states, the former by alcohol dehydrogenase, the latter by acetaldehyde dehydrogenase. The increased NADH/NAD $^{+}$ ratio can, in principle, affect all NAD $^{+}$ -dependent dehydrogenases in the liver cell. Although many studies have examined the acute effects of ethanol on hepatocyte

Abbreviations used: ketoleucine, 4-methyl-2-oxovaleric acid. Other abbreviations are given in the legend to Fig. 1.

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metabolism, alterations caused by the large influx of acetate resulting from ethanol oxidation have not been thoroughly addressed. In the present study, quantification of flow through pathways associated with acetate metabolism was facilitated by the inclusion of ethanol, acetate and ketoleucine in the substrate mixture. Ketoleucine, which is derived from the transamination of leucine, was of further interest due to the unique role of the liver in branched-chain ketoacid catabolism. Metabolism of the branched-chain amino acids is initiated largely by rat muscle (Wahren *et al.*, 1976). The ketoacids are released into the blood and taken up by the liver for further oxidation (Livesey & Lund, 1980). Most previous studies have examined ketoleucine metabolism and the effects of ethanol on liver cells in the presence of few or no other substrates. The present study quantified the distribution of ketoleucine carbon and the perturbations due to acute ethanol administration in hepatocytes exposed to a complex substrate mixture. Our data support the view that under some metabolic conditions there are effectively two pools of acetyl-CoA (Von Glutz & Walter, 1975; DesRosiers *et al.*, 1988; Leiser, 1988) and of oxaloacetate (Heath & Rose, 1985) in the mitochondrion, but that there is only a single pool of CO_2 in rat hepatocytes (Balboni & Lehninger, 1986; Marsolais *et al.*, 1987). In addition, the flux values determined from the fits to the label incorporation data were used to calculate the amount of energy in ATP equivalents which was dissipated by 'futile' cycling in four substrate cycles.

EXPERIMENTAL

Hepatocytes were isolated from male Sprague-Dawley rats weighing 250–380 g by the method of Berry & Friend (1969) as modified by Wood & Blum (1982). The animals were kept on a 12 h light/12 h dark cycle, with Purina rat chow and water available *ad libitum*. Surgery on sodium pentobarbital-anaesthetized animals was performed between 09:30 and 10:00 h (3.5–4 h into the light period). Viability as assessed by Trypan Blue exclusion was routinely greater than 85% and did not decrease during the incubation period.

Incubations

Incubations were performed in triplicate in 50 ml polycarbonate flasks, and were initiated by adding 1.0 ml of washed hepatocyte suspension to 2.5 ml of substrate-supplemented bicarbonate buffer, pH 7.4. In each experiment, paired flasks were run with and without 10 mM-ethanol. Components of the final incubation mixture were 5–8 mg/ml hepatocyte protein, 0.05 mM-defatted, dialysed bovine serum albumin, 10 mM-glucose, 1.0 mM-ribose, 3.5 mM-alanine, 1.25 mM-acetate, 2.0 mM-glutamate, 2.0 mM-aspartate, 3.0 mM-ketoleucine, 145 mM- Na^+ , 6 mM- K^+ , 1.2 mM- Mg^{2+} , 127 mM- Cl^- , 24 mM- HCO_3^- , 1.2 mM- SO_4^{2-} , 1.2 mM-buffered HPO_4^{2-} , 1.0 mM-DL-carnitine, 5.0 mM-Hepes buffer and 10 mM-ethanol (when present). Each flask also contained tracer amounts of one of the following radiolabelled substrates: [$1\text{-}^{14}\text{C}$]- or [$6\text{-}^{14}\text{C}$]glucose, [$1\text{-}^{14}\text{C}$]- or [$\text{U}\text{-}^{14}\text{C}$]ribose, [$1\text{-}^{14}\text{C}$]- or [$\text{U}\text{-}^{14}\text{C}$]alanine, [$1\text{-}^{14}\text{C}$]- or [$2\text{-}^{14}\text{C}$]acetate, [$1\text{-}^{14}\text{C}$]- or [$\text{U}\text{-}^{14}\text{C}$]glutamate, [$1\text{-}^{14}\text{C}$]- or [$4\text{-}^{14}\text{C}$]aspartate, [$1\text{-}^{14}\text{C}$]-, [$2\text{-}^{14}\text{C}$]- or [$\text{U}\text{-}^{14}\text{C}$]ketoleucine, or [$1\text{-}^{14}\text{C}$]ethanol. Final specific activities in d.p.m./nmol were: glucose 18–38, ribose 138–325, alanine 71–95, aspartate 121–159,

glutamate 92–160, acetate 797–1020, ketoleucine 23–91, and ethanol 18–108. Flasks were gassed with 95% O_2 /5% CO_2 for 15 s, capped, and incubated in a 37 °C shaking water bath (80 oscillations/min) for 0, 20 or 40 min. For measurement of glycogen content and radioactivity, incubations were terminated by adding 1 ml of incubation suspension to 6 ml of ice-cold 83% (v/v) ethanol (Wood & Blum, 1982). For all other measurements, incubations were terminated by adding 0.3 ml of 13% (w/v) HClO_4 to the capped flasks and cooling to 0 °C. $^{14}\text{CO}_2$ was collected by addition of 0.2 ml of hyamine hydroxide to the centre wells and incubation for 1 h at 25 °C with shaking (Passman *et al.*, 1956). The HClO_4 suspensions were then neutralized with KOH and the centrifuged supernatants used for all assays except glycogen.

Preparation of radiolabelled ketoleucine

L-[$\text{U}\text{-}^{14}\text{C}$]- and L-[$2\text{-}^{14}\text{C}$]ketoleucine were synthesized from the appropriately labelled leucine derivatives, according to the method of Rudiger *et al.* (1972), as modified by Odessey & Goldberg (1979).

Measurements

Incorporation of radioactivity into glucose and amino acids was measured as described by Crawford & Blum (1983). Glycogen content and radioactivity were determined as described by Wood & Blum (1982). Radiolabelled lactate, urea content, alanine content, lactate content, glucose content and oxygen consumption were measured as described by Rabkin & Blum (1985). Radiolabel incorporation into lipid glycerol and fatty acids was quantified using an adaption of the hexane/isopropanol extraction of Hara & Radin (1978). The amount of ^{14}C in positions 1 and 2–4 of acetoacetate and 3-hydroxybutyrate was determined using a method based on the enzymic procedure of Mewes *et al.* (1979). Measurement of label incorporation from ethanol into acetate utilized the Conway microdiffusion chamber (Obrink, 1955). Radiolabelled urea was measured by incubation of samples with urease and determination of the $^{14}\text{CO}_2$ liberated (Hems & Saez, 1983). Ethanol content was determined with alcohol dehydrogenase according to Cornell & Veech (1983). Acetate content was measured using the enzymic assay of Guynn & Veech (1975). Acetoacetate and 3-hydroxybutyrate contents were determined with 3-hydroxybutyrate dehydrogenase by the methods of Mellanby & Williamson (1974). Glutamate and aspartate content were measured enzymically (Bergmeyer *et al.*, 1974; Berni & Bergmeyer, 1974). Isovaleryl carnitine was determined by fast atom bombardment-mass spectrometry using a VG 7070EQ mass spectrometer; as described by Roe *et al.* (1984). Details of these assays are available (Baranyai, 1988). Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Measurements of radioactivity

Radioactivity was counted in a model 3375 Packard TriCarb liquid-scintillation spectrometer with automatic external standard for quench correction. Hydrofluor scintillant (National Diagnostics, Highland Park, NJ, U.S.A.) was used for determination of glucose, lipids and lactate; other samples were counted in Safety-Solve scintillant (Research Products International, Elk Grove Village, IL, U.S.A.).

Materials

Collagenase (approx 150 units/mg) and Flozyme Glucose Reagent were from Worthington Biochemical Corp., Freehold 2, NJ, U.S.A. Sodium pentobarbital was from Barber Veterinary Supply Co., Inc. Hyamine hydroxide was from National Diagnostics. Absolute ethanol was purchased from Aaper Alcohol and Chemical Co., Shelbyville, KY, U.S.A. Glucose was from Mallinkrodt, St. Louis, MO, U.S.A., and 3-hydroxybutyrate was from Boehringer-Mannheim Corp. New York, NY, U.S.A. Bovine serum albumin (fraction V), all other incubation mixture components and all enzymes used for metabolite assays were from Sigma Chemical Co., St. Louis, MO, U.S.A. [$1\text{-}^{14}\text{C}$]- and [$6\text{-}^{14}\text{C}$]glucose, [$\text{U-}^{14}\text{C}$]ribose, [$\text{U-}^{14}\text{C}$]alanine, [$1\text{-}^{14}\text{C}$]- and [$2\text{-}^{14}\text{C}$]acetate, [$1\text{-}^{14}\text{C}$]- and [$\text{U-}^{14}\text{C}$]glutamate, [$2\text{-}^{14}\text{C}$]leucine, [^{14}C]urea, and [$\text{carboxyl-}^{14}\text{C}$]tri olein were from Research Products International. All other radioactive compounds were from Amersham International. Triolein and [$9\text{-}^2\text{H}$]isovalerylcarnitine were generously donated by Dr. Robert Bell and Dr. Charles R. Roe, respectively. Other chemicals were of the highest grade commercially available.

PRELIMINARY CONSIDERATIONS

The technique of analysis employed in these studies relies upon only two assumptions: that the system is near isotopic and metabolic steady state according to defined criteria (Blum & Stein, 1982; Leiser, 1988) and that the metabolic model accurately portrays pathways relevant to metabolism of the substrates provided.

Choice of substrates

Substrate mixture components were chosen to provide well-distributed inputs and outputs over the metabolic network. While net consumption of glutamate and aspartate would be low due to restricted uptake (Haussinger & Gerok, 1984), it was anticipated that label incorporation data from these amino acids would help constrain interpretation of flux patterns in the 'lower' portion of the metabolic model. Ketoleucine was of interest not only because of the importance of understanding branched chain oxoacid metabolism in liver, but also since it would contribute to information regarding acetyl-CoA pool structure and ketone body metabolism. Substrate concentrations had to be large enough to avoid significant depletion during the 40 min incubation and to minimize dilution of specific activity of any substrates which were also produced by the cells. Glucose, ribose, alanine, aspartate and glutamate concentrations were chosen based on these considerations and in accordance with amounts used in previous work (Crawford & Blum, 1983; Rabkin & Blum, 1985; J. Leiser & J. J. Blum, unpublished work). Ketoleucine concentration was set at 3.0 mM since preliminary experiments indicated that this was the minimal amount needed to prevent rate-limiting depletion. Although the concentration of ketoleucine in rat arterial blood is about 0.05 mM (Livesey & Lund, 1980), the K_m for the rat liver branched-chain ketoacid dehydrogenase is 10–15 μM (Parker & Randle, 1978). Therefore, the higher concentration of ketoleucine should not further increase flux through this enzyme. Ethanol at 10 mM in blood produces mild intoxication in humans (Gilman *et al.*,

1985). Since rats are able to tolerate higher blood ethanol concentrations than humans (Barry, 1979), 10 mM can be considered a low dose. It is, however, sufficient to alter the redox state of the cells (Christenson & Higgins, 1979).

Metabolic scheme

The metabolic model used to analyse the data (Fig. 1) is consistent with current information regarding the compartmental structure, subcellular location of enzymes and metabolites, and enzyme complement of rat hepatocytes. It includes all pathways relevant to metabolism of the substrate mixture components. Except for deletion of the fructokinase pathway, the upper portion of the model is identical to that employed by Rabkin & Blum (1985). The lower part of the model is considerably more complex than previous schemes. In earlier models, it was assumed for simplicity that metabolically-generated $^{14}\text{CO}_2$ was infinitely diluted by unlabelled HCO_3^- in the medium. Since our study and those of others (Tomera *et al.*, 1982; Hems & Saez, 1983) indicate that this approximation is inadequate, we did not assume such equilibration and also allowed for the possibility of separate mitochondrial and cytosolic pools of CO_2 . In careful studies on isolated rat liver mitochondria, Lopes-Cardozo *et al.* (1978) concluded that there was effectively a single pool of acetyl-CoA, whereas Von Glutz & Walter (1975) concluded that there were two pools. Provision was made, therefore, for possible non-equilibration of mitochondrial acetyl-CoA. Pathways for ketoleucine metabolism and ketone body production were added. Reactions involving acetate and fatty acid metabolism were essentially as depicted by Rabkin & Blum (1985), with the addition of mitochondrial acetyl-CoA hydrolase. Acetyl-CoA hydrolase activity in rat liver cell fractions has been reported to be 70% mitochondrial, 20% cytosolic and 10% lysosomal (Seufert *et al.*, 1978). Ethanol input was directly into mitochondrial acetate, since acetaldehyde dehydrogenase is located within the mitochondria. Formation of isovalerylcarnitine was included since significant amounts of branched chain acylcarnitines have been detected in whole liver (Bohmer & Bremer, 1968; Choi *et al.*, 1977), and isolated mitochondria incubated with carnitine and ketoleucine release a small quantity of isovalerylcarnitine (Solberg & Bremer, 1970). Recently, L-carnitine administration has been found to be therapeutic in the treatment of isovaleric acidemia resulting from genetic deficiency of isovaleryl-CoA dehydrogenase (Roe *et al.*, 1984). The excess isovaleryl-CoA is conjugated with carnitine and the resulting isovalerylcarnitine is excreted in the urine. In the present study, 1.0 mM-DL-carnitine was included in the substrate mixture to replenish the carnitine lost during isolation of the hepatocytes (Christiansen & Bremer, 1976).

Amino acid metabolism was modelled in considerably more detail than in previous models. In addition to the three amino acids supplied, catabolism of other amino acids could result from breakdown of endogenous protein. Proteolysis was modelled as input into alanine, glutamate or aspartate, with input or output of other amino acids included indirectly as components of the fluxes associated with the three substrate amino acids. Separate cytosolic and mitochondrial pyruvate pools and cytosolic and mitochondrial alanine and aspartate aminotransferase reactions (DeRosa & Swick, 1975)

Fig. 1. Best-fit models for data obtained from control hepatocytes

Label incorporation measurements taken from Baranyai (1988) were used to obtain best-fit estimates of steady-state fluxes for the 0–20 min interval, while the data in Table 1 were used for the 20–40 min interval. Each pair of numbers represents values for fluxes, expressed as nmol/20 min per mg of protein from cells incubated with the standard substrate mixture. The upper number is the optimal-fit flux during the 0–20 min incubation interval; the lower number corresponds to the 20–40 min interval. A single pair of numbers at a bidirectional reaction indicates that only net flux through those pathways could be determined; small arrows show the direction of net carbon flow at these nodes. Metabolite abbreviations used: AcAcCoA, acetoacetyl-CoA; AcCoA_C, AcCoA_{M1} and AcCoA_{M2}, cytosolic and mitochondrial acetyl-coenzyme A; ACET_C and ACET_M, cytosolic and mitochondrial acetate; ACETI and ACETO, acetate input and output; ALA, intracellular alanine; ALAI and ALAO, alanine input and output; ASP, intracellular aspartate; ASPI and ASPO, aspartate input and output; CIT, citrate; DHAP, dihydroxyacetone phosphate; E4P, erythrose 4-phosphate; EALA, alanine input from endogenous protein; EGLUT, glutamate input from endogenous protein; FDP, fructose diphosphate; F6P, fructose 6-phosphate; FUM, fumarate; GAP, glyceraldehyde

were also included. The citric acid and urea cycles were modelled as described by Rabkin & Blum (1985).

Since studies on label distribution in the liver of intact rats (Heath & Rose, 1985) indicated that there were likely to be two pools of mitochondrial oxaloacetate (OAA), provision was made for such compartmentation. For all metabolites with potentially separate pools, the initial fits were performed with very large fluxes between pools, i.e. with effectively a single pool.

Analysis of data

Label incorporation data were expressed as nmol/20 min interval per mg of protein. Radioactivity present in products in zero time samples was subtracted to correct for background in the various assays. Since alanine concentration decreased appreciably during the incubation, the d.p.m. remaining as alanine at 20 and 40 min were used to compute the appropriate background values for this substrate.

The four sets of corrected label incorporation data (control 0–20 min and 20–40 min and ethanol-treated 0–20 min and 20–40 min) were then analysed by applying previously developed techniques (reviewed by Blum & Stein, 1982) to the biochemical pathways depicted in Fig. 1. Briefly, the reactions were described by a set of simultaneous linear equations detailing the flow of radioactivity into and out of each carbon atom of each intermediate in the system, under steady-state conditions. A computer program (presented in detail in Leiser, 1988) was used to solve the specific activity of every carbon atom in the scheme for a given set of trial values of the fluxes. The rate of label incorporation from any carbon of the substrates into each of the products could then be calculated and compared to the experimental measurements. A model was fitted by repeatedly adjusting trial flux values until an optimal fit (see below) was obtained. The model was completely determined by a maximum of 57 independent flux values. The number was reduced to an average of 37 because only net flux through some bidirectional flux pairs was ascertained. The considerable excess of experimental measurements (77–81 per fit) over independent parameters ensured a stringent test of the model.

Calculation of acetate specific activity

When ethanol was present, large amounts of acetate were produced (see Table 3). If the ethanol was unlabelled, this acetate decreased the specific radioactivity of [1-¹⁴C]- or [2-¹⁴C]acetate when either of these was the labelled substrate. When the tracer was [1-¹⁴C]-ethanol, labelled acetate was produced, creating a pool of acetate of unknown specific radioactivity which could then be utilized and transferred into other measured products. Procedures used to account for these effects, which had not been encountered in previous studies, are presented in detail in the Appendix.

RESULTS

Tables 1 and 2 present the experimental and computed data for incorporation of ¹⁴C into the metabolites shown when hepatocytes were incubated with the standard substrate mixture, with a single substrate labelled as indicated. These data are for the 20–40 min interval after the addition of hepatocytes to the substrate mixture in the absence (Table 1) or presence (Table 2) of ethanol. Data for the 0–20 min intervals are available in Baranyai (1988). Rates of production or consumption of several substrates and products are listed in Table 3.

Ethanol metabolism

Ethanol was oxidized at a linear rate of 266 ± 56 nmol/min per mg of protein ($n = 5$), in accordance with literature reports for hepatocytes isolated from fed rats (Williamson & Tischler, 1979). The general pattern of label incorporation from [1-¹⁴C]ethanol matched that of [1-¹⁴C]acetate.

Ketoleucine metabolism

Control cells metabolized ketoleucine linearly with time (Table 3). Ethanol caused a small but significant decrease in this rate. The end products of ketoleucine metabolism are acetoacetate and acetyl-CoA in a 1:1 proportion. Hepatocytes in this study were highly ketogenic, producing about 88 nmol of ketone bodies/mg of protein (acetoacetate + 3-hydroxybutyrate) during a 20 min interval. Most of this was from ketoleucine metabolism; label incorporation from [U-¹⁴C]ketoleucine into total ketone bodies was 75 nmol/20 min per mg of protein. Lesser amounts of ketone bodies were also formed from acetate (and ethanol). These values agree with reported rates (Lund, 1978, 1981; Patel *et al.*, 1981). Appearance of label from [2-¹⁴C]- and [U-¹⁴C]ketoleucine in several products in addition to CO₂ and ketone bodies demonstrated that acetyl-CoA derived from the ketoacid had metabolic fates other than complete oxidation in the citric acid cycle. Label incorporation into glutamate approached or exceeded CO₂ production. Fatty acid formation from [2-¹⁴C]ketoleucine (which is metabolized to [1-¹⁴C]acetyl-CoA) ranged from 2 to 5.5 nmol/20 min per mg of protein or roughly 2–6% of total label incorporation from [2-¹⁴C]ketoleucine. These values are comparable to the 5% incorporation of ketoleucine-derived acetyl-CoA into lipid reported by others (Lund, 1981). Radiolabelled urea was produced from [1-¹⁴C]ketoleucine, indicating reincorporation of ¹⁴CO₂. There was about 4% as much labelled urea as ¹⁴CO₂ in each of the four conditions. Ketoleucine oxidation rates calculated from evolution of ¹⁴CO₂ from [1-¹⁴C]ketoleucine were, therefore, slightly low. Samples were analysed for acylcarnitines formed from the various coenzyme A intermediates of ketoleucine metabolism. Only isovalerylcarnitine was detected; it accounted for

3-phosphate; GLUI and GLUO, glucose input and output; G6P, glucose 6-phosphate; GLUT, intracellular glutamate; GLUTI and GLUTO, glutamate input and output; GLYI, glucose input from glycogen degradation; GLYO, newly-synthesized glycogen; HMGCoA, hydroxymethylglutaryl-CoA; ISOCT, isocitrate; IVC, isovalerylcarnitine; IVCoA, isovaleryl-coenzyme A; KB, ketone bodies; KIC, ketoleucine; LACO, lactate output; LFA, lipid fatty acid; MAL_c and MAL_m, cytosolic and mitochondrial malate; OAA_c, OAA_{m1} and OAA_{m2}, cytosolic and mitochondrial oxaloacetate; OG, oxoglutarate; PEP, phosphoenolpyruvate; PYR_c and PYR_m, cytosolic and mitochondrial pyruvate; R15P, ribose 5-phosphate; RU5P, ribulose 5-phosphate; S7P, sedoheptulose 7-phosphate; SUCC, succinate; XU5P, xylulose 5-phosphate. Fluxes in the model are designated by *V*; see text for further details.

Table 1. Experimental and computed ^{14}C label incorporation into metabolic products by 20–40 min in control cells

Upper numbers are incorporation data given as means $\pm$ s.e.m. (avg. $n = 4$). Lower values are predictions from optimal fit fluxes shown in Fig. 1. N.M., not measured; *, not used in calculating fit parameter (see text for details); <, upper limit; KB, ketone bodies; IVC, isovalerylcarnitine.

Labelled substrate	Incorporation (nmol/20 min per mg of protein)													
	CO ₂	Glucose	Lactate	Aspartate	Glutamate	Alanine	Acetate	Urea	KB:1	KB:2	Fatty acids	Glycerol	Glycogen	IVC
[1- ¹⁴ C]Glucose	2.82±0.38 2.81	N.M. 35.2	5.27±1.96 5.74	2.07±0.94* 1.78	1.02±0.54 0.54	4.39±1.06 2.85	N.M. 0.15	N.M. 0.05	< 0.20* 0.05	< 0.18* 0.10	0.17±0.06 0.04	0.79±0.14 0.86	5.98±1.12 6.85	N.M. 0
[6- ¹⁴ C]Glucose	1.74±0.24 1.58	N.M. 35.2	N.M. 6.59	N.M. 2.03	N.M. 0.62	N.M. 3.27	N.M. 0.17	N.M. 0.03	N.M. 0.04	N.M. 0.11	N.M. 0.05	N.M. 0.75	N.M. 6.84	N.M. 0
[1- ¹⁴ C]Ribose	1.78±0.47 1.83	22.6±2.2 19.9	3.12±0.17 3.14	1.05±0.59 0.98	0.46±0.76 0.20	2.28±1.21 1.56	N.M. 0.05	N.M. 0.04	< 0.16* 0.04	< 0.23* 0.03	0.01±0.01 0.01	0.51±0.08 0.48	2.98±0.83 3.86	N.M. 0
[U- ¹⁴ C]Ribose	1.78±0.47 1.52	N.M. 18.9	N.M. 3.87	N.M. 1.20	N.M. 0.27	N.M. 1.92	N.M. 0.07	N.M. 0.03	N.M. 0.05	N.M. 0.03	N.M. 0.02	N.M. 0.50	N.M. 3.68	N.M. 0
[1- ¹⁴ C]Alanine	47.0±1.2 47.5	9.60±1.46 11.5	27.6±3.0 21.5	22.2±2.9 20.8	1.72±0.32 1.37	N.M. 79.2	N.M. 0	0.97±0.14 1.12	N.M. 1.24	N.M. 0	N.M. 0	N.M. 0.38	2.63±0.40 2.23	N.M. 0
[U- ¹⁴ C]Alanine	23.0±1.4 21.9	22.8±3.2 22.2	23.6±2.3 26.8	24.7±2.9 22.3	6.91±1.09 5.00	N.M. 82.9	< 1.4* 1.29	0.43±0.11 0.51	N.M. 0.78	N.M. 0.67	N.M. 0.37	N.M. 0.75	4.50±0.69 4.32	N.M. 0
[1- ¹⁴ C]Acetate	9.19±0.82 10.2	2.10±0.21 2.33	1.52±0.41 1.07	0.66±0.11 0.45	4.36±0.37 4.53	0.62±0.06 0.53	N.M. 18.3	N.M. 0.23	2.06±0.19 1.93	1.90±0.24 1.68	2.65±0.34 2.41	0.03±0.03 0.08	N.M. 0.45	N.M. 0
[2- ¹⁴ C]Acetate	1.74±0.16 1.88	6.47±0.32 6.04	3.75±0.79 2.81	1.16±0.22 0.88	4.31±0.47 5.47	1.98±0.31 1.39	N.M. 18.4	N.M. 0.04	< 0.08* 0.06	3.04±0.26 3.40	2.43±0.49 2.43	0.17±0.04 0.20	N.M. 1.17	N.M. 0
[1- ¹⁴ C]Glutamate	9.63±0.73 10.6	N.M. 0.09	3.92±0.56* 0.04	N.M. 0.19	N.M. 3.53	N.M. 0.02	N.M. 0	0.37±0.20 0.34	N.M. 0.37	N.M. 0	N.M. 0	N.M. 0	N.M. 0.02	N.M. 0
[U- ¹⁴ C]Glutamate	5.86±0.29 5.68	N.M. 2.48	6.44±1.33* 1.15	N.M. 0.43	N.M. 4.14	N.M. 0.57	N.M. 0.02	0.15±0.01 0.15	N.M. 0.17	N.M. 0.01	N.M. 0.01	N.M. 0.08	N.M. 0.48	N.M. 0
[1- ¹⁴ C]Ketoleucine	92.4±4.1 94.4	N.M. 0.76	N.M. 0.35	N.M. 1.66	N.M. 0.18	N.M. 0.17	N.M. 0	3.53±0.58 3.01	N.M. 3.31	N.M. 0	N.M. 0	N.M. 0.03	N.M. 0.15	N.M. 0
[2- ¹⁴ C]Ketoleucine	34.1±3.0 34.2	5.38±0.87 7.82	7.01±2.49 3.61	1.25±0.10 1.50	19.6±2.3 15.3	1.38±0.17 1.79	N.M. 16.3	N.M. 0.77	6.30±1.83 8.26	5.76±0.79 7.42	3.78±1.07 4.61	0.20±0.03 0.26	N.M. 1.52	N.M. 0.75
[U- ¹⁴ C]Ketoleucine	20.3±1.5 22.5	4.33±0.50 4.82	5.21±1.74 2.24	1.10±0.15 1.02	6.18±1.31 5.64	1.02±0.13 1.11	N.M. 5.46	N.M. 0.65	2.21±0.46 1.96	69.5±2.7 55.4	1.28±0.42 1.55	0.17±0.01 0.16	N.M. 0.94	0.70±0.30 0.63

Table 2. Experimental and computed ^{14}C label incorporation into metabolic products by 20–40 min in ethanol-treated cells

Upper numbers are incorporation data given as means $\pm$ s.e.m. (avg. $n = 4$). Lower values are predictions from optimal fit fluxes shown in Fig. 2. N.M., not measured; *, not used in calculating fit parameter (see text for details); <, upper limit; KB, ketone bodies; IVC, isovalerylcarnitine.

Labelled substrate	Incorporation (nmol/20 min per mg of protein)													
	CO ₂	Glucose	Lactate	Aspartate	Glutamate	Alanine	Acetate	Urea	KB:1	KB:2	Fatty acids	Glycerol	Glycogen	IVC
[1- ¹⁴ C]Glucose	2.03±0.24 2.07	N.M. 38.8	3.91±1.78 2.30	N.M. 0.50	1.27±0.72 0.41	N.M. 1.13	N.M. 0	N.M. 0.04	<0.19* 0.04	<0.18* 0.01	<0.05* 0	0.59±0.07 0.57	7.95±0.86 7.92	N.M. 0
[6- ¹⁴ C]Glucose	0.94±0.18 0.99	N.M. 35.8	N.M. 4.80	N.M. 1.02	N.M. 0.85	N.M. 2.36	N.M. 0	N.M. 0.02	N.M. 0.02	N.M. 0.03	N.M. 0	N.M. 0.77	N.M. 7.32	N.M. 0
[1- ¹⁴ C]Ribose	1.47±0.13 1.46	28.8±1.6 28.7	1.90±0.52 1.65	0.26±0.09 0.36	0.38±0.12 0.23	0.67±0.14 0.81	N.M. 0	N.M. 0.03	<0.15* 0.03	<0.22* 0.01	<0.02* 0	0.40±0.06 0.42	6.45±1.65 5.87	N.M. 0
[U- ¹⁴ C]Ribose	1.52±0.18 1.37	N.M. 26.3	N.M. 3.17	N.M. 0.68	N.M. 0.45	N.M. 1.56	N.M. 0	N.M. 0.03	N.M. 0.04	N.M. 0.01	N.M. 0	N.M. 0.58	N.M. 5.38	N.M. 0
[1- ¹⁴ C]Alanine	43.9±2.0 44.4	16.5±2.7 18.3	8.76±2.06 7.16	12.6±2.0 10.8	3.59±0.19 3.49	N.M. 27.3	N.M. 0	0.83±0.07 1.08	N.M. 1.22	N.M. 0	N.M. 0	N.M. 0.59	3.61±0.43 3.75	N.M. 0
[U- ¹⁴ C]Alanine	17.6±1.2 18.2	31.8±2.3 31.3	9.77±1.63 10.7	11.9±2.2 11.3	8.85±0.72 7.64	N.M. 28.7	<3.30* 0.01	0.52±0.03 0.44	N.M. 0.55	N.M. 0.16	N.M. 0.03	N.M. 1.02	7.62±1.02 6.39	N.M. 0
[1- ¹⁴ C]Acetate	3.24±0.68 3.09	1.27±0.26 0.96	0.27±0.07 0.26	0.26±0.06 0.12	3.42±0.47 3.20	0.14±0.01 0.13	N.M. 0	N.M. 0.08	5.96±1.07 5.33	5.05±0.52 5.24	1.65±0.32 1.73	0.02±0.004 0.03	N.M. 0.20	N.M. 0
[2- ¹⁴ C]Acetate	0.39±0.03 0.40	3.42±0.44 2.36	0.78±0.21 0.64	0.27±0.07 0.20	3.51±0.44 3.64	0.30±0.10 0.32	N.M. 0	N.M. 0.01	2.25±0.39 0.01	9.39±0.74 10.5	1.68±0.32 1.73	0.08±0.01 0.08	N.M. 0.48	N.M. 0
[1- ¹⁴ C]Glutamate	7.28±0.53 7.23	N.M. 0.10	1.88±0.88* 0.03	N.M. 0.08	N.M. 5.49	N.M. 0.01	N.M. 0	0.17±0.10 0.26	N.M. 0.29	N.M. 0	N.M. 0	N.M. 0	N.M. 0.02	N.M. 0
[U- ¹⁴ C]Glutamate	3.65±0.26 3.66	N.M. 2.10	1.47±0.41* 0.57	N.M. 0.22	N.M. 6.23	N.M. 0.28	N.M. 0	0.08±0.03 0.11	N.M. 0.12	N.M. 0	N.M. 0	N.M. 0.07	N.M. 0.43	N.M. 0
[1- ¹⁴ C]Ketoleucine	82.3±2.6 84.0	N.M. 1.15	N.M. 0.31	N.M. 0.88	N.M. 0.36	N.M. 0.15	N.M. 0	3.74±0.33 3.04	N.M. 3.42	N.M. 0	N.M. 0	N.M. 0.04	N.M. 0.24	N.M. 0
[2- ¹⁴ C]Ketoleucine	15.0±1.8 13.9	4.49±0.24 4.33	<4.4 1.17	0.50±0.06 0.55	19.8±3.0 14.4	N.M. 0.57	N.M. 2.68	N.M. 0.34	17.1±1.9 24.0	23.3±3.0 23.6	5.46±1.36 5.87	0.13±0.02 0.14	N.M. 0.88	N.M. 1.08
[U- ¹⁴ C]Ketoleucine	15.3±1.0 16.6	2.68±0.22 2.69	<5.2 0.73	0.61±0.22 0.39	6.95±0.77 5.19	N.M. 0.36	N.M. 0.89	N.M. 0.57	4.10±0.30 4.58	69.2±2.8 58.1	2.01±0.45 1.96	0.11±0.01 0.09	N.M. 0.55	0.91±0.09 0.90
[1- ¹⁴ C]Ethanol	9.07±1.17 10.8	3.13±0.59 3.35	0.93±0.10 0.90	0.34±0.16* 0.43	9.89±0.51 11.1	0.06±0.04* 0.44	273±28 243	N.M. 0.27	11.5±1.9 18.6	18.7±0.3 18.3	7.78±1.84 6.03	0.09±0.02 0.11	N.M. 0.68	1.85±0.28 1.81

Table 3. Rates of production or consumption of metabolites by control and ethanol-treated hepatocytes

Flux rates are means $\pm$ S.E.M., $n = 5$. Negative values indicate net consumption. Statistical significance was tested with a multifactor analysis of variance, $P < 0.05$. * Indicates ethanol-treated significantly different from control; † indicates significant difference between time intervals.

Time interval (min)	Rate of production/consumption (nmol/mg of protein)	
	Control	+ Ethanol
Acetate		
0-20	-28.3 $\pm$ 2.2	165.6 $\pm$ 4.5*
20-40	-25.3 $\pm$ 2.9	162.9 $\pm$ 5.1*
Acetoacetate		
0-20	73.8 $\pm$ 2.0	54.2 $\pm$ 1.4*
20-40	70.2 $\pm$ 2.3	55.6 $\pm$ 3.1*
Alanine		
0-20	-110.7 $\pm$ 9.6	-125.9 $\pm$ 10.9
20-40	-73.0 $\pm$ 7.7†	-65.3 $\pm$ 8.3†
3-Hydroxybutyrate		
0-20	16.9 $\pm$ 0.5	32.5 $\pm$ 1.0*
20-40	17.3 $\pm$ 1.9	31.2 $\pm$ 2.2*
Lactate		
0-20	71.5 $\pm$ 12.9	81.2 $\pm$ 10.8
20-40	55.2 $\pm$ 9.8	24.8 $\pm$ 2.9*†
Urea		
0-20	51.3 $\pm$ 1.4	50.4 $\pm$ 2.3
20-40	59.8 $\pm$ 2.8	57.4 $\pm$ 2.8
Ketoleucine		
0-20	-96.8 $\pm$ 3.5	-86.0 $\pm$ 3.4*
20-40	-92.2 $\pm$ 4.1	-82.1 $\pm$ 2.6*

about 2% of the total ketoleucine oxidized. The sum of label incorporation from [2-¹⁴C]- and [U-¹⁴C]ketoleucine into other measured products agreed with the amount oxidized, as determined by ¹⁴CO₂ formation from [1-¹⁴C]ketoleucine. Transamination of ketoleucine to leucine (not included in Fig. 1) in control and ethanol-treated hepatocytes occurred at a rate of 9.0 $\pm$ 0.4 nmol/20 min per mg of protein during the first 20 min interval and was slightly lower in the second time interval. These results agree with literature reports that the rate of leucine formation from ketoleucine by isolated hepatocytes is less than 10% of the rate of ketoleucine decarboxylation (Williamson *et al.*, 1979).

Effects of ethanol on metabolism

Acetate was consumed by control cells, but was produced by ethanol-treated cells (Table 3). 3-Hydroxybutyrate and acetoacetate were produced at constant rates both in the presence and absence of ethanol. Ethanol decreased acetoacetate production by approximately 20%, but nearly doubled that of 3-hydroxybutyrate. Total ketone body concentrations were unaffected. The ratio of 3-hydroxybutyrate to acetoacetate, often used as an index of the mitochondrial oxidation/reduction state, increased from 0.2 in control hepatocytes to 0.6 in ethanol-treated cells. This 3-fold increase with ethanol is comparable to published results (Rawat, 1968; Sugden & Williamson, 1982; Davis & Baugh, 1985).

Glucose, glycogen, glutamate and aspartate concen-

trations were unchanged throughout the 40 min incubation in both control and ethanol-treated cells (results not shown). Glycogen content was 946 $\pm$ 27 nmol glucose equivalents/mg protein as expected (Hems *et al.*, 1978). Appearance of label from acetate, alanine and glutamate in glucose, concurrent with incorporation of glucose carbon into amino acids and CO₂, indicated simultaneous gluconeogenic and glycolytic fluxes. The ratio of ¹⁴CO₂ from [1-¹⁴C]glucose to ¹⁴CO₂ from [6-¹⁴C]glucose was always greater than one, showing operation of the pentose phosphate shunt; this ratio was increased in the presence of ethanol.

Contrary to what might be expected from the change in cytosolic redox state, lactate concentration decreased in the presence of ethanol (Table 3). Although the rate of alanine consumption declined during the second time interval in both control and ethanol-treated cells, ethanol did not affect net alanine utilization. Ethanol did, however, reduce the rate of label incorporation from [1-¹⁴C]- and [U-¹⁴C]alanine into lactate; the magnitude of this effect was comparable to the decrease in net lactate production.

Significant amounts of urea were produced at a constant rate by both control and ethanol-treated hepatocytes (Table 3), indicating that adequate cellular ATP levels were maintained in both conditions, in agreement with direct measurements of ATP concentrations by Stowell & Crow (1985). Ethanol did not alter rates of urea production or label incorporation from [1-¹⁴C]- and [U-¹⁴C]alanine, [1-¹⁴C]- and [U-¹⁴C]glutamate, or [1-¹⁴C]ketoleucine into urea.

¹⁴CO₂ production from [1-¹⁴C]- and [6-¹⁴C]glucose, [U-¹⁴C]alanine, [1-¹⁴C]- and [2-¹⁴C]acetate, [1-¹⁴C]- and [U-¹⁴C]glutamate and [2-¹⁴C]- and [U-¹⁴C]ketoleucine were decreased in ethanol-treated cells, indicating possible inhibition of the citric acid cycle.

There were no significant alterations of label appearance in fatty acids in the presence of ethanol. Incorporation of [1-¹⁴C]- or [2-¹⁴C]acetate or of [U-¹⁴C]- or [2-¹⁴C]ketoleucine into fatty acids tended to be higher with ethanol.

Oxygen consumption rates were 899 $\pm$ 19 nmol of oxygen atoms/20 min per mg of protein for control cells ($n = 5$) and 874 $\pm$ 13 nmol of oxygen atoms/20 min per mg of protein for ethanol-treated cells ($n = 5$). These rates were substantially greater than those observed in previous studies (Crawford & Blum, 1983; Rabkin & Blum, 1985), presumably due to the presence of ketoleucine in the substrate mixture.

Statistical analysis of data

The [1-¹⁴C]- and [4-¹⁴C]aspartate data (incorporation into CO₂, glucose, lactate, glutamate, alanine and urea) were not used in fitting the models. Measurements from these tracers were inconsistent with one another (Leiser, 1988); calculations of total aspartate utilization from the two tracers also disagreed. It is possible that impurities were present in one or both of the labelled compounds. Several of the lactate incorporation measurements were also neglected, due to technical difficulties with the assay. Upper limits for several other measurements were calculated when small numbers and/or scatter in the data prevented conclusive measurements. Values calculated by the models were always less than these limits. Notice that in Tables 1 and 2 several of the S.E.M. values are over half of the mean, an indication of positive

squares parameter, E , as defined by Blum & Stein (1982). Flux values resulting in minimal values for E are presented in Figs. 1 and 2. E is essentially equivalent to the total unexplained variation and hence, a minimum value of E is a satisfactory indication that the best fit to the data has been achieved. The quality of this fit, however, was analysed as described by Burdick (1985).

Goodness-of-fit was assessed with a weighted least

Data for the 0–20 min interval were taken from Baranyai (1988), while those for the 20–40 min interval are from Table 2. See the legend to Fig. 1 for further details.

(see below). The optimal fit parameters were: 0–20 min control, $E = 176$; 20–40 min control, $E = 135$; 0–20 min ethanol-treated, $E = 219$; 20–40 min ethanol-treated, $E = 175$. A satisfactory fit is achieved when each calculated value is within one standard deviation of the corresponding measured value. For experiments repeated in quadruplicate, this is four times the number of fitted measurements, or approximately 300. Thus, a satisfactory fit was obtained for each model.

Methods for statistical analysis of the interaction between label incorporation data and the model developed to fit those data have been presented elsewhere (D. S. Burdick in Rabkin & Blum, 1985). An F -distribution test using square root transformed data showed that random errors could not account for label incorporation into any of the products in any of the four models ($P < 10^{-5}$).

A second statistical consideration was whether the models explained a significant proportion of the variation between label incorporation measurements from the different substrates. This was examined by evaluating the ratio, r , of explained variation to total variation. The r value, including the oxygen consumption data, was > 0.99 for all four models, indicating that most of the information contained in each data set was accounted for.

The degree of constraint associated with individual flux values in the fitted models varies from flux to flux since there is a complex relationship between the flux values, the arrangement of pathways in the metabolic scheme, the distribution of labelled inputs and outputs, and the precision of the label incorporation measurements. The acceptable range of values for each flux had previously been estimated by varying a given flux from its best-fit value until the fit to the measured data became unacceptable, i.e. until E increased by 5% (Crawford & Blum, 1983; Rabkin & Blum, 1985). This method of error range analysis was recognized to be incomplete, since the magnitude of the 5% error range depends upon which other fluxes, designated as 'counter-fluxes,' are allowed to vary and thus compensate for changes in the independent flux being examined (Leiser, 1988). Since counter-fluxes consist of both independent and dependent fluxes, a complete error analysis would require allowing every remaining flux to vary, at a prohibitive cost in time. Instead, error range analyses were performed by varying each independent flux value against a single or several counter-fluxes. A full analysis of the error range for each independent flux and of the counter-fluxes used to assess the error range is presented in Baranyai (1988) and is available upon request from the authors.

DISCUSSION

Validity of steady-state assumption

Although the analysis used is based on the assumption that the system reaches metabolic and isotopic steady-state soon after the incubation begins, flux calculations remain valid for systems in which changes in metabolite concentrations occur slowly relative to carbon flow through the pools (Borowitz *et al.*, 1977; Leiser, 1988). In this study, ethanol oxidation, ketoleucine decarboxylation and oxygen uptake were constant from the earliest time examined (5 min) through 40 min of incubation in both control and ethanol-treated cells (results not shown). Rates of metabolite production or consumption

(Table 3) were linear with time, with the exception of alanine consumption by both control and ethanol-treated cells and lactate production by ethanol-treated cells. These rates decreased significantly with time, but the degree of decline was consistent with a quasi-steady-state treatment in which all computed flux values are averages for each 20 min interval.

Best-fit flux values computed from the label incorporation measurements predict the net amounts of glucose, glycogen, glutamate, aspartate, acetate (ethanol-treated cells), lactate, urea and ketone bodies that should have been produced, and the amounts of alanine, acetate (control cells) and oxygen that should have been consumed during the incubations. Agreement between predicted and observed values was generally satisfactory (Table 4). Oxygen consumption rates were accurately predicted in all but the 0–20 min interval for control cells. Discrepancies between observed and predicted rates for amino acids will be discussed subsequently.

Metabolic compartmentation

The present model included provision for cytosolic/mitochondrial compartmentation of CO_2 . In each of the four models there was large bidirectional flow between CO_2 pools, indicating essentially complete equilibration in agreement with the results of Balboni & Lehninger (1986) and Marsolais *et al.* (1987).

Initially, provision was made for the possible existence of both cytosolic and mitochondrial pools of acetate. Acetate derived from ethanol entered the mitochondrial pool. It was found, however, that the data for each of the four conditions could best be fitted with complete equilibration between the pools. This accords well with the report that acetate is freely permeable to mitochondria (Patel *et al.*, 1981). As noted above, there have been conflicting reports concerning the existence of two pools of acetyl-CoA within rat liver mitochondria (Von Glutz & Walter, 1975; Lopes-Cardozo *et al.*, 1978). Examination of the label incorporation data of our study indicated that a single pool of mitochondrial acetyl-CoA probably would not be sufficient to explain our results. Carbon atom 1 of acetyl-CoA became radiolabelled when either $[1-^{14}\text{C}]$ acetate or $[2-^{14}\text{C}]$ ketoleucine was the labelled input. For the 0–20 min interval in control cells, the ratio of label from $[2-^{14}\text{C}]$ ketoleucine to label from $[1-^{14}\text{C}]$ acetate appearing in ketone bodies averaged 6.2, while the ratio into CO_2 , aspartate, glutamate, alanine and lipid fatty acid was 2.9 ± 0.4 . For the 20–40 min interval in control cells, the ratio was 3.0 ± 0.4 for all measured products. The ratio was also constant for both time intervals in ethanol-treated cells.

We formulated the two pool configuration such that acetyl-CoA from ketoleucine entered pool AcCoA_{M1} , while acetyl-CoA derived from acetate (and ethanol) entered pool AcCoA_{M2} (Figs. 1 and 2). This configuration is consistent with evidence that in rat liver mitochondria acetyl-CoA from palmitate was preferentially used for ketone body formation, while acetyl-CoA from pyruvate oxidation was preferentially incorporated into citrate (Von Glutz & Walter, 1975).

The degree of equilibration between two pools depends on the magnitude of the inputs and outputs to and from the pools relative to the rates of exchange between the pools (see Leiser, 1988, for a more quantitative discussion). In three of the four conditions studied, there was essentially complete equilibration between the

Table 4. Predicted and observed metabolic flux values for control and ethanol-treated hepatocytes

Values predicted by the model (upper numbers) are listed beside the appropriate flux symbols (see legend to Fig. 1). Experimentally measured values are listed below these, next to the name of the metabolite. Label incorporation measurements have been rounded to the nearest whole number.

Flux parameters and metabolites	Control cells		Ethanol-treated cells	
	0–20 min	20–40 min	0–20 min	20–40 min
$V_{12} - V'_{12}$	1	5	0	8
Glycogen	11 ± 18	–35 ± 23	20 ± 15	–31 ± 16
$V'_G - V_G$	52	52	63	77
Glucose	60 ± 46	92 ± 48	126 ± 35	75 ± 18
$V'_{ALA} - V_{ALA} - V_{EALA}$	–151	–130	–133	–96
Alanine	–111 ± 10	–73 ± 8	–126 ± 11	–65 ± 8
$V'_{GLT} - V_{GLT} - V_{EGLT}$	14	–51	38	5
Glutamate	21 ± 5	30 ± 7	26 ± 4	50 ± 7
$V_{ASP} + V_{EASP} - V'_{ASP}$	30	7	62	73
Aspartate	–19 ± 14	–5 ± 14	–15 ± 6	–3 ± 12
$V'_{AC} - V_{AC}$	–24	–5	164	158
Acetate	–28 ± 2	–25 ± 3	166 ± 5	163 ± 5
V_{LDH}	92	93	74	50
Lactate	72 ± 13	81 ± 11	55 ± 10	25 ± 3
V_{GDH}	84	94	78	82
Urea	51 ± 1	60 ± 3	50 ± 2	52 ± 3
V_{KB}	118	114	136	135
Ketone bodies	91 ± 3	88 ± 4	87 ± 2	87 ± 5
Oxygen	509	775	745	815
consumption	881 ± 18	918 ± 35	854 ± 19	894 ± 11

AcCoA_{M1} and AcCoA_{M2} pools, i.e. they behaved as a single pool. The 0–20 min data for control cells could not, however, be fitted with the assumption of a single pool; when [1-¹⁴C]- or [2-¹⁴C]acetate was the labelled input, the ratio of specific activity of AcCoA_{M1} to that of AcCoA_{M2} was 0.57, while with [2-¹⁴C]- or [U-¹⁴C]-ketoleucine this ratio was 0.79. Leiser (1988), in a study using a substrate mixture containing glucose, ribose, fructose, alanine, aspartate, glutamate, acetate and oleate, also required two incompletely mixed pools of mitochondrial acetyl-CoA to fit his data; the ratio of AcCoA_{M1} to AcCoA_{M2} specific activity for acetate as the labelled substrate was 0.70. DesRosiers *et al.* (1988) recently performed a study in which rat livers were perfused with glucose, lactate, pyruvate and ketoleucine in the presence or absence of oleate and octanoate. When [1-¹⁴C]acetate was the labelled input, the ratio of the specific activities of the carbons derived from acetyl-CoA in acetoacetate to those in citrate was 0.63. The close agreement between the results obtained in the present study and in that of Leiser & Blum (unpublished work; see Leiser, 1988) with those obtained by DesRosiers *et al.* (1988) on perfused liver demonstrate that under certain conditions mitochondrial acetyl-CoA derived from pyruvate and ketoleucine or fatty acids does not behave as a single pool. Enzyme–enzyme interactions *in situ* may be far more prevalent than has been thought from studies *in vitro* using very low protein concentrations. Srivastava & Bernhard (1986) have presented evidence that ‘direct transfer’ of intermediates from one enzyme to another may occur. Whether this accounts for the presence of two pools of acetyl-CoA in rat liver mitochondria remains to be determined.

Since OAA is impermeable to the mitochondrial membrane and must be transported as citrate, malate or

aspartate, the initial metabolic scheme included one cytosolic and one mitochondrial pool of OAA. A fully satisfactory fit to the data could not be obtained with this configuration for the 0–20 or 20–40 min control conditions. In particular, the incorporation of label from acetate or alanine into aspartate was difficult to fit. As noted above, several earlier studies (Mullhofer *et al.*, 1977; Katz & Rognstad, 1978) indicated that there might be two pools of OAA in the mitochondrion. In a more recent study, Heath & Rose (1985) infused [¹⁴C]-bicarbonate to a steady isotopic state into rats in the basal state induced by halothane anaesthesia. Measurements of specific radioactivities of blood bicarbonate, alanine, glycerol and lactate, of liver alanine and lactate, and of individual carbon atoms in blood glucose and liver aspartate, citrate and malate were combined with published rates of gluconeogenesis, liver uptake of gluconeogenic precursors and citric acid cycle flux. Although provision was made for CO₂ fixation by pyruvate carboxylase, phosphoenolpyruvate carboxykinase, isocitrate dehydrogenase and malic enzyme, their results could not be explained if there was only one well-mixed pool of OAA in the mitochondria. Incomplete mixing of mitochondrial OAA had to be assumed, with channelling of OAA formed from pyruvate towards gluconeogenesis. When such compartmentation was incorporated into the metabolic model of the present study (Fig. 1) the fit improved markedly (*E* decreased from 400 to 180 for the 20–40 min interval for control cells). In the ethanol-treated cells, however, a satisfactory fit was obtained whether there were one or two mitochondrial pools of OAA. Increased flux through the OAA pools, as is evident upon comparison of the flux values shown in Fig. 2 with those in Fig. 1, could account for the effective equilibration of these pools in the

Table 5. Contribution of substrate cycling to ATP consumption in control and ethanol-treated hepatocytes

For details of abbreviations see legend to Fig. 1.

Substrate cycle	Parameter	Control cells		+ Ethanol	
		0–20 min	20–40 min	0–20 min	20–40 min
Glucose/G6P	ATP consumption	60	62	46	60
	ATP cons/prod (%) [*]	2.2	2.1	1.4	1.7
Glycogen/G6P	ATP consumption	11	17	11	20
	ATP cons/prod (%)	0.4	0.6	0.3	0.6
F6P/FDP	ATP consumption	335	432	162	220
	ATP cons/prod (%)	12	15	4.8	6.2
PEP/PYR/OAA	ATP consumption [†]	133	215	177	209
	ATP cons/prod (%)	4.9	7.3	5.3	5.9

^{*} ATP consumed by cycle/total cellular ATP production. Total ATP production listed in Table 6.[†] ATP consumed by the futile mode depicted in Fig. 3(d) and Fig. 4(d). See text for details.**Table 6. Rates of utilization of ATP during incubation of control and ethanol-treated hepatocytes**

See legend to Fig. 1 for definitions of flux symbols.

Reaction		ATP flux			
		Control cells		+ Ethanol	
		0–20 min	20–40 min	0–20 min	20–40 min
Glucokinase	V_G	60	62	46	60
Ribokinase	V_R	34	32	33	40
Glycogen synthetase	V_{12}^*	12	22	11	28
Phosphofructokinase	$V_3^{\dagger}$	335	432	162	220
Lactate dehydrogenase	$3V_{LDH}^{\dagger}$	277	278	224	152
Phosphoenolpyruvate carboxykinase	V_{PCK}^*	186	224	224	294
Acetyl-CoA thiokinase	$2V_{AKC}^{\S}$	1	6	3	3
Glycerol-phosphate dehydrogenase	$3V_{15}^{\dagger}$	14	19	18	20
Malic enzyme	$3(V'_{ME} - V_{ME})^{\dagger}$	111	–147	72	51
Pyruvate carboxylase	V_{PC}	133	215	177	209
Malate dehydrogenase	$3(V'_{MDHM} - V_{MDHM})^{\dagger}$	368	–2	281	208
Citrate lyase	V_{CL}	3	5	6	11
β -Methyl-crotonyl-CoA carboxylase	V_{KKB}	96	103	92	92
Fatty acid synthesis	$49/8 V_{FA}^{\parallel}$	23	50	42	74
3-Hydroxybutyrate dehydrogenase	$R \times V_{KB}^{\dagger, \P}$	83	80	231	230
Acetyl-CoA thiokinase	$2V_{AKM}^{\S}$	113	170	136	149
Urea synthesis	V_{GDH}^{**}	335	375	313	329
Glyceraldehyde-P dehydrogenase	$3(V'_6 - V_6)^{\dagger}$	176	208	246	339
Phosphoglycerate kinase	$V'_6 - V_6$	59	70	82	113
Total utilization of ATP		2418	2202	2399	2622

^{*} One UTP or GTP equivalent to one ATP.[†] Lower limits from error analysis.[‡] One NAD(P)H = 3 ATP.[§] ATP cleaved to AMP = 2 ATP consumed.^{||} Fatty acid synthetase: $(14 \text{ NADPH}/8 \text{ AcCoA}) \times 3 = 42/8 \text{ ATP}$, assuming average of 16 carbon atoms/fatty acid molecule.[¶] Control cells: $R = 3\text{-hydroxybutyrate}/\text{AcCoA} = 0.23 \times 3 \text{ ATP}/\text{NADH} = 0.7$; with ethanol: $R = 3\text{-hydroxybutyrate}/\text{AcCoA} = 0.57 \times 3 \text{ ATP}/\text{NADH} = 1.7$.^{**} Synthesis of one urea requires 4 ATP.

Table 7. Rates of production of ATP during incubation of control and ethanol-treated hepatocytes

See legend to Fig. 1 for definitions of flux symbols.

Reaction		ATP Flux			
		Control cells		+ Ethanol	
		0–20 min	20–40 min	0–20 min	20–40 min
Glucose 6-P dehydrogenase	$3V_1$	47	32	43	25
6-Phosphogluconate dehydrogenase	$3V_1$	47	32	43	25
Pyruvate dehydrogenase	$3V_{PDH}$	50	77	0	4
Alcohol dehydrogenase	$3V_E$	0	0	699	684
Aldehyde dehydrogenase	$3V_E$	0	0	699	684
Isocitrate dehydrogenase	$3V_{ID}$	270	315	199	198
Oxoglutarate dehydrogenase	$3V_{OGS}$	229	468	85	184
Succinate dehydrogenase	$2(V_{SM} - V'_{SM})^*$	153	312	57	122
Malate dehydrogenase	$3(V_{MDHC} - V'_{MDHC})$	960	600	673	690
Glutamate dehydrogenase	$3V_{GDH}$	251	281	235	247
Isovaleryl-CoA dehydrogenase	$2V_{KKB}^*$	193	207	184	185
Ketoleucine dehydrogenase	$3V_{KIC}$	296	312	280	281
Pyruvate kinase	V_{PK}	172	154	142	181
Succinyl-CoA synthetase	$V_{OGS}^\dagger$	76	156	28	61
Total production		2699	2946	3367	3571
Net production		281	744	968	949

* One $FADH_2 = 2$ ATP.

† One UTP or GTP equivalent to one ATP.

All other reactions are dehydrogenases: one $NAD(P)H = 3$ ATP.

presence of ethanol. Thus, as with the acetyl-CoA pools, the magnitudes of the input and output rates relative to the exchange rates between the pools determine whether or not they are completely equilibrated.

Glucose/glucose 6-phosphate substrate cycle

Net gluconeogenic flux occurred in each of the time intervals for both control and ethanol-treated cells, with alanine as the major gluconeogenic precursor. As expected from earlier studies (Katz & Rognstad, 1976; Crawford & Blum, 1983; Hue, 1982; Rabkin & Blum, 1985), there was simultaneous entry of glucose into the system via glucokinase (V_G in Figs. 1 and 2). Since glucose content did not change significantly from the initial level of 10 mM, the rate of glucose phosphorylation would be expected to remain constant (Bontemps *et al.*, 1978). Indeed, flux through V_G was approximately 60 nmol/20 min per mg of protein in three of the four conditions examined, but decreased to 46 nmol/20 min per mg of protein in the 0–20 min interval for ethanol-treated cells. Ethanol caused a similar 30% decrease in glucokinase flux in perfused rat livers (Topping *et al.*, 1979); inhibition of glucokinase activity by ethanol has been reported (Wimhurst & Harris, 1976). The glucose 6-phosphatase reaction (V'_G) also may be dependent upon factors other than substrate level; evidence has been presented for heterogeneous glucose 6-phosphate pools (Kalant *et al.*, 1987). In the present study, however, it was not necessary to invoke two pools of glucose 6-phosphate. Flux through V'_G was constant at about 112 nmol/20 min per mg of protein in three of the four conditions, but apparently increased by about 20% in the 20–40 min interval for ethanol-treated cells. The

mechanism of this small increase cannot be addressed by this study.

Substrate cycling has been quantified using radiolabel dilution methods. These methods are limited by assumptions regarding isotopic equilibration (Hue, 1981). Such assumptions were not required in the present study since quantification of substrate cycling is an automatic consequence of a fit to the label incorporation data (reviewed by Stein & Blum, 1978; Leiser & Blum, 1987). The amount of ATP equivalents dissipated by each substrate cycle is presented in Table 5. Overall cellular ATP consumption and production are listed in Tables 6 and 7, respectively.

ATP 'wastage' by cycling between glucose and glucose 6-phosphate was approximately 2% of net ATP production in each of the four conditions examined (Table 5). These results are in agreement with those of Crawford & Blum (1983), Rabkin & Blum (1985) and Leiser & Blum (unpublished work; see Leiser, 1988), who calculated ATP consumption at this locus to be about 4% of net production in control, glucagon-treated and ethanol-treated cells. Katz & Rognstad (1976) have estimated that recycling of glucose would dissipate less than 5% of the ATP synthesized by hepatocytes from rats fed a commercial diet. Thus, the ATP used by this cycle appears to be a relatively low and constant percentage of ATP production in several metabolic states of the rat.

Glycogen/glucose 6-phosphate substrate cycle

The glycogen synthase (V_{12} in Figs. 1 and 2) and glycogen phosphorylase (V'_{12}) system appears to be a classical example of efficient metabolic control, since

activation of the synthase is strongly coupled to inhibition of the phosphorylase (Hers, 1981). However, cycling between glycogen and glucose 6-phosphate has been observed (Stalmans *et al.*, 1974; Hue *et al.*, 1975; Katz *et al.*, 1979; Crawford & Blum, 1983; Rabkin & Blum, 1985; Leiser, 1988). In all four conditions of the present study, simultaneous synthesis and degradation of glycogen occurred with no change in the overall level. Individual flux values were not tightly constrained and the error range analysis indicated that either net synthesis or net degradation could be occurring at rates too low to be detectable by measurement of total glycogen content. ATP loss at this node was negligible (Table 5).

Fructose 6-phosphate (F6P)/fructose 1,6-bisphosphate (FDP) substrate cycle

The F6P/FDP cycle is catalysed by a pair of irreversible enzymes: phosphofructokinase (PFK) and fructose diphosphatase (FDPase) (V_3 and V'_3 in Figs. 1 and 2). These enzymes are regulated by a number of allosteric and covalent mechanisms (reviewed by Hue & Rider, 1987). Fructose 2,6-bisphosphate is a potent activator of liver PFK (Pilkis *et al.*, 1981a) and a competitive inhibitor of FDPase (Pilkis *et al.*, 1981b). Ethanol decreased flux through PFK in hepatocytes isolated from fed rats (Rognstad & Katz, 1976). Incubation of hepatocytes from fed rats with 10 mM-ethanol in the presence of 10 mM-glucose caused a rapid increase in the concentration of glycerol 3-phosphate and a slow, continuous decrease in the concentration of fructose 2,6-bisphosphate (Van Schaftingen *et al.*, 1984). These effects were explained by enhancement of glycerol 3-phosphate production due to the ethanol-induced increase in the cytosolic NADH/NAD⁺ ratio; the glycerol 3-phosphate was demonstrated to stimulate FDPase-2 and inhibit PFK-2.

In the present study, cycling between F6P and FDP consumed 12–15% of the ATP produced by control cells, but only 5–6% of ATP produced by ethanol-treated cells (Table 5). A comparable decrease in cycling at this locus in the presence of ethanol has been observed by others (Rognstad & Katz, 1976; Topping *et al.*, 1979). In our study, values for ($V'_3 - V_3$) were tightly constrained, but the individual flux rates could not be determined accurately, preventing evaluation of effects on the individual enzymes. The significant lowering of ATP wastage at the F6P/FDP cycle by ethanol is, however, consistent with an inhibitory effect of ethanol on flux through PFK (Van Schaftingen *et al.*, 1984).

Pentose phosphate shunt

Ribose contributed an appreciable fraction of the carbon used for gluconeogenesis and glycogen synthesis. Ribose utilization was not significantly affected by ethanol and was the same in the first and second time intervals (Figs. 1 and 2). Although bidirectional fluxes through the transketolase pathways of the pentose cycle (V_9 and V_{11}) were large and could not be determined individually, the net fluxes were tightly determined. Net flow from the transketolase and transaldolase reactions ($V_9 - V'_9 + V'_{10} - V_{10}$) accounted for approximately 40% of the net gluconeogenic flux into F6P. Although the percentage contribution of the pentose phosphate pathway to gluconeogenesis varied with different substrate mixtures, the rate of ribose utilization and the magnitudes of the net fluxes through the transaldolase and transketolase steps of the pentose phosphate cycle

were comparable in each metabolic state (Crawford & Blum, 1983; Rabkin & Blum, 1985; J. Leiser & J. J. Blum, unpublished work, see Leiser, 1988).

Phosphoenolpyruvate/pyruvate/oxaloacetate substrate cycle

The intersection of metabolic pathways involving phosphoenolpyruvate (PEP), pyruvate (PYR) and OAA is the most complex substrate cycle of the gluconeogenic/glycolytic pathways. It consists of reactions catalysed by pyruvate kinase (V_{PK}), pyruvate carboxylase (V_{PC}), malic enzyme (V_{ME}) and phosphoenolpyruvate carboxykinase (V_{PCK}), as well as both cytosolic (V_{MDHC}) and mitochondrial (V_{MDHM}) malate dehydrogenases. Alanine (V_{ALTC} , V_{ALTM}) and aspartate (V_{ASTC} , V_{ASTM}) amino-transferase reactions, and cytosolic/mitochondrial transport of pyruvate, citrate, aspartate and malate are also included (Figs. 1 and 2). Glucose synthesis from substrates such as pyruvate, lactate and alanine requires conversion of mitochondrial pyruvate to OAA by pyruvate carboxylase or conversion of cytosolic pyruvate to malate by malic enzyme. If pyruvate kinase is not completely inactivated during gluconeogenesis, substrate cycling will occur. There have been numerous reports of pyruvate recycling under a variety of physiological conditions (Katz & Rognstad, 1976; Rabkin & Blum, 1985; J. Leiser & J. J. Blum, unpublished work, see Leiser, 1988).

In all four conditions of the present study, there was net gluconeogenic flux concurrent with flow through pyruvate kinase. Cycling at the PEP/PYR/OAA crossroads was quantitatively analysed using procedures developed by Stein & Blum (1978) and extended by Leiser & Blum (1987). The steady-state flux pattern at this node (Figs. 3a and 4a) was decomposed into five fundamental modes (Fig. 3b–f for control cells and Fig. 4b–f for ethanol-treated cells). The non-futile mode (Figs. 3b and 4b) represents the most efficient pattern of flow through the PEP/PYR/OAA cycle for the input and output flux values in the steady-state configuration. Three of the four futile modes (c, e, and f) are energetically neutral, with no net production or utilization of ATP. Futile mode (d), however, results in expenditure of one ATP molecule per turn of the cycle. Although fluxes through pyruvate kinase and the phosphoenolpyruvate carboxykinase appeared to increase in the ethanol-treated cells, V_{PK} and V_{PCK} were components of the energetically neutral mode (f). Thus, despite variations in flux through individual enzymes and alterations in flow through some of the futile modes (c and f), ATP consumption by the PEP/PYR/OAA cycle remained constant at about 5–7% of the ATP produced in each of the four conditions examined (Table 5).

Lipid glycerol

The rate of label incorporation into lipid glycerol (V_{15}) was essentially constant in each of the four conditions studied, averaging about 6 nmol/20 min per mg of protein. These results indicate no appreciable enhancement of lipid glycerol synthesis by acute ethanol administration.

Acetate, ethanol and ketoleucine metabolism

Results regarding acetate and acetyl-CoA compartmentation have been discussed above. The best-fit models required mitochondrial acetyl-CoA hydrolase

(a) Steady-state flux configuration, taken from Fig. 1. Flux values are nmol/20 min per mg of protein; the upper number is for the first 20 min period and the lower number is for the second 20 min interval. The steady-state configuration can be decomposed into the five modes shown in (b)–(f). (b) The nonfutile mode; (c)–(f), the purely futile modes associated with the substrate cycle. Abbreviations are as in Fig. 1.

(V_{AHM}) and acetyl-CoA thiokinase (V_{AKM}) pathways. Simultaneous flow through these opposing reactions indicated cycling between acetate and acetyl-CoA, as has been observed elsewhere (Rabkin & Blum, 1985; Jessop *et al.*, 1986). Inclusion of cytosolic hydrolase or thiokinase did not improve the fit. Lipogenesis from cytosolic acetate was not required; flow from citrate lyase (V_{CL}) was sufficient to account for the low rates of fatty acid synthesis (V_{FA}). Flux through V_{FA} was higher in the second interval in both control and ethanol-treated hepatocytes, as observed in other studies (McGarry *et al.*, 1978; Rabkin & Blum, 1985). The slight increase in V_{FA} in the presence of ethanol was probably not significant, in accordance with evidence that synthesis of fatty acids *de novo* is not enhanced by ethanol (Barboriak & Menahan, 1979). Although cells in this study were provided with carnitine and a large supply of acetyl-CoA from ethanol, acetate and ketoleucine, inclusion of cytosolic/mitochondrial exchange of acetyl-CoA via carnitine acetyltransferase did not appreciably improve the fit, consistent with the low activity of this enzyme in mitochondria from fed rats (Lund & Bremer, 1983).

Although the rate of ethanol oxidation was about 10-

fold higher than the acetate consumption rate in control cells (Table 4), label incorporation from [$1\text{-}^{14}\text{C}$]ethanol was consistently only three times that from [$1\text{-}^{14}\text{C}$]acetate, suggesting that exogenous acetate utilization was increased by ethanol. Indeed, flux through V_{AC} increased from 44 nmol/20 min per mg of protein in control cells to 92–102 nmol in ethanol-treated cells. This was probably due to the rise in acetate levels resulting from ethanol oxidation; acetate uptake by perfused livers increases with increasing acetate concentration (Jessop *et al.*, 1986). A rise in acetate concentration and hence in acetyl-CoA levels could also account for the 15% increase in V_{KB} caused by ethanol, which appears to be significant according to the error range analysis presented in Baranyai (1988) even though there was no detectable increase in total ketone body concentration (Table 4).

Label from [$1\text{-}^{14}\text{C}$]ethanol appeared in CO_2 , glucose, lactate, glutamate, ketone bodies and lipid fatty acid, as well as acetate (Table 2), indicating that some of the acetate produced from ethanol was further metabolized, rather than simply being released. In the study of Crawford & Blum (1983), incorporation of ethanol into any product other than acetate was negligible. The

Fig. 4. The PEP/PYR/OAA crossroads in ethanol-treated cells

(a) Steady-state flux configuration, taken from Fig. 2. (b) The nonfutile mode; (c)–(f), purely futile modes. Flux values are nmol/20 min per mg of protein; the upper number is for the first 20 min period and the lower number is for the second 20 min interval. Abbreviations are as in Fig. 1.

metabolic state of their cells was quite different from that in the present study: the substrate mixture containing glycerol was considerably more reducing, the rate of ethanol oxidation was submaximal, flux through the citric acid cycle was very low, and there was no detectable ketogenesis. Thus, the metabolic fate of the acetate produced from ethanol depends upon the nature and concentrations of the components of the substrate mixture provided.

Citric acid cycle

Flux through the citric acid cycle was near capacity (Lindros, 1972) in the control cells and increased slightly during the 20–40 min interval in both control and ethanol-treated cells. The increase was greatest from oxoglutarate through malate and was probably related to alterations in input and output of these metabolites. In all four conditions examined, there were high rates of bidirectional flow between succinate and malate in the mitochondria and between malate and oxaloacetate in both the cytosol and mitochondria. Net transfer of malate was out of the mitochondria into the cytosol.

Ethanol, as noted above, increased the net flow of acetate into pool AcCoA_{M2} , apparently due to a

substantial decreased in flux through acetyl-CoA hydrolase (V_{AHM}) with little or no change through acetyl-CoA thiokinase (V_{AKM}). Input of acetyl-CoA to pool AcCoA_{M2} via pyruvate dehydrogenase was completely suppressed by ethanol, presumably because of the high intramitochondrial acetyl-CoA/CoA and NADH/NAD⁺ ratios (Cohen *et al.*, 1981). An increase in pool size of AcCoA_{M2} might be anticipated since the increase in formation of acetyl-CoA in this pool from acetate plus its decreased utilization for citrate synthesis (V_{CS}) exceeded the loss of input from pyruvate. Such an increase in AcCoA_{M2} pool size could account for the marked reduction in net transfer of acetyl-CoA from pool AcCoA_{M1} to AcCoA_{M2} (V_{AAEX}) in the presence of ethanol.

Ethanol has been reported to inhibit the tricarboxylic acid cycle by as much as 75% in livers perfused only with 5 mM-alanine and 10 mM-ethanol (Williamson *et al.*, 1969). Flux through the citric acid cycle is dependent upon the availability of oxaloacetate and acetyl-CoA (Williamson & Cooper, 1980). The considerably more modest effect of ethanol observed in the present study is probably attributable to the maintenance of high levels of mitochondrial acetyl-CoA due to inclusion of ketoleucine and acetate in the substrate mixture.

Alanine transamination and pyruvate pool structure

There was substantial label flow into and out of alanine, with net consumption of this amino acid. It was hoped that the large number of measurements of label flow made in this study would enable specification of the fluxes between alanine and the cytosolic and mitochondrial pools of pyruvate (V_{ALTC} , V_{ALTM} , V_{PPTX}). In fact, however, it was not possible to determine these fluxes; even the directions of flow between ALA, PYR_C , and PYR_M could not be specified. The representations shown in Figs. 1 and 2 are determined only in so far as they are consistent with the net inputs from ALA and PYR_C and with the net output from PYR_M . Despite this indeterminacy, it was necessary to include the two separate pyruvate pools with some alanine input into each. The fluxes through V_{ALTM} , V_{ALTC} and V_{PPTX} could be altered over wide ranges relative to their best fit values with scarcely any effect elsewhere in the model.

Cellular energy metabolism

The flux values shown in Figs. 1 and 2 were used to compute ATP consumption and production rates during each 20 min interval (Tables 6 and 7). Net ATP production by control cells appeared to be greater in the 20–40 min than in the 0–20 min interval, but the value in the first interval may be because the model underestimated oxygen consumption during this period. Ethanol increased net ATP production (by about 25% in the 20–40 min interval). The alcohol dehydrogenase and aldehyde dehydrogenase reactions contributed 40% of the total ATP produced in the presence of ethanol. Acute ingestion of ethanol has been reported to increase liver ATP levels (French, 1966), as would be expected from the oxidation of NADH. However, other studies have indicated constant or decreased amounts of ATP in livers of rats given ethanol by stomach tube or injection (Hernandez-Munoz *et al.*, 1978; Gillam & Ward, 1986). Our results indicate that the increased ATP production from the ethanol oxidation reactions was counteracted, in part, by decreased flux through other NADH-producing reactions.

Ketoleucine metabolism

Ketoleucine decarboxylation and ketone body production from it occurred at rates comparable to those reported in the literature (Lund, 1981). The oxidation rate was constant throughout the 40 min incubation, indicating that the substrate level remained saturating for the branched-chain ketoacid dehydrogenase. Transamination of ketoleucine to leucine occurred at less than 10% of the decarboxylation rate, in accord with the low levels of transaminase present in rat liver (Shinnick & Harper, 1976). Label from ketoleucine appeared in products other than ketone bodies and CO_2 , indicating that acetyl-CoA from ketoleucine had fates other than complete oxidation in the citric acid cycle. Isovalerylcarnitine was a minor product, accounting for less than 2% of the ketoleucine decarboxylated.

The major contribution of ketoleucine to our study was elucidation of the pool structure of acetyl-CoA in the mitochondrion. Comparison of label incorporation data from ketoleucine and acetate indicated that there was compartmentation of mitochondrial acetyl-CoA, with preferential use of acetyl-CoA derived from ketoleucine for ketone body production and acetyl-CoA

from acetate more closely associated with pyruvate dehydrogenase and the citric acid cycle.

Summary of metabolic effects of ethanol

Acute ethanol administration affects metabolic reaction pathways by increasing the cytosolic and mitochondrial NADH/NAD⁺ ratios (Reitz, 1979). Cells in this study oxidized ethanol at rates comparable to those observed *in vivo*, indicating adequate regeneration of both cytosolic and mitochondrial NAD⁺. Fluxes through several dehydrogenases were inhibited, consistent with the increased NADH/NAD⁺ ratio in the presence of ethanol: pyruvate dehydrogenase (V_{PDH}) was completely inactivated, while flows through ketoleucine dehydrogenase (V_{KIC}), glutamate dehydrogenase (V_{GDH}), citrate synthase (V_{CS}) and isocitrate dehydrogenase (V_{ID}) were mostly inhibited. The ratio of acetoacetate to 3-hydroxybutyrate decreased from about 5:1 to 2:1. However, flux rates through other enzymes reputed to be affected by NADH production from ethanol oxidation were unchanged. These included glyceraldehyde 3-phosphate dehydrogenase (V_6/V_6'), glucose 6-phosphate dehydrogenase (V_1), and glycerophosphate dehydrogenase (V_{15}). Lactate production (V_{LDH}), which would be expected to increase if the effect of NADH on the lactate dehydrogenase is the only factor, actually decreased.

Effects related to the increase in acetate concentration in ethanol-treated cells included a 15% stimulation of the rate of ketone body synthesis, and an increase in exogenous acetate utilization (Patel *et al.*, 1981; Jessop *et al.*, 1986).

Fluxes through several pathways reputed to be altered by ethanol remained unchanged in the present study. Net glycogen output, ribose utilization (V_R) and flow through the pentose phosphate shunt were the same in control and ethanol-treated cells. Chronic ethanol administration has been reported to increase glucose 6-phosphate dehydrogenase activity, suggesting activation of the pentose phosphate cycle (Luoma & Vorne, 1976). However, acute exposure of liver slices to ethanol did not affect this pathway (Majchrowicz & Quastel, 1963). Although both chronic and acute ethanol administration have been reported to cause accumulation of triacylglycerols in liver (Reitz, 1979), oxidation, synthesis, esterification or liver uptake of fatty acids or triacylglycerol secretion as well as alterations in glycerol metabolism may be involved. In this study, incorporation of label into lipid glycerol (V_{15}) was essentially constant in each condition examined. Oxygen consumption also was unaffected by ethanol. Studies using perfused livers or isolated hepatocytes from fed rats, provided with no substrates other than ethanol, have reported decreased oxygen utilization (Israel *et al.*, 1979), increased oxygen utilization (Yuki & Thurman, 1980), or no change (Stowell & Crow, 1985). Decreased urea production after an acute dose of ethanol (Stubbs & Krebs, 1975) and increased aspartate and glutamate production from alanine (Krebs *et al.*, 1973) have also been reported. There were no changes in net glutamate or aspartate levels or in urea production with ethanol treatment in the present study.

Substrate cycling at the four cycles examined accounted for 14% of the cellular ATP production in the presence of ethanol, compared to 22% in control cells. ATP loss by cycling between glucose/G6P and glycogen/G6P was low in all four conditions examined. Cycling at

the PEP/PYR/OAA crossroads resulted in significant ATP 'wastage', which was not altered by ethanol. The decreased ATP expenditure by substrate cycle reactions in the ethanol-treated cells was due to attenuation of flow through the F6P/FDP cycle.

Despite evidence that ethanol significantly altered the redox state of the cells in this study, there were relatively few changes in metabolic flux patterns. Although flux through specific enzymes, such as pyruvate dehydrogenase or lactate dehydrogenase, was quite different in the presence of ethanol, alterations in pathways associated with these reactions, such as the tricarboxylic acid cycle or PEP/PYR/OAA cycle, were minor. These results illustrate that ethanol effects observed in isolated cells or perfused livers provided with a single substrate are not necessarily applicable to hepatocytes metabolizing ethanol in the presence of a relatively rich substrate mixture which more closely approximates the supply of nutrients to the liver *in vivo*.

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APPENDIX

Calculation of acetate specific activity

The modelling analysis employed in this study had never been applied to a system in which significant label dilution or reutilization occurred. The inclusion of both ethanol and acetate in the substrate mixture required a method to calculate the specific radioactivity of the internal acetate pool when the radiolabel from [1-¹⁴C]- or [2-¹⁴C]acetate was diluted by acetate production from unlabelled ethanol, or when the external acetate became labelled by [1-¹⁴C]ethanol.

A critical consideration in these calculations was the model structure used to represent inputs and outputs into the intracellular acetate pool. This structure is depicted in Fig. A1. Ethanol enters directly into the internal acetate pool, since acetate derived from ethanol metabolism is produced inside the mitochondria where aldehyde dehydrogenase is localized. Flow between cytosolic/mitochondrial acetate and external acetate allows for mixing of acetate derived from ethanol with the acetate present in the substrate mixture. The

intracellular acetate derived from both ethanol and external acetate is the pool which is connected with other pathways of intermediary metabolism.

The following simplifying assumptions were made: (1) Output via flux V_{AKC} (cytosolic acetyl-CoA thiokinase) was presumed to be negligible. Results from the 0–20 min and 20–40 min control models supported this. (2) Rates V_{AHM} and V_{AKM} (mitochondrial acetyl-CoA hydrolase and thiokinase, respectively) were assumed to be small relative to the rate of ethanol input, V_E . Measurements of ethanol utilization and label incorporation from [1-¹⁴C]ethanol into acetate indicated that a substantial amount of ethanol was oxidized, and that nearly all of the label appeared in acetate. (3) Most acetate output (V'_{AC}) came from ethanol. This assumption was based on data indicating negligible label incorporation from [1-¹⁴C]- or [U-¹⁴C]alanine into acetate. (4) There is rapid equilibration between mitochondrial and cytosolic acetate. There was no need to invoke separate

Fig. A1. Acetate pool structure

ACETATE_{ext} = extracellular acetate, ACET_{C,M} = equilibrated cytosolic and mitochondrial acetate, AcCoA_M and AcCoA_C = mitochondrial and cytosolic acetyl-CoA, respectively. Flux symbols as in text of Appendix.

pools in the control models of this study, or in a previous model of ethanol metabolism (Crawford & Blum, 1983). Furthermore, acetate is transported into the mitochondrial compartment by passive diffusion (Patel *et al.*, 1981).

The rate of ethanol utilization, V_E , was calculated by measuring ethanol concentrations during the 40 min incubation period ($V_E = 266 \pm 56$ nmol/20 min per mg of protein). Acetate output, V'_{AC} , was calculated from the rate of label incorporation from [1-¹⁴C]ethanol into acetate (assumptions 3 and 4) ($V'_{AC} = 257 \pm 27$ nmol/20 min per mg of protein). The net acetate production rate ($V'_{AC} - V_{AC}$) was equal to the change in acetate concentration. The rate was constant over each 20 min interval (Table 3). Thus, ($V'_{AC} - V_{AC}$) = 163 ± 10 nmol/20 min per mg of protein. Then $V_{AC} = (V'_{AC} - \text{production rate}) = (257 - 163) = 94$ nmol/20 min per mg of protein.

These calculated flux values and the acetate concentration measurements were then used to determine the specific activity of external acetate, $SA_{Acet,ext}$, when acetate was radiolabelled in the presence of unlabelled ethanol. At $t = 0$, the external acetate concentration, $[Acet]_{ext}$, was 198 nmol/mg of protein, with a specific activity of 1. During the 0–20 min interval, this acetate was diluted by the ethanol which entered Acet_{ext} by rate V'_{AC} . The corrected $SA_{Acet,ext}$ was then calculated using eqn. 1:

$$SA_{Acet,ext} = \frac{([Acet]_{ext,t=0} \times SA_{Acet,ext}) + (V'_{AC,t=0} \times SA_{Acet,C,M})}{[Acet]_{ext,avg,0-20 \text{ min}}} \quad (1)$$

As a first approximation, the specific activity of the intracellular acetate pool, $SA_{Acet,C,M}$, was set to 0.15 d.p.m./nmol. Then,

$$SA_{Acet,ext} = \frac{\left(\frac{198 \text{ nmol}}{\text{mg prot.}} \times \frac{1.0 \text{ d.p.m.}}{\text{nmol}}\right) + \left(\frac{257 \text{ nmol}}{\text{mg prot.}} \times \frac{0.15 \text{ d.p.m.}}{\text{nmol}}\right)}{277 \text{ nmol/mg prot.}} = 0.85 \text{ d.p.m./nmol.}$$

When ethanol was the tracer, both ethanol and acetate were radiolabelled. However, the modelling program allows only one substrate to be labelled at a time. This limitation was circumvented by computing an imaginary specific activity for ethanol, SA_E^i , which would account for the total counts entering the internal acetate pool. This imaginary specific activity would have a value greater than unity. During the 0–20 min interval,

$$SA_E^i = \frac{(V_E \times SA) + (V_{AC} \times SA_{Acet,ext,t=0})}{V_E} \quad (2)$$

where ($V_{AC} \times SA_{Acet,ext}$) represents the re-utilization of ethanol-derived labelled acetate. The external acetate specific activity was calculated from eqn. 1, with $SA_{Acet,C,M} = 0.80$ d.p.m./nmol as a first approximation:

$$SA_{Acet,ext} = \frac{198 \text{ nmol/mg prot.} \times 0.85 \text{ d.p.m./nmol}}{277 \text{ nmol/mg prot.}} = 0.61 \text{ d.p.m./nmol.}$$

Then,

$$SA_E^i = \frac{\left(\frac{266 \text{ nmol}}{\text{mg prot.}} \times \frac{1.0 \text{ d.p.m.}}{\text{nmol}}\right) + \left(\frac{94 \text{ nmol}}{\text{mg prot.}} \times \frac{0.61 \text{ d.p.m.}}{\text{nmol}}\right)}{266 \text{ nmol/mg prot.}} = 1.2 \text{ d.p.m./nmol.}$$

An initial fit to the label incorporation data was obtained using these specific activities. The flux parameters V_E and V'_{AC} were maintained within the measured range and V_{AC} was kept within $\pm 10\%$ of the calculated value. The predicted internal acetate pool specific activity was 0.23 for labelled acetate and 0.89 for labelled ethanol. These values were then used in eqns. 1 and 2 to compute new external acetate and effective ethanol specific activities for use in the modelling program. This iterative process was repeated six times until the specific activity of the internal acetate pool no longer changed. The final SA_E^i was 1.3, with $SA_{Acet,C,M} = 0.87$. The final $SA_{Acet,ext}$ was 0.99, with $SA_{Acet,C,M} = 0.30$. These values were within 20% of the initial approximations which were based on inferences regarding relative flux rates associated with these substrates. Analogous calculations were made to determine the specific activities for the 20–40 min interval, which were $SA_E^i = 1.4$, with $SA_{Acet,C,M} = 0.97$ and $SA_{Acet,ext} = 0.98$, with $SA_{Acet,C,M} = 0.28$. These results indicate that there was significant labelling of external and internal acetate from [1-¹⁴C]ethanol. When the labelled substrate was [1-¹⁴C]- or [2-¹⁴C]acetate, the specific activity of the external acetate pool was decreased very little by unlabelled ethanol, but dilution of the intracellular pool was substantial.