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Acute Effects of Ethanol on the Perfused Rat Liver

STUDIES ON LIPID AND CARBOHYDRATE METABOLISM, SUBSTRATE CYCLING AND PERFUSATE AMINO ACIDS

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1. Livers from fed rats were perfused *in situ* with whole rat blood containing glucose labelled uniformly with ^{14}C and specifically with ^{3}H at positions 2, 3 or 6. 2. When ethanol was infused at a concentration of 24 μmol/ml of blood the rate of utilization was 2.8 μmol/min per g of liver. 3. Ethanol infusion raised perfusate glucose concentrations and caused a 2.5-fold increase in hepatic glucose output. 4. Final blood lactate concentrations were decreased in ethanol-infused livers, but the mean uptake of lactate from erythrocyte glycolysis was unaffected. 5. Production of ketone bodies (3-hydroxybutyrate+3-oxobutyrate) and the ratio [3-hydroxybutyrate]/[3-oxobutyrate] were raised by ethanol. 6. Formation of $^{3}H_2O$ from specifically ^{3}H-labelled glucoses increased in the order [6-^{3}H]<[3-^{3}H]<[2-^{3}H]. Production of $^{3}H_2O$ from [2-^{3}H]glucose was significantly greater than that from [3-^{3}H]glucose in both control and ethanol-infused livers. Ethanol significantly decreased $^{3}H_2O$ formation from all [^{3}H]glucoses. 7. Liver glycogen content was unaffected by ethanol infusion. 8. Production of very-low-density lipoprotein triacylglycerols was inhibited by ethanol and there was a small increase in liver triacylglycerols. Very-low-density-lipoprotein secretion was negatively correlated with the ratio [3-hydroxybutyrate]/[3-oxobutyrate]. Perfusate fatty acid concentrations and molar composition were unaffected by perfusion with ethanol. 9. Ethanol decreased the incorporation of [U-^{14}C]glucose into fatty acids and cholesterol. 10. The concentration of total plasma amino acids was unchanged by ethanol, but the concentrations of alanine and glycine were decreased and ([glutamate]+[glutamine]) was raised. 11. It is proposed that the observed effects of ethanol on carbohydrate metabolism are due to an increased conversion of lactate into glucose, possibly by inhibition of pyruvate dehydrogenase. The increase in gluconeogenesis is accompanied by diminished substrate cycling at glucose–glucose 6-phosphate and at fructose 6-phosphate–fructose 1,6-bisphosphate.

The liver plays a key role in the maintenance of blood glucose concentration *in vivo* and is also the principal site of ethanol utilization in the body. The effects of ethanol on glucose metabolism vary considerably with nutritional state. After a prolonged fast in human subjects, ethanol ingestion causes a profound hypoglycaemia (Field *et al.*, 1963; Freinkel *et al.*, 1963). This effect is commonly ascribed to a direct inhibition of gluconeogenesis, as ethanol lowers glucose production in perfused livers from starved rats (Krebs *et al.*, 1969; Williamson *et al.*, 1969). However, other work has indicated that in man, after a short fast, ethanol ingestion potentiates the hyperglycaemia caused by glucose ingestion (O'Keefe & Marks, 1977) and may also by itself increase blood glucose and insulin (Svendsen *et al.*, 1978) and decrease plasma fatty acids (Crouse *et al.*, 1968).

Studies *in vivo* (M. G. Clark *et al.*, 1974) and *in vitro* with isolated hepatocytes and the perfused rat liver (Clark *et al.*, 1973, 1975), using ^{14}C- and ^{3}H-labelled glucoses, have shown that so-called 'futile' substrate cycles exist in hepatic glucose metabolism. The possible roles of these cycles in energy expenditure for thermoregulation and blood glucose homoeostasis have been extensively reviewed (Katz & Rognstad, 1976; Newsholme & Crabtree, 1976; Newsholme & Gevers, 1967). In view of the reported differences in the literature on the effects of ethanol on blood glucose, we decided to investigate the acute effects of ethanol on the metabolism and

Abbreviation: VLD lipoproteins, very-low-density lipoproteins (d<1.006).

substrate cycling of livers from fed rats perfused with whole blood. A preliminary account of some of this work has been presented (Topping *et al.*, 1979).

Materials and Methods

Liver perfusions

Livers from adult male hooded Wistar rats (300–320g body wt.) were perfused *in situ* with 100ml of defibrinated whole rat blood by a modification of the method of Mayes & Felts (1966). The perfusate was dialysed for 24h at 0–4°C against two changes (of 2 litres each) of a modified Krebs & Henseleit (1932) buffer containing glucose (10mM), mixed amino acids (500mg/litre) and 50% of the specified concentration of Ca^{2+}. The pO_2 of the perfusate was monitored continuously and maintained at 90–100mmHg (12–13 kPa) and the rate of perfusion was 1.2–1.4ml of blood/min per g of liver.

At the start of the experiment (which was designated zero time), ethanol was infused to maintain a concentration of 24μmol/ml of blood, and 2μCi of [U-^{14}C]glucose, labelled additionally with 15μCi of [2-^{3}H]-, [3-^{3}H]- or [6-^{3}H]-glucose (all purchased from The Radiochemical Centre, Amersham, Bucks., U.K.), in 1ml of 0.15M-NaCl was added to the perfusate.

Sampling techniques

Samples of the perfusate were taken at zero time and then at 15min intervals. The sample volumes taken were: zero time, 11ml; 15min, 2ml; 30min, 5ml; 45min, 2ml; and 60min, 11ml. Each sample was rapidly chilled in ice until further processing. At the end of the experiment a small portion of liver was taken, quickly blotted dry, weighed and frozen in liquid N_2 for determination of glycogen. The remainder of the liver was similarly taken, weighed and then homogenized with 18vol. of chloroform/methanol (2:1, v/v) for analysis of lipids and radioactivity.

In calculating net rates of production or utilization of perfusate metabolites by the liver, corrections were applied for additions and withdrawals as described previously (Topping & Mayes, 1972, 1976*a*). For glucose output and lactate uptake, calculations were based on the perfusate volume after sample removal for each time interval.

Analytical techniques

Liver glycogen was isolated by the method of Good *et al.* (1933), and the glucose, released after hydrolysis and neutralization, was measured by a glucose oxidase method (Boehringer Mannheim Pty. Ltd., Mt. Waverley, Victoria 3149, Australia). This enzymic preparation was also used to measure perfusate glucose. L(+)-Lactate and ketone bodies in blood were determined enzymically by the methods of Hohorst (1963) and Williamson *et al.* (1962) respectively. Blood ethanol was measured by g.l.c. by using Poropak Q (Waters Associates, Milford, MA, U.S.A.).

Serum was obtained by centrifugation of the blood at 4°C and 2ml layered under 0.15M-NaCl in cellulose nitrate tubes. VLD lipoproteins were prepared ultracentrifugally and extracted with chloroform/methanol (2:1, v/v) as described previously (Topping & Mayes, 1972). Fatty acids were extracted from 0.1ml of serum by the method of Trout *et al.* (1960) and methylated with diazomethane. The fatty acyl methyl esters were separated by g.l.c. on diethylene glycol succinate (3% on Gas-Chrom Q). Serum (1ml), containing norleucine as an internal standard, was deproteinized with saturated picric acid. The supernatant was applied to a column (3.5cm×1.0cm) containing Dowex AG 50 (X4; H^+ form). Amino acids were eluted with 3M-NH_3 and converted into the isobutyl-*N*-heptafluorobutyramide derivatives by the method of Mackenzie & Tenaschuk (1974) for g.l.c. separation with OV 101 on Gas-Chrom Q.

After partition of the chloroform/methanol extracts of liver and VLD lipoproteins with 0.4vol. of 0.03M-HCl, portions of the chloroform layer were taken for assay of triacylglycerols as described previously (Topping & Mayes, 1972). Whole perfusate (1ml) was deproteinized with 4ml of ethanol and the supernatant passed through ion-exchange columns (Clark *et al.*, 1975). Water, glucose, lactate, pyruvate and amino acids were successively eluted and the radioactivity was counted (Katz *et al.*, 1974).

To measure the incorporation of radioactivity into liver lipids, portions of the chloroform extract were evaporated to dryness under N_2 and saponified by a modification of the method of Exton *et al.* (1972). Cholesterol, fatty acids and glycerol were separated and the radioactivity was counted.

Statistical methods

The statistical significance of differences between the experimental groups was obtained by the analysis of variance (Brownlee, 1949). A value of $P<0.05$ was taken as the criterion of statistical significance. All values are shown as the means±S.E.M. for the numbers of observations in parentheses.

Results

The weights of livers in the control and ethanol-treated groups did not differ significantly and averaged 12.86±0.30g. In livers where ethanol was infused concentrations remained constant during the perfusion at 23.9±1.2μmol/ml and the rate of utilization was 2.80±0.18μmol of ethanol/min per g of liver. No acetaldehyde was detected in any of the perfusate samples.

Table 1. *Concentrations of perfusate glucose and lactate and output of glucose and uptake of lactate in perfused rat liver*
Livers from fed rats were perfused with 100ml of whole rat blood at a flow rate of 1.2–1.4ml/min per g of liver. Ethanol was infused to maintain a concentration of 24μmol/ml of blood. Concentrations of glucose and lactate were determined as described in the text. Uptake of lactate and output of glucose were calculated for each time interval after sample removal by assuming a rate of perfusate glycolysis of 1.85μmol/h per ml of blood (Topping & Mayes, 1972). Negative values indicate uptake and positive values indicate output. All data are shown as means±S.E.M. $*P<0.05$, $***P<0.001$, for control versus ethanol-infused livers at the appropriate time interval.

Time of perfusion (min)	Glucose: Concentration (μmol/ml of blood)		Glucose: Output (μmol/h per g of liver)		Lactate: Concentration (μmol/ml of blood)		Lactate: Uptake (μmol/h per g of liver)	
	Control	Ethanol	Control	Ethanol	Control	Ethanol	Control	Ethanol
0	8.4±0.3	9.1±0.6	—	—	3.51±0.20	3.62±0.52	—	—
15	7.2±0.2*	8.8±0.6	−23.1±4.5*	+3.1±6.9	—	—	—	—
30	6.7±0.3***	9.3±0.5	+0.1±3.9***	+30.0±4.6	2.42±0.11	2.38±0.11	−43.0±8.5	−41.5±6.7
45	6.5±0.3***	10.0±0.6	+3.3±3.8***	+27.3±5.2	—	—	—	—
60	6.5±0.3***	10.7±0.7	+9.7±3.8***	+28.3±3.4	2.00±0.18*	1.34±0.19	−32.2±2.1	−38.1±6.1
No. of observations ...	9	9	9	9	6	6	6	6

Carbohydrate metabolism

Concentrations of blood glucose and lactate and the respective rates of glucose output and lactate uptake by the liver are shown in Table 1.

In control livers the concentrations of blood glucose reached equilibrium at 6.5μmol/ml of blood. After the infusion of ethanol, blood glucose concentrations did not fall, but rose linearly from 15min after zero time. Calculation of hepatic glucose output, based on the differences in concentration and the rate of erythrocyte glycolysis (Topping & Mayes, 1972, 1976*a*), revealed that, up to 30min after zero time, control livers removed glucose from the perfusate and thereafter there was a net release. In livers infused with ethanol there was a net hepatic release of glucose at all sampling intervals and this was constant after 30min. These effects of ethanol were highly significant statistically.

Concentrations of blood lactate fell during the perfusion in control livers, reaching a final concentration of approx. 2μmol/ml of blood (Table 1). There was a similar fall in the ethanol-infused livers, except that the final concentration was significantly lower than in the controls. The rate of uptake of lactate by the liver did not differ between the two groups at either interval (Table 1). Attention is drawn to the fact that, in ethanol-infused livers, hepatic glucose output was approximately equal to lactate uptake. The final concentration of liver glycogen did not differ between the two groups and averaged 188±13 (17)μmol of glucose/g of liver.

Metabolism of [U-^{14}C]glucose and of [2-^{3}H]-, [3-^{3}H]- and [6-^{3}H]-glucose

Uptakes of [^{14}C]- and [^{3}H]-glucose from the perfusate are shown in Fig. 1. The pattern of labelled-glucose metabolism in control livers was very similar to that obtained previously in livers perfused with fresh whole rat blood or with a buffer containing 3.5% (w/v) albumin and 25% (v/v) aged bovine erythrocytes (Clark *et al.*, 1975). Thus, in the absence of ethanol, there was an apparent utilization of 38.5% of [U-^{14}C]glucose, 37% of [6-^{3}H]glucose, 44% of [3-^{3}H]glucose and 55% of [2-^{3}H]glucose (Fig. 1). In the presence of ethanol the corresponding values were decreased to 26, 25, 35 and 46% (Fig. 1). These metabolic changes were highly significant statistically.

Rates of release of $^{3}H_2O$ from specifically ^{3}H-labelled glucoses are shown in Fig. 2. In the control perfusions 55% of [2-^{3}H]glucose, 38% of [3-^{3}H]-glucose and 31% of [6-^{3}H]glucose were converted into $^{3}H_2O$ (Fig. 2*a*). The total yields of $^{3}H_2O$ were significantly decreased by ethanol to 44, 19.5 and 16% of initial radioactivity (Fig. 2*b*).

The ^{3}H/^{14}C ratios for perfusate glucose are shown in Fig. 3. Zero-time values for each of [2-^{3}H]-, [3-^{3}H]- and [6-^{3}H]-glucose radioactivity relative to [U-^{14}C]glucose were arbitrarily set at 1.0. During recirculation of the perfusate for 60min in the absence of liver, the ratios for [2-^{3}H]-, [3-^{3}H]- and [6-^{3}H]-glucose were unchanged by erythrocyte glycolysis in the presence or absence of ethanol. During control perfusions the ^{3}H/^{14}C ratios declined to 0.88 and 0.94 for [3-^{3}H]- and [6-^{3}H]-glucose respectively (Fig. 3*a*). Neither ratio was affected by ethanol infusion (Fig. 3*b*). The final perfusate ratio [2-^{3}H]glucose/[U-^{14}C]glucose was 0.73 and 0.65 in control and ethanol-infused livers respectively.

Accurate interpretation of these radioisotopic data

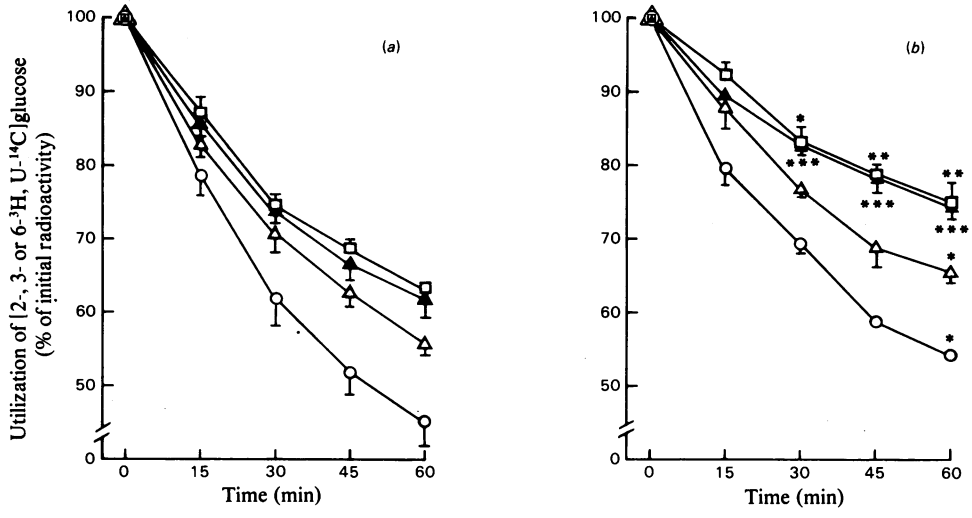

Fig. 1. *Metabolism of [U-^{14}C]glucose and [2-^{3}H]-, [3-^{3}H]- or [6-^{3}H]-glucose by perfused rat liver in the absence (a) and presence (b) of 24mM-ethanol*

Livers from fed rats were perfused with whole rat blood containing 2μCi of [U-^{14}C]glucose (▲, $n = 9$) and 15μCi of [2-^{3}H]- (○, $n = 3$), [3-^{3}H]- (△, $n = 3$) or [6-^{3}H]- (□, $n = 3$) glucose at a flow rate of 1.2–1.4ml/min per g of liver. Radioactivity remaining in glucose at each sampling time was determined as described in the text. Data are shown as means ± S.E.M. for n observations. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, for control versus ethanol-infused livers at the appropriate time interval.

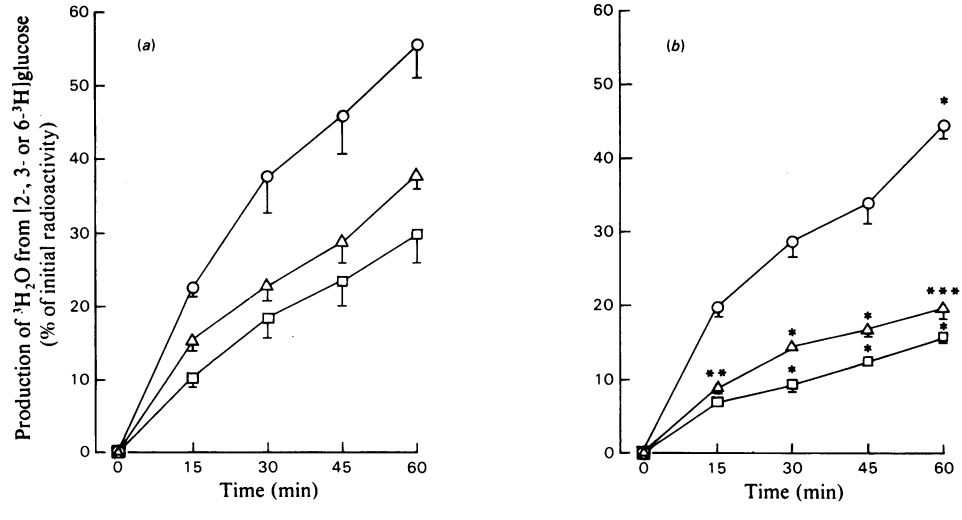

Fig. 2. *Production of $^{3}H_2O$ from [2-^{3}H]-, [3-^{3}H]- or [6-^{3}H]-glucose by perfused rat liver in the absence (a) and presence (b) of 24mM-ethanol*

Livers from fed rats were perfused with whole rat blood containing 15μCi of (○) [2-^{3}H]-, (△) [3-^{3}H]- or (□) [6-^{3}H]-glucose at a flow rate of 1.2–1.4ml/min per g of liver. Radioactivity in $^{3}H_2O$ at each sampling time was determined as described in the text. The data are shown as means ± S.E.M. for three observations. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, for control versus ethanol-infused livers at the appropriate time interval.

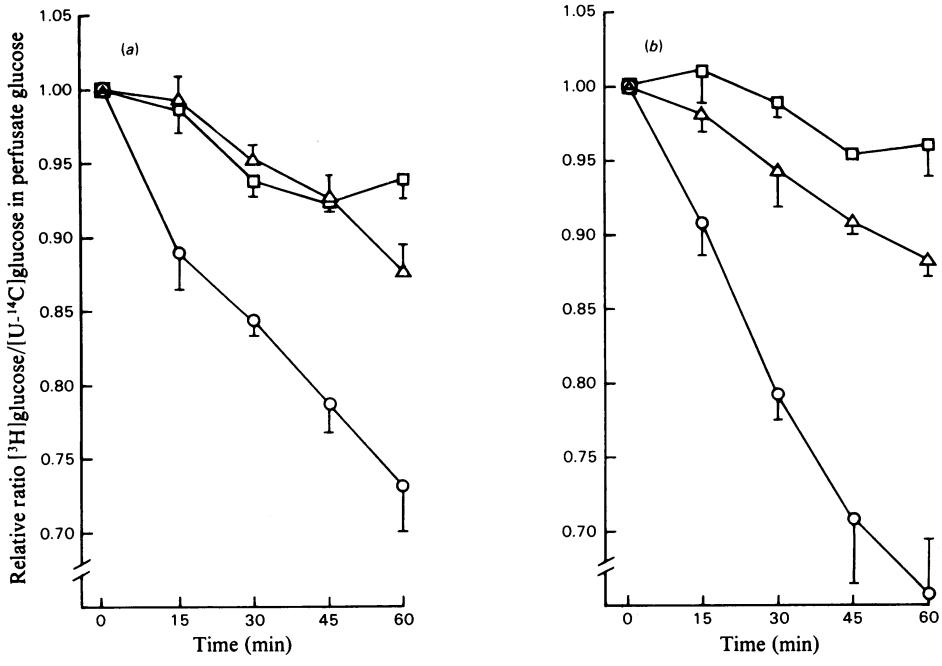

Fig. 3. *$^3H/^{14}C$ ratios in perfusate glucose during the metabolism of [U-^{14}C]glucose and [2-^3H]-, [3-^3H]- or [6-^3H]-glucose by perfused rat liver in the absence (a) and presence (b) of 24mM-ethanol*

Livers from fed rats were perfused with whole rat blood containing 2μCi of [U-^{14}C]glucose and 15μCi of (○) [2-^{3}H]-, (△) [3-^{3}H]- or (□) [6-^{3}H]-glucose at a flow rate of 1.2–1.4ml/min per g of liver. Radioactivity remaining in glucose at each sampling time was determined as described in the text. The data are shown as means ± S.E.M. for three observations.

is complicated by the metabolism of perfusate glucose by erythrocyte glycolysis. Recirculation of defibrinated whole rat blood in the perfusion apparatus in the absence of liver resulted in the catabolism of 1.79±0.23 (3)μmol of glucose/h per ml of blood. This rate of glycolysis accounted for the metabolism of approx. 160μmol of glucose/perfusion when correction was made for the change, caused by sampling, in perfusate volume from 100ml at zero time to 80ml at the end of the experiment.

When erythrocytes are incubated with [2-^{3}H]- or [3-^{3}H]-glucose the major product is ^3H_2O. This accounts for nearly 100% of the metabolized substrate with [3-^{3}H]glucose and over 80% with [2-^{3}H]-glucose. The remaining label from [2-^{3}H]glucose is found as [^{3}H]lactate. This indicates that equilibration of glucose 6-phosphate and fructose 6-phosphate is not complete and ^{3}H on position 1 of fructose 6-phosphate (from [2-^{3}H]glucose 6-phosphate) is retained during erythrocyte glycolysis (Katz & Rognstad, 1976). Erythrocyte glycolysis of [6-^{3}H, U-^{14}C]glucose produces [3-^{3}H,U-^{14}C]lactate as the major product (D. G. Clark, unpublished work). The ^{3}H is lost as ^3H_2O when lactate is oxidized in the tricarboxylic acid cycle (although some can be retained during gluconeogenesis via oxaloacetate and phosphoenolpyruvate). The retention of ^{3}H from [3-^{3}H]lactate during gluconeogenesis is a further complicating factor in the expression of the radioisotopic data, but this can probably be ignored, as Rognstad & Wals (1976) have demonstrated in hepatocytes from starved hamsters that over 85% of ^{3}H from [3-^{3}H]lactate is lost during gluconeogenesis.

A further complication is the increased hepatic production of glucose in the presence of ethanol (Table 1). However, this problem was overcome by calculation of the rates of ^3H_2O production and [^{14}C]- and [^{3}H]-glucose uptake from glucose specific radioactivities. The latter were calculated from average radioactivities (d.p.m.) and concentrations at each 15min period of perfusion. These corrected rates of total glucose metabolism and ^3H_2O production were averaged for the whole experiment and are shown in Table 2. Correction was then applied for erythrocyte glycolysis at 1.85μmol of glucose/h per ml of blood (Topping & Mayes, 1972, 1976*a*), assuming equal yields of ^3H_2O from [2-^{3}H]-, [3-^{3}H]- and [6-^{3}H]-glucose (see above). Subtraction of these gave rates of [^{14}C]- or [^{3}H]-glucose uptake and ^3H_2O formation caused by hepatic metabolism. To facilitate comparison with other data, the rates were converted into μmol/h per g of liver.

Table 2. *Metabolism of* [2-^3H]-, [3-^3H]-, [6-^3H]- *and* [U-^{14}C]-*glucose in perfused rat liver*

Livers from fed rats were perfused with 100ml of whole rat blood at a flow rate of 1.2–1.4ml/min per g of liver. Ethanol was infused to maintain a concentration of 24μmol/ml of blood. Concentrations of blood glucose and radioactivity in perfusate [U-^{14}C]-, [2-^{3}H]-, [3-^{3}H]- and [U-^{14}C]-glucose and ^3H_2O were determined as described in the text. Rates of glucose disappearance and ^3H_2O production were obtained from glucose specific radioactivity calculated from the mean glucose concentration and radioactivity for each 15min sampling interval and averaged for the whole perfusion. In calculating the rate of liver metabolism alone, it was assumed that erythrocyte glycolysis consumed 1.85μmol of glucose/h per ml of blood and that the principal product from each specifically labelled glucose was ^3H_2O. Data are shown as means±S.E.M. *$P<0.05$, ***$P<0.001$, for control versus ethanol-infused livers.

			Apparent glucose metabolism			Apparent ^3H_2O formation		
Radioactivity in glucose	Ethanol	No. of observations	Total (μmol/h)	Liver (μmol/h)	Liver (μmol/h per g)	Total (μmol/h)	Liver (μmol/h)	Liver (μmol/h per g)
[U-^{14}C]	–	9	400±44	182±21	14.7±1.7	—	—	—
	+	9	285±25	134±21*	10.1±1.6*	—	—	—
[2-^{3}H]	–	3	778±108	603±235	45.0±8.8	779±65	604±67	45.7±4.0
	+	3	602±29	425±27	30.0±1.3	620±48	443±43*	31.3±2.9*
[3-^{3}H]	–	3	393±3	219±2	17.6±0.4	341±2	170±2	13.6±2.8
	+	3	402±28	239±26	17.2±2.2	252±36	47±14***	3.6±1.0***
[6-^{3}H]	–	3	362±16	189±12	16.0±1.3	289±24	114±27	9.7±2.3
	+	3	281±59	174±27.6	14.0±1.8	166±27	22±11*	1.9±0.9*

The metabolism of glucose by the liver in both control and ethanol-infused livers followed the order [2-^{3}H]>[3-^{3}H]>[6-^{3}H]>[U-^{14}C]. In both groups close agreement was obtained between metabolism of and production of ^3H_2O from [2-^{3}H]glucose. With [3-^{3}H]- and [6-^{3}H]-glucose, production of ^3H_2O was considerably less than total metabolism. Infusion of ethanol did not alter the metabolism of [3-^{3}H]- or [6-^{3}H]-glucose, but lowered that of [2-^{3}H]glucose. Production of ^3H_2O from all specifically ^{3}H-labelled glucoses was significantly decreased by ethanol, and that from [3-^{3}H]- and [6-^{3}H]-glucose was virtually abolished.

A similar calculation to the above was made (based on mean specific radioactivity) of the incorporation of [U-^{14}C]glucose into liver acylglycerol glycerol, sterols and fatty acids. In the presence of 24μmol of ethanol/ml of blood, incorporation into acylglycerol glycerol was unaffected, with mean values of 0.60±0.05 (7) and 0.54±0.04 (7) μmol of glucose equivalents/h per g of liver in control and ethanol-infused livers respectively. Infusion of ethanol decreased incorporation into fatty acids from 6.72±1.50 (7) to 2.46±0.39 (7) μmol of glucose equivalents/h per g of liver, and into sterols from 0.79±0.11 (7) to 0.32±0.03 (7) μmol of glucose equivalents/h per g of liver. These differences were highly significant statistically ($P<0.01$ and $P<0.001$ respectively).

Lipid metabolism and ketogenesis

At zero time plasma fatty acid concentrations were similar in both groups, averaging 0.18μmol/ml. During the perfusion fatty acid concentrations declined,

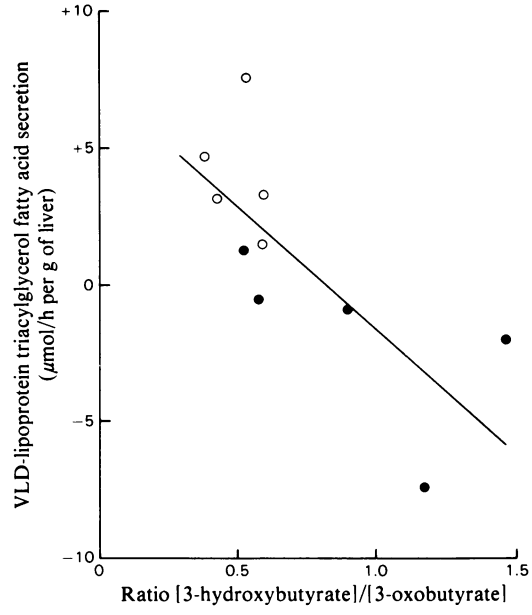

Fig. 4. *Rate of VLD-lipoprotein triacylglycerol fatty acid secretion and the ratio* [3-*hydroxybutyrate*]/[3-*oxobutyrate*] *in perfused rat liver*

Livers from fed rats were perfused with whole rat blood at a flow rate of 1.2–1.4ml/min per g of liver. Ethanol was infused to maintain a concentration of 24μmol/ml of blood and the rate of VLD-lipoprotein triacylglycerol fatty acid secretion and the ratio [3-hydroxybutyrate]/[3-oxobutyrate] were determined as described in the text. Negative values indicate net uptake. ○, Control; ●, +ethanol; $r=-0.72$, $P<0.05$.

reaching final values of 0.083±0.012 (6) and 0.115±0.016 (6) μmol/ml for control and ethanol-infused rats respectively. These final concentrations did not differ significantly, and neither did the molar composition of perfusate fatty acids, which averaged 35.5±0.8% of $C_{16:0}$, 6.8±0.9% of $C_{16:1}$, 19.1±0.6% of $C_{18:0}$, 24.2±0.4% of $C_{18:1}$, 9.1±0.4% of $C_{18:2}$ and 6.2±0.8% of $C_{20:4}$ for both groups combined.

In control livers the mean rate of production of the ketone bodies (3-hydroxybutyrate+3-oxobutyrate) declined from 5.2±1.2 (6) μmol/h per g of liver during the period 0–30min to 2.4±0.5 (6) μmol/h per g of liver at 30–60min. In ethanol-infused livers the initial rate of ketogenesis was lower (but not significantly) than in controls, at 2.8±0.1 (6) μmol/h per g of liver. However, the rate of ketone-body formation during the period 30–60min was significantly ($P<0.05$) higher in the presence of ethanol, i.e. 4.3±0.7 (6) μmol/h per g of liver. In both control and ethanol-infused livers the ratio [3-hydroxybutyrate]/[3-oxobutyrate] remained constant during the perfusion at 0.55±0.06 (6) and 0.99±0.18 (6) respectively. This increase in ratio was significant statistically ($P<0.05$).

The mean rate of secretion of VLD-lipoprotein triacylglycerol fatty acids was 4.2±1.2 (5) μmol/h per g of liver. Ethanol infusion significantly ($P<0.05$) lowered VLD-lipoprotein secretion, and the mean rate of −1.3±1.9 (5) indicated that in fact there was apparent net uptake of triacylglycerol fatty acids by the liver. A significant ($r=-0.72$, $P<0.05$) negative correlation was obtained between the rate of VLD-lipoprotein triacylglycerol fatty acid secretion and the ratio [3-hydroxybutyrate]/[3-oxobutyrate] (Fig. 4).

The total content of triacylglycerol fatty acids in the system (i.e. net VLD-lipoprotein secretion+liver content) did not differ between the two groups in those perfusions where both were measured simultaneously and the mean value was 312.2±27.1 (10) μmol of triacylglycerol fatty acid.

Table 3. *Concentrations of plasma amino acids in perfused rat liver*

Livers from fed rats were perfused with whole blood at a flow rate of approx. 1.2–1.4ml/min per g of liver. Ethanol was infused to maintain concentrations of 24μmol/ml of blood. After 60min of perfusion plasma amino acids were determined by g.l.c. as described in the text. As glutamate and glutamine co-chromatograph, they are indicated as such. Values are shown as means±S.E.M. for five observations. **$P<0.01$ for control versus ethanol-infused livers.

Addition to perfusate ...	Amino acid (μmol/ml of plasma)	
	None (control)	Ethanol
Alanine	0.30±0.02**	0.18±0.03
Glycine	0.20±0.02**	0.10±0.01
Valine	0.52±0.03	0.45±0.07
Threonine	0.20±0.02	0.17±0.02
Serine	0.14±0.02	0.13±0.01
Leucine	0.63±0.04	0.54±0.05
Isoleucine	0.41±0.02	0.36±0.03
Proline	0.09±0.03	0.09±0.02
Aspartate	0.06±0.01	0.06±0.01
Phenylalanine	0.06±0.01	0.06±0.01
Glutamate+glutamine	0.79±0.04**	1.23±0.08
Lysine	0.20±0.02	0.27±0.04
Arginine	0.07±0.03	0.09±0.02
Total	3.67±0.21	3.69±0.19

Plasma amino acids

The mean concentrations of total plasma amino acids at the end of perfusion did not differ between the two groups (Table 3). However, in ethanol-infused liver the plasma concentrations of alanine and glycine were significantly lower and [glutamate+glutamine] was higher than in controls.

Discussion

The rate of utilization of ethanol by the perfused liver in these experiments was well within published values of hepatic alcohol dehydrogenase (EC 1.1.1.1) activity. Only at much higher concentrations of ethanol is it considered likely that alternative pathways of metabolism become operative (Lieber & DeCarli, 1970; Grunnet *et al.*, 1973).

The products of ethanol oxidation after oxidation of acetaldehyde are acetate and NADH. Thus ethanol causes an increase in the hepatic ratio [NADH]/[NAD^+] (Krebs *et al.*, 1969) and in liver [acetyl-CoA] (Kondrup & Grunnet, 1973), and it is reasonable to ascribe the observed metabolic effects of ethanol to a greatly enhanced flux of acetyl-CoA and reducing equivalents into the liver.

Metabolic effects of ethanol

In the rat *in vivo*, the gastrointestinal tract is a net producer of lactate under a variety of nutritional conditions (Topping & Mayes, 1971), and it is now recognized that lactate is a preferred metabolic fuel for oxidation by the rat liver in the fed state (D. G. Clark *et al.*, 1974; Salmon *et al.*, 1974). Thus, in addition to giving improved metabolic regulation and O_2 consumption by the perfused liver (Mayes & Felts, 1976), the use of homologous whole blood containing glycolysing erythrocytes provides a constant supply of lactate under conditions close to the physiological (Topping & Mayes, 1971).

After conversion into pyruvate, lactate may enter the tricarboxylic acid cycle via pyruvate dehydrogenase (EC 1.2.4.1) or the gluconeogenic sequence of reactions after carboxylation by pyruvate carboxylase

(EC 6.4.1.1). In the present experiments it would appear that the effects of ethanol were to redirect lactate arising from erythrocyte glycolysis to glucose formation. Thus the rate of hepatic glucose output with ethanol nearly equalled that of erythrocyte lactate production. It is unlikely that there was any change in glycogenolysis, as the concentration of liver glycogen was unchanged. Alanine and glycine are gluconeogenic substrates for the liver (Exton, 1972), and therefore the fall in their perfusate concentrations and that of lactate suggests that ethanol may have additionally stimulated gluconeogenesis. Although glutamate+glutamine are potentially gluconeogenic, Ross *et al.* (1967) found that in perfused liver only glutamine was effective. In our analytical system, glutamate and glutamine co-chromatographed, but in perfused liver little or no glutamate is detected in the perfusate (Tolman *et al.*, 1972). The increase may therefore reflect a rise in glutamine owing to enhanced transamination of alanine in the presence of ethanol. Our data support an enhanced formation of glucose by the liver, and the lower yields of ^3H_2O from [3-^{3}H]- and [6-^{3}H]-glucose and the smaller incorporation of [U-^{14}C]-glucose into liver fatty acids and sterols indicate an impairment of glycolysis up to, and beyond, pyruvate.

A possible regulatory site for these effects is pyruvate dehydrogenase. This enzyme exists in an active (non-phosphorylated) and in an active (phosphorylated) form. The factors regulating interconversion have been extensively reviewed (Denton & Hughes, 1978; Denton *et al.*, 1975). Activation is favoured by falls in the mitochondrial ratios [NADH]/[NAD$^+$] and [acetyl-CoA]/[CoA]. Ethanol has been shown to increase the ratio [NADH]/[NAD$^+$] both *in vivo* (e.g. Hernandez-Muñoz *et al.*, 1978) and *in vitro* (Krebs *et al.*, 1969; Siess & Wieland, 1976). Ethanol increases liver acetyl-CoA, and Siess *et al.* (1978) have shown that this change is mirrored by an increase in the mitochondrial ratio [acetyl-CoA]/[CoA].

It is highly probable that these ratios were raised in the latter part of the experiment, as ketogenesis was increased and the ratio [3-hydroxybutyrate]/[3-oxobutyrate] was raised throughout the perfusion. Ketogenesis appears to occur in perfused liver when the supply of acetyl-CoA exceeds the oxidative capacity of the tricarboxylic acid cycle (Mayes & Felts, 1967). Thus it appears that the effects of ethanol on glucose production may be substantially explained by an inactivation of pyruvate dehydrogenase and possibly by a stimulation of gluconeogenesis at pyruvate carboxylase (EC 6.4.1.1). The latter suggestion is supported by the observation that alanine increases gluconeogenesis in perfused livers from starved rats (Williamson *et al.*, 1969), although the effect is abolished by fatty acids. Acetate also enhances glucose synthesis in hepatocytes from starved rats (Whitton *et al.*, 1979). It has been reported that diets high in ethanol increase the activities of two gluconeogenic enzymes in liver and kidney in the rat (Jauhonen *et al.*, 1978) and decrease gluconeogenic amino acids in plasma in sheep (G. B. Belling & B. J. Potter, unpublished work) and liver in rats (Mørland *et al.*, 1979). In rat liver, as in the present study, both alanine and glycine were decreased, with no change in serine, although glycine is metabolized via the latter (Mørland *et al.*, 1979).

Substrate cycling

The existence of 'futile' (i.e. ATP-dissipating) substrate cycles in glucose metabolism has been established *in vivo* and *in vitro* at glucose–glucose 6-phosphate and fructose 6-phosphate–fructose 1,6-bisphosphate. However, exact quantification of their physiological significance has been difficult. In the present experiments accurate interpretation of the data is complicated (as it was in part of the study by Clark *et al.*, 1975) by erythrocyte glycolysis. As indicated, the major product from [2-^{3}H]- and [3-^{3}H]-glucose is ^3H_2O, whereas that from [U-^{14}C]-glucose is [^{14}C]lactate. The metabolism, by erythrocytes, of [U-^{14}C]glucose and [^{3}H]glucose does not alter perfusate [^{3}H]-[^{14}C]-glucose values unless the lactate is recycled to glucose (Clark *et al.*, 1975). ^3H_2O is formed from [^{3}H]lactate (derived from [6-^{3}H]glucose) during oxidation in the tricarboxylic acid cycle, although a small amount of the label is retained during gluconeogenesis (Rognstad & Wals, 1976).

Ethanol substantially altered the metabolism of [U-^{14}C]-, [3-^{3}H]- and [6-^{3}H]-glucose. With the assumption that the retention of ^{3}H from [3-^{3}H]lactate during gluconeogenesis was minimal (Rognstad & Wals, 1976), the production of ^3H_2O and utilization of [^{14}C]glucose was calculated by subtraction of the rate of erythrocyte glycolysis. Thus ethanol inhibited the utilization of [U-^{14}C]glucose and [6-^{3}H]glucose and, when corrected for erythrocyte glycolysis, virtually abolished ^3H_2O formation from both [3-^{3}H]glucose and [6-^{3}H]glucose.

In an earlier study on 'futile' cycling by the perfused liver the ratios ^{3}H/^{14}C in perfusate glucose were used to show cycling at glucose–glucose 6-phosphate and fructose 6-phosphate–fructose 1,6-bisphosphate (Clark *et al.*, 1975). In the present study the increased yield of ^3H_2O from [2-^{3}H]glucose relative to [3-^{3}H]glucose in control livers demonstrates an extensive cycle at glucose–glucose 6-phosphate. Similarly, the data obtained with ^3H_2O formation from [3-^{3}H]glucose demonstrate cycling at fructose 6-phosphate–fructose 1,6-bisphosphate (Clark *et al.*, 1973). To our knowledge this is the first time that, in perfused liver, ^3H_2O yields have been used to show substrate cycling.

As Rognstad & Katz (1976) have observed in isolated hepatocytes, the cycle fructose 6-phosphate–fructose 1,6-bisphosphate was greatly decreased by ethanol. However, under these conditions we have still observed a substantial cycle at glucose–glucose 6-phosphate. These radioisotopic data indicate that, with ethanol, there was very little metabolism of glucose below the level of fructose 6-phosphate in the glycolytic pathway. Calculation of the rate of cycling at glucose–glucose 6-phosphate suggested that this was also decreased in our fed livers by 30% (Table 2). Newsholme & Crabtree (1976) have suggested that the ethanol-induced hypoglycaemia of starvation is due to an inhibition of this cycle. However, preliminary calculation of the data based on percentage change in initial radioactivity corrected for alterations in glucose concentration indicated a slight increase in ^3H_2O formation from [2-^{3}H]glucose in the presence of ethanol (Topping *et al.*, 1979). An explanation for this discrepancy might lie in the fact that there is incomplete equilibration between glucose 6-phosphate and fructose 6-phosphate (Katz & Rognstad, 1976), and some [2-^{3}H]glucose may be converted into [1-^{3}H]-glucose after metabolism to fructose 6-phosphate. This transfer, apart from underestimating the rate of ^{3}H removal, would also raise the apparent specific radioactivity of [2-^{3}H]glucose. As good agreement was obtained between data for [3-^{3}H]- and [6-^{3}H]-glucose by both means of calculation, it is therefore possible that the cycle glucose–glucose 6-phosphate was essentially unchanged.

The assumptions used in estimating the rates of substrate cycling are similar to those applied to studies with other preparations, including isolated hepatocytes (Clark *et al.*, 1973; Katz & Rognstad, 1976), where changes in glucose concentration also occurred. An additional correction was applied in the present study for the rate of erythrocyte glycolysis, which was constant in both groups. However, it should be emphasized that it remains only an estimate, and further studies with specifically labelled lactates are necessary to quantify the changes exactly.

Hers (1976) has discussed the concept that the liver may maintain blood glucose at the cycle glucose–glucose 6-phosphate solely in response to changes in substrate. During ethanol oxidation perfusate glucose concentrations rose continuously with no sign of equilibration, and this finding supports previous observations that the perfused liver cannot autoregulate blood glucose in the face of an augmented supply (Topping & Mayes, 1976*a*). Our data do not support Hers' (1976) hypothesis, and it would appear that, for equilibration to occur, an exogenous stimulus (such as fructose) is required (Topping & Mayes, 1976*a*).

There appear to be large discrepancies in the yields of ^3H_2O from [3-^{3}H]- and [6-^{3}H]-glucose compared with the net utilization of these glucoses. For example, with ethanol there was no apparent formation of ^3H_2O from [6-^{3}H]glucose, yet nearly 10% of [6-^{3}H]glucose was metabolized by the liver. This difference, and that with [3-^{3}H]glucose, may be explained by incorporation into triacylglycerol and also glycogen. ^{14}C and ^{3}H from [U-^{14}C,6-^{3}H]glucose are known to be incorporated into both species, but ^{3}H from [2-^{3}H]glucose is not due to loss of ^{3}H at glucose phosphate isomerase (EC 5.3.1.9) (Katz & Rognstad, 1976).

Lipid metabolism

It has been well documented that ethanol administration *in vivo* causes hepatic triacylglycerol accumulation (Lieber *et al.*, 1971). In the present studies we found that ethanol completely inhibited VLD-lipoprotein secretion, with a corresponding increase in liver triacylglycerol fatty acids. As most of the fatty acids would have been derived from synthesis *de novo* under the experimental conditions (Mayes & Topping, 1974; Topping & Mayes, 1976*b*), it is possible that this reflected an inhibition of lipogenesis. Selmer & Grunnet (1976) have reported that, in isolated hepatocytes, ethanol diminishes lipogenesis by approx. 20%. Such a small change would not account for the complete loss of triacylglycerol secretion, particularly as the total triacylglycerol content of the system did not differ between control and ethanol-infused livers. Although it has been established that the isolated liver perfused with whole blood does not metabolize intact triacylglycerol-rich particles, there is a substantial catabolism of lipoprotein remnants (Gardner & Mayes, 1977). These products of VLD-lipoprotein and chylomicron hydrolysis may be present in the plasma of blood-donor rats. One of the major changes during their metabolism is a transfer of fatty acids from triacylglycerols to phospholipids. Such a transfer would account for the present decrease in VLD-lipoprotein triacylglycerols.

Various explanations have been put forward for the ethanol-induced fatty liver. For example, Abrams & Cooper (1976*a*,*b*) have suggested that it may reflect greater delivery of fatty acids to the liver secondary to enhanced blood flow. In the present experiments, inhibition of VLD-lipoprotein secretion by ethanol was observed at constant blood flow. More recently Hernandez-Muñoz *et al.* (1978) have proposed that ethanol alters the hepatic redox state, particularly the [*sn*-glycerol 3-phosphate]/[dihydroxyacetone phosphate] ratio, and have correlated this with liver triacylglycerol accumulation. In the present experiments a similar correlation was observed between liver redox state and VLD-lipoprotein secretion.

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