Inhibition of Hepatic Gluconeogenesis by Ethanol

By H. A. KREBS, R. A. FREEDLAND, R. HEMS AND MARION STUBBS

Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, and Metabolic Research Laboratory, Nuffield Department of Clinical Medicine, Radcliffe Infirmary, University of Oxford

(Received 5 November 1968)

1. Gluconeogenesis from 10mM-lactate in the perfused liver of starved rats is inhibited by ethanol. The degree of inhibition reached a maximum of 66% at 10mM-ethanol under the test conditions and decreased at higher ethanol concentrations. The concentration-dependence of the inhibition is paralleled by the concentration-dependence of the activity of alcohol dehydrogenase. The enzyme is also inhibited by ethanol concentrations above 10mM. 2. Gluconeogenesis from pyruvate is not inhibited by ethanol. 3. The degree of the inhibition of gluconeogenesis from lactate by ethanol depends on the concentration of lactate and other oxidizable substances, e.g. oleate, in the perfusion medium. 4. Ethanol also inhibits, to different degrees, gluconeogenesis from glycerol, dihydroxyacetone, proline, serine, alanine, fructose and galactose. 5. The inhibition of gluconeogenesis from lactate by ethanol is reversed by acetaldehyde. 6. Pyrazole, a specific inhibitor of alcohol dehydrogenase, also reverses the inhibition of gluconeogenesis by ethanol. 7. Gluconeogenesis in kidney cortex, where the activity of alcohol dehydrogenase is very low, is not inhibited by ethanol. 8. Kidney cortex, testis, ovary, uterus and certain tissues of the alimentary tract were the only rat tissues, apart from the liver, that showed measurable alcohol dehydrogenase activity. 9. The concentrations of pyruvate in the liver were decreased to about one-fifth by ethanol. 10. The concentration of lactate in the perfused liver was about 3mM below that of the perfusion medium 30min. after the addition of 10mM-lactate. 11. The great majority of the findings support the view that the inhibition of gluconeogenesis by ethanol is caused by the alcohol dehydrogenase reaction, which decreases the $[free\ NAD^+]/[free\ NADH]$ ratio. The decrease lowers the concentration of pyruvate and this is the immediate cause of the inhibition of gluconeogenesis from lactate, alanine and serine: the fall in the concentration of pyruvate lowers the rate of the pyruvate carboxylase reaction, one of the rate-limiting reactions of gluconeogenesis. The cause of the inhibition of gluconeogenesis from other substrates is discussed.

Next to the nervous system, the liver is the main target organ of the toxic effects of ethanol. Recent work (Isselbacher & Greenberger, 1964*a,b*; Forsander, Rih & Suomalainen, 1958; Smith & Newman, 1959; Reboucas & Isselbacher, 1961; Field, Williams & Mortimore, 1963; Freinkel *et al.* 1965; Lieber, 1967*a,b*) suggests that the primary effect of ethanol on the liver is its interaction with alcohol dehydrogenase, which causes a rapid conversion of NAD^+ into NADH. The resulting shift of the $[NAD^+]/[NADH]$ ratio is taken to be a major causal factor in the disturbances of carbohydrate metabolism associated with chronic alcoholism, which manifests itself as the hypoglycaemia of the underfed or starved alcoholic (Madison, 1968). The hypoglycaemia is taken to be due to an inhibition of gluconeogenesis.

Until 1967 the evidence on the inhibition of gluconeogenesis was not unequivocal. Freinkel *et al.* (1965) state: 'although the available data are consistent with our working hypothesis...' (that alcohol-induced hypoglycaemia is due to impaired gluconeogenesis) '...a causal relationship has not been established'. Lochner, Wulff & Madison (1967) wrote: 'although the inferential *in vivo* evidence, which suggests that ethanol induced hypoglycaemia is the consequence of impaired hepatic gluconeogenesis, is supported by *in vitro* studies, whether ethanol induced hypoglycaemia results from a decrease in the output of glucose from the liver and/or an acceleration of peripheral glucose utilisation has not been elucidated'.

Experiments by Lochner *et al.* (1967) and by Madison, Lochner & Wulff (1967) on liver of starved

dogs *in vivo*, involving measurements of the hepatic glucose output, have provided strong support for the view that ethanol caused a partial inhibition of gluconeogenesis.

Experiments on the isolated perfused rat liver reported in the present paper demonstrate conclusively that ethanol can cause major inhibitions of hepatic gluconeogenesis. Apparently discrepant reports of previous investigators can be accounted for by the fact that the inhibition does not occur in all circumstances. It depends on the nature of the gluconeogenic precursor and, in a special way, on the concentration of ethanol: above 10mm ethanol is less inhibitory than at lower concentrations.

EXPERIMENTAL

Technique of liver perfusion and analytical methods. The method of liver perfusion and the analytical procedures for the determination of glucose, lactate, pyruvate and other metabolites were as previously described (Hems, Ross, Berry & Krebs, 1966; Ross, Hems & Krebs, 1967a; Ross, Hems, Freedland & Krebs, 1967b). Ethanol was determined enzymically by the method of Dickinson & Dalziel (1967) with yeast alcohol dehydrogenase. It was necessary to dialyse the enzyme to remove traces of ethanol. The reaction mixture consisted of 1.5 ml. of M-tris-acetate buffer, pH 9.5, containing 0.3 mm-EDTA, 0.20 ml. of 1% (w/v) NAD⁺, 30 μ l. of dialysed enzyme (100 mg. in 3.4 ml. of original solution, the volume of which increased somewhat on dialysis), the unknown solution and water to make a total volume of 3.1 ml. The NADH formed was estimated from spectrophotometric measurements of E_{340} . Readings were taken at 5, 10 and 15 min.

Acetaldehyde was freshly redistilled.

Glucose was determined in the perfusion medium at 10 min. or 15 min. intervals. The rate of gluconeogenesis was calculated from the linear part of the progress curve, which usually extended for more than 60 min. after the addition of substrate (provided that the substrate was not used up earlier). Unless otherwise stated, the rats were starved for 48 hr. The rates of gluconeogenesis are expressed throughout in μ moles/min./g. wet wt. of liver and given as means $\pm$ s.e.m. with numbers of observations in parentheses.

Assay of alcohol dehydrogenase activity. The procedure was based on that of Greenberger, Cohen & Isselbacher (1965). The tissue was homogenized with 9 parts (v/w) of 0.1 M-sodium phosphate buffer, pH 7.2, in an all-glass or Teflon-glass homogenizer. The homogenates were centrifuged at 38700g and the supernatant was used for the assay. The cuvettes contained 0.1 ml. of 0.5% NADH, 0.1 ml. of 0.1 M-acetaldehyde and 0.1 ml. or 0.2 ml. of enzyme solution. The amount of enzyme was so adjusted as to give a change between 0.008 and 0.050 E_{340} unit/min. With liver the supernatant was diluted tenfold. The total volume in the cuvette was made up to 3.1 ml. with 0.1 M-sodium phosphate buffer, pH 7.2. A control without acetaldehyde was set up for each assay. Measurements were made with a Hilger-Gilford spectrophotometer and a Beckman DU monochromator.

Disappearance of ethanol from the perfusion medium. The

ethanol concentrations stated in the text and tables refer to the initial concentrations. These gradually fall, partly because of evaporation of ethanol in the oxygenator and partly because of metabolic conversion in the liver. The rate of evaporation in the perfusion system without the liver was about 11%/hr. The rate of metabolic removal was on average 0.93 μ mole/min./g. wet wt. of liver. Almost all the alcohol removed metabolically was recovered as acetate. The combined removal by evaporation and oxidation decreased the initial ethanol concentration of 10 mm by about one-third after 1 hr. The calculations of the rates of gluconeogenesis were based on the changes within the first 30–40 min. after the simultaneous addition of ethanol and substrates, which was made 38 min. after the beginning of the perfusion.

Definition of 'free' nicotinamide nucleotides. Throughout this paper $[NAD^+]/[NADH]$ refers to the ratio of the concentrations of the free (as opposed to the protein-bound) nicotinamide nucleotides, as determined experimentally on the basis of the equilibrium equation of dehydrogenase systems:

$$\frac{[NAD^+]}{[NADH]} = \frac{1}{K} \frac{[\text{oxidized substrate}]}{[\text{reduced substrate}]}$$

By definition, then, the ratio of the free nicotinamide nucleotides is an experimental entity, the significance of which is discussed by Williamson, Lund & Krebs (1967) and Krebs & Veech (1969).

RESULTS

Effect of ethanol on gluconeogenesis from lactate. As shown in Table 1, a 47% inhibition was found when the ethanol concentration was 2.5 mm. The inhibition reached a maximum of 66% at 10 mm-ethanol and decreased when the ethanol concentration was raised, being only 33% at about 40 mm. This concentration effect of ethanol is paralleled by its action on alcohol dehydrogenase, as liver alcohol dehydrogenase is also inhibited by higher

Table 1. *Effect of ethanol on the rate of gluconeogenesis from lactate in the perfused rat liver*

The rats were starved for 48 hr. The initial concentration of lactate was 10 mm. The results are given as means $\pm$ s.e.m. with numbers of observations in parentheses. The rates were calculated from the linear part of the progress curve, which usually extended for more than 60 min. after the addition of substrate.

Concn. of ethanol (mm)	Glucose formation (μ moles/min./g. wet wt. of tissue)	Inhibition by ethanol (%)
0	1.06 $\pm$ 0.09 (12)	
1.25	0.89 $\pm$ 0.13 (3)	16
2.5	0.56 $\pm$ 0.04 (5)	47
5	0.42 $\pm$ 0.06 (7)	60
10	0.36 $\pm$ 0.05 (7)	66
20	0.49 $\pm$ 0.03 (4)	54
40	0.71 $\pm$ 0.05 (4)	33

Table 2. *Time-course of the removal of pyruvate and the formation of glucose and lactate in the presence and absence of ethanol in perfused liver*

The values refer to μ moles in the total perfusion medium. At 38 min. 750 μ moles of pyruvate and (in Expt. 1 only) 750 μ moles of ethanol were added to the medium (150 ml.). The first sample was taken 2 min. later to allow mixing of the added substrates to take place. The rats were starved for 48 hr.

Time (min.)	...	...	...	...	...	40	50	60	70	80	90	100
Expt. 1: 5 mm-ethanol; liver wt. 6.31 g.	Pyruvate found (μ moles)					638	274	34	7	4	7	4
	Lactate found (μ moles)					192	338	441	518	406	431	464
	Glucose found (μ moles)					82	152	246	294	294	316	318
	[Lactate]/[pyruvate] ratio					0.30	1.23	13.0	74	101	62	116
Expt. 2: no ethanol; liver wt. 7.16 g.	Pyruvate found (μ moles)					720	331	105	30	22	13	13
	Lactate found (μ moles)					264	503	578	513	365	271	209
	Glucose found (μ moles)					158	225	275	374	466	604	575
	[Lactate]/[pyruvate] ratio					0.37	1.52	5.50	17.1	16.6	20.8	16.1

concentrations of ethanol (Theorell & Bonnichsen, 1951; Theorell & McKinley-McKee, 1961; Dalziel & Dickinson, 1966). In the experiments of Dalziel & Dickinson (1966) the maximum rate of the alcohol dehydrogenase reaction (with horse liver enzyme at 23.5°) occurred at 8 mM. The experiment recorded in Table 1 shows that the maximum inhibition of gluconeogenesis by ethanol occurs likewise between 5 mM- and 20 mM-ethanol. This indicates that the concentration-dependence of rat liver alcohol dehydrogenase is similar to that of horse liver alcohol dehydrogenase. The parallelism between inhibition of gluconeogenesis and the maximum rate of alcohol dehydrogenase may be taken as evidence that the inhibition of gluconeogenesis is a direct function, not of ethanol, but of the action of alcohol dehydrogenase, and that ethanol as such is not inhibitory. The inhibition occurs only when ethanol, under the influence of alcohol dehydrogenase, converts NAD⁺ into NADH.

Effect of ethanol on gluconeogenesis from pyruvate. The effect of ethanol on gluconeogenesis from pyruvate is shown in Table 2. Pyruvate was removed by the perfused liver with great rapidity, forming both glucose and lactate. The initial rates of pyruvate removal were about the same with and without 5 mM-ethanol. Within 30 min. almost all the added pyruvate had disappeared. The concentration of lactate rose rapidly during the early stages of perfusion, but, whereas in the absence of ethanol it began to fall 20 min. after the addition of the pyruvate, it continued to rise in the presence of ethanol as long as pyruvate was available and then remained approximately stationary. The initial rates of gluconeogenesis were about the same with and without ethanol, but there was a striking difference in the rates after the disappearance of added pyruvate. In the absence of ethanol glucose formation continued at about the same rate, with lactate serving as the precursor. In the presence of ethanol the rate of lactate utilization was very

low (as expected on the basis of the results given in Table 1), and the concentrations of both glucose and lactate remained almost constant after 30 min. Thus the initial rates of glucose formation from pyruvate were not much affected by 5 mM-ethanol, but the total amount of glucose synthesized from the added pyruvate was much decreased because the lactate formed in the initial stages of perfusion was not later converted into glucose in the presence of ethanol. Since pyruvate is the first of the chain of intermediates leading from lactate to glucose, and since in the presence of an excess of pyruvate the stages between pyruvate and glucose are not inhibited by ethanol, it follows that the stage of gluconeogenesis from lactate that is inhibited by ethanol is the conversion of lactate into pyruvate.

Factors modifying the inhibition of gluconeogenesis from lactate by ethanol. The degree of inhibition of gluconeogenesis from lactate by 10 mM-ethanol, recorded in Table 1, applies only to the experimental condition specified. When the lactate concentration was raised to 30 mM the inhibition by 10 mM-ethanol was much lower: 18% instead of 66% (Table 3). This is to be expected if the critical factor responsible for the inhibition is the low concentration of pyruvate. Increasing the concentration of lactate causes an increased steady-state concentration of pyruvate in the perfusion medium, owing to regulatory mechanisms in the liver that adjust the [lactate]/[pyruvate] ratio to values between 8 and 20 (Schimassek, 1963). After 30 min. incubation the pyruvate concentration in the medium on addition of 30 mM-lactate was 0.18 mM, i.e. about 20 times that with 10 mM-lactate. The high concentration of lactate itself was somewhat inhibitory in the absence of ethanol, the rate being about 25% lower at 30 mM-lactate than at 10 mM-lactate.

When glucagon and oleate were added together with lactate and the highest rates of gluconeogenesis occurred (Ross *et al.* 1967b), the inhibitory effect of

Table 3. *Effect of lactate concentration and other factors on the inhibition of gluconeogenesis by ethanol*

The experimental conditions were as described for Table 1, except for the metabolite concentrations. The results are given as means $\pm$ S.E.M. with numbers of observations in parentheses.

Additions to the perfusion medium	Rate of gluconeogenesis (μ moles/min./g. wet wt. of tissue)		Inhibition by ethanol (%)
	Without ethanol	With 10 mm-ethanol	
L-Lactate (10 mm)	1.06 ± 0.09 (12)	0.36 ± 0.05 (7)	66
L-Lactate (30 mm)	0.81 ± 0.09 (3)	0.67 ± 0.05 (3)	18
L-Lactate (10 mm) + oleate (2 mm) + glucagon (0.5 μ g./ml.)	2.32 ± 0.15 (4)	1.49 ± 0.16 (5)	36
L-Alanine (10 mm)	0.66 ± 0.12 (6)	0.49 ± 0.12 (5)	26
L-Alanine (10 mm) + oleate (2 mm)	0.46 ± 0.11 (2)	0.19 ± 0.04 (4)	59

Table 4. *Effect of ethanol on the rate of gluconeogenesis from various substrates in the perfused rat liver*

The concentrations of the added substrates and of ethanol were 10 mm. The experimental conditions were as described in Table 1. The results are given as means $\pm$ S.E.M. with numbers of observations in parentheses.

Substrate	Rate of gluconeogenesis (μ moles/min./g. wet wt. of tissue)		Inhibition by ethanol (%)
	Without ethanol	With ethanol	
None	0.14 ± 0.03 (5)	0.05	65
Lactate	1.06 ± 0.09 (12)	0.42 ± 0.06 (7)	60
Glycerol	0.48 ± 0.03 (5)	0.17 ± 0.04 (4)	65
Dihydroxyacetone	2.07 ± 0.30 (6)	0.97 ± 0.13 (3)	53
D-Fructose	2.68 ± 0.25 (4)	1.90 ± 0.09 (3)	29
L-Proline	0.55 ± 0.10 (3)	0.16 ± 0.03 (3)	71
L-Serine	0.98 ± 0.04 (3)	0.73 ± 0.06 (3)	25
L-Alanine	0.66 ± 0.12 (6)	0.49 ± 0.12 (5)	26
Galactose	0.36 ± 0.11 (3)	< 0.1	> 72

ethanol was also diminished. The cause of this effect of oleate and glucagon remains to be explored. With alanine as the precursor oleate increased the inhibition by ethanol.

Effect of ethanol on gluconeogenesis from other precursors. Ethanol (10 mm) inhibits, to different degrees, gluconeogenesis from glycerol, dihydroxyacetone, proline, serine, alanine, fructose and galactose (Table 4). With serine and alanine the inhibitions are relatively small (25 and 26%). In view of what has already been said, inhibitions by ethanol may be expected whenever pyruvate is an intermediate, because the steady-state concentration of pyruvate is liable to be lowered by interaction with the NADH formed through the alcohol dehydrogenase reaction. In fact, in the presence of ethanol, serine caused a substantial formation of lactate at an initial rate (during the first 30 min.) of about 0.8μ mole/min/g. wet wt. During the latter periods of perfusion the rate fell and eventually lactate was taken up by the liver. Alanine also formed lactate initially in the presence of ethanol, but the rate was lower than with serine (0.3μ mole/min./g. wet wt.).

The strong inhibition of gluconeogenesis from galactose is already known from the work of Isselbacher & Krane (1961) and Salaspuro (1966). This inhibition can be readily explained by the effect of ethanol on the redox state of the NAD system. Although, on balance, the conversion of galactose into glucose does not represent an oxidoreduction, it is known (Kalckar & Maxwell, 1956; Maxwell, 1957) that NAD is an essential component of the epimerase and that the epimerase is inhibited by NADH (Isselbacher & Krane, 1961; Robinson, Kalckar, Troedsson & Sanford, 1966).

Reversal by acetaldehyde of inhibition of gluconeogenesis by ethanol. Acetaldehyde, being volatile, rapidly disappears in the oxygenator, and to maintain a somewhat constant concentration acetaldehyde has to be added at frequent intervals. In the following experiments sufficient aldehyde (750 μ moles/150 ml. of medium) was added every 10 min. to bring the concentration from zero to 5 mm. When added together with 10 mm-lactate the rate of gluconeogenesis was 0.87 ± 0.11 (4) μ mole/min./g. wet wt. Acetaldehyde caused a slight inhibition (18%) of gluconeogenesis from lactate, but

ethanol was not inhibitory in the presence of acetaldehyde.

Gluconeogenesis from glycerol was increased by the addition of acetaldehyde from 0.48 ± 0.03 (5) to 0.84 ± 0.08 (4) $\mu\text{mole/min./g. wet wt.}$

Within 15 min. of the addition of acetaldehyde there was an accumulation of 0.8–1.0 mm-ethanol in the medium. Afterwards the concentration of ethanol gradually fell to 0.1 mm in 90 min. Much acetate appeared in the medium: it reached a concentration of 7 mm at 90 min.

It is not possible to predict whether or not acetaldehyde would counteract the effect of ethanol in the perfused liver because acetaldehyde can react in the liver with NAD in two ways: either donating electrons to NAD^+ and forming NADH and acetate, or accepting electrons from NADH and forming NAD^+ and ethanol, the two reactions being catalysed by different enzymes.

Experiments with liver homogenates showed that the reaction of acetaldehyde with NAD depends on the relative concentrations of NAD^+ and NADH. As may be expected, when NAD^+ was added alone it was readily reduced, the rate being dependent on the NAD^+ concentration between 0.05 and 2.1 mm, and when NADH was added alone the reduction of acetaldehyde prevailed. With a mixture of NAD^+ and NADH the reduction generally prevailed, but NAD^+ decreased the rate of reduction.

As the changes in the redox state of the cytoplasmic NAD couple of the tissue are reflected by the [lactate]/[pyruvate] ratio of the perfusion medium, the direction of the action of acetaldehyde in the liver can be assessed from measurements of this ratio. In experiments not reported here in full, the ratio always fell on addition of acetaldehyde, for example from 24 to 12.

Removal of the inhibitory effects of ethanol by pyrazole. According to Theorell & Yonetani (1963) and Theorell (1967) pyrazole (1,2-diazole) is one of the most effective inhibitors of alcohol dehydrogenase. As expected, addition of 0.1 M-pyrazole to the perfusion medium completely removed the inhibitory effect of ethanol on gluconeogenesis from lactate (Table 5). At 0.01 mm-pyrazole most of the inhibition was removed. In the absence of ethanol 0.1 mm-pyrazole had no detectable effects. These results confirm the conclusion that it is not the presence of ethanol, but the interaction between ethanol and alcohol dehydrogenase, that causes the inhibition of gluconeogenesis. Several pyrazole derivatives tested at 1 mm (1,3-dimethylpyrazole, pyrazole-3,5-dicarboxylic acid and 5-methylpyrazole-3-carboxylic acid) did not counteract the inhibition by 10 mm-ethanol.

Effect of ethanol on gluconeogenesis in kidney cortex. Gluconeogenesis by rat kidney-cortex slices incubated with 10 mm-lactate as described by Krebs,

Table 5. *Effects of pyrazole on gluconeogenesis from lactate in the presence of ethanol*

The initial concentrations of lactate and ethanol were 10 mm. The experimental conditions were as described for Table 1. The results are given as means $\pm$ S.E.M. with the numbers of observations in parentheses.

Concn. of pyrazole (mm)	Rate of gluconeogenesis ($\mu\text{moles/min./g. wet wt. of tissue}$)
0	0.36 ± 0.05 (7)
0.01	0.80 ± 0.13 (4)
0.1	1.30 ± 0.11 (3)

Table 6. *Alcohol dehydrogenase activities in rat tissues*

The assay was carried out at pH 7.2 and 37° in the direction acetaldehyde $\rightarrow$ ethanol as described in the text. The results are given as means $\pm$ S.E.M. with numbers of observations in parentheses. No measurable activity (i.e. $< 0.1 \mu\text{mole/min./g. wet wt.}$) was found in brain cortex, pancreas, submaxillary gland, lung, spleen, seminal vesicle, bladder, renal inner medulla, epididymal fat, mucosa of ileum and jejunum, diaphragm, heart muscle and skeletal muscle.

Tissue	NADH oxidized ($\mu\text{moles/min./g. wet wt. of tissue}$)
Liver	20.3 ± 3.13 (7)
Kidney cortex	2.14 ± 0.48 (6)
Gastric mucosa (body)	0.445 ± 0.10 (4)
Gastric mucosa (fundus)	1.50 ± 0.42 (4)
Duodenal mucosa	0.392 ± 0.091 (3)
Caecal mucosa	0.308 ± 0.11 (3)
Testis	0.269 ± 0.047 (3)
Ovary	0.133 ± 0.024 (3)
Uterus (whole)	0.192 ± 0.037 (3)

Bennett, de Gasquet, Gascoyne & Yoshida (1963) was not affected by 10 mm-ethanol. It is relevant in this context that the activity of alcohol dehydrogenase of rat kidney cortex is no more than 10% of that of rat liver (Table 6). Failure of ethanol to influence the rate of gluconeogenesis bears out the conclusion that the inhibitory effect of ethanol depends on the activity of alcohol dehydrogenase.

Activity of alcohol dehydrogenase in rat tissues. Systematic assays of alcohol dehydrogenase in human tissues have been reported by Schmidt & Schmidt (1960). The distribution of alcohol dehydrogenase among rat tissues is similar to that of human tissues. Measurable activities were found only in liver, kidney cortex, testis and certain tissues of the intestinal tract (Table 6). On a weight-for-weight basis the activity found in rat liver was not higher than that reported for human liver.

Effect of ethanol on lactate removal in liver of well-fed rats. It is not practicable to measure gluconeogenesis in liver of well-fed rats directly because the

increment of carbohydrate is too small in relation to the carbohydrate stored in the liver. It is, however, possible to measure accurately the uptake of lactate. The maximum rate of glucose synthesis can then be calculated on the assumption (which has proved valid in liver of starved rats; Hems *et al.* 1966) that the formation of glucose is the only major reaction of lactate. The experiments recorded in Table 7 show that the uptake of lactate in liver of well-fed rats is about 70% of that observed in liver of starved rats and this is almost completely inhibited by 10 mM-ethanol.

Effect of ethanol on the concentrations of lactate and pyruvate in the liver. The experiments shown in Table 2 confirm earlier findings (Forsander, Rih, Salaspuro & Menp, 1965; Forsander, 1968) that ethanol raises the [lactate]/[pyruvate] ratio. It is known that under some conditions the value of this ratio within the tissue is very similar to that of the medium (Schimassek, 1963, 1968). This applies, for example, to the liver of well-fed rats when the normal balance has not been upset by major additions of lactate or pyruvate. The distribution of lactate and pyruvate between tissue and medium after addition of 10 mM-lactate with and without ethanol is shown in Table 8. In these experiments the tissue concentrations of lactate and pyruvate

were determined in the freeze-clamped material 30 min. after the addition of lactate to the medium. A correction was made for extracellular space as determined with ¹²⁵I-labelled albumin. The tissue concentrations of lactate were considerably (about 3 mM) lower than the concentrations in the medium. For pyruvate the absolute differences between tissue and medium were small and not significant ($P > 0.1$), but the effect of ethanol on the pyruvate concentration was highly significant for both medium and tissue ($P < 0.001$). Ethanol decreased the concentration of pyruvate in the tissue to about one-quarter and in the medium to about one-tenth. The concentration of lactate in both tissue and medium was higher in the presence of ethanol, presumably because of the inhibition of lactate utilization, but in spite of this lower rate, the concentration gradient of lactate between tissue and medium was about the same with and without ethanol. Previous work (Hems, Stubbs & Krebs, 1968) has shown that the entry of di- and tri-carboxylic acids and of glutamate into liver tissue is relatively slow. The experiments recorded in Table 8 indicate that the entry of lactate into liver cells is also restricted. Although it is much faster than the entry of bi- or ter-valent anions it is not sufficiently rapid to establish equal concentrations in tissue and medium. An alternative explanation of the concentration differences is the assumption that gradients are actively maintained, as with other small ions such as Na⁺, K⁺ and Cl⁻.

Table 7. *Effect of ethanol on lactate removal in liver of well-fed rats*

The initial lactate concentration of the medium was 10 mM. The experimental conditions were as described for Table 4. The results are given as means $\pm$ S.E.M. with the numbers of observations in parentheses.

Concn. of ethanol (mM)	Lactate change (μ moles/min./g. wet wt. of tissue)
0	-1.52 $\pm$ 0.25 (4)
10	+0.09 $\pm$ 0.04 (4)

DISCUSSION

Inhibition by ethanol of gluconeogenesis from lactate. The experiments establish a parallelism between the inhibitory effects of ethanol on gluconeogenesis and the activity of alcohol dehydrogenase. Inhibition of gluconeogenesis by

Table 8. *Effects of ethanol on the concentrations of lactate and pyruvate in perfused rat liver tissue and in the perfusion medium*

The freeze-clamped tissue and the perfusion medium were analysed 30 min. after the addition of the substrates. The values for the tissue were corrected for the extracellular space, which was determined with ¹²⁵I-labelled albumin to be 22.2 $\pm$ 0.10% (4) at the end of the perfusion. Most of the extracellular space consists of the vascular system and its contents. The rats were starved for 48 hr. The results are given as means $\pm$ S.E.M. with numbers of observations in parentheses.

Substrate(s) added	Concentrations 30 min. after addition of substrate(s)					
	In liver			In medium		
	[Lactate] (mM)	[Pyruvate] (mM)	[Lactate] [Pyruvate]	[Lactate] (mM)	[Pyruvate] (mM)	[Lactate] [Pyruvate]
L-Lactate (10 mM)	3.86 $\pm$ 0.31 (6)	0.32 $\pm$ 0.03 (6)	12.1	6.69 $\pm$ 0.62 (6)	0.43 $\pm$ 0.05 (6)	15.5
L-Lactate (10 mM) + ethanol (10 mM)	4.56 $\pm$ 0.31 (6)	0.076 $\pm$ 0.024 (8)	60	8.22 $\pm$ 0.45 (8)	0.036 $\pm$ 0.010 (8)	228

concentrations of ethanol above 10mM is less than at lower concentrations, just as the activity of liver alcohol dehydrogenase is lower at high alcohol concentrations. Inactivation of the enzyme by pyrazole removes the inhibition of gluconeogenesis by ethanol. This parallelism is conclusive proof that it is not the presence of alcohol, but the interaction of alcohol with alcohol dehydrogenase, that causes the inhibition of gluconeogenesis. The experiments lend strong support to the concept that the primary effect of ethanol on gluconeogenesis is on the alcohol dehydrogenase reaction, which causes a shift of the $[NAD^+]/[NADH]$ ratio.

It is not at once evident why a fall in the value of this ratio should inhibit gluconeogenesis because, like glycolysis, gluconeogenesis can occur within a wide range of $[lactate]/[pyruvate]$ ratios. This follows from the facts that the rates of gluconeogenesis are about equal when either 10mM-lactate or 10mM-pyruvate is added (Ross *et al.* 1967a) and that the rate remains constant when the $[lactate]/[pyruvate]$ ratio of the medium (which roughly though not precisely parallels the $[NAD^+]/[NADH]$ ratio of the tissue) changes with time (Table 2). The relation between the change in the $[NAD^+]/[NADH]$ ratio and the rate of gluconeogenesis is not a direct one. The rise of the $[lactate]/[pyruvate]$ ratio is, under many conditions, caused mainly by a fall in pyruvate concentration rather than a rise in lactate concentration. Pyruvate concentration is one of the factors limiting the rate of the pyruvate carboxylase reaction, a key step in gluconeogenesis from lactate and certain other precursors. According to Scrutton & Utter (1965) the K_m of chick liver pyruvate carboxylase is $0.44 \times 10^{-3}M$, i.e. higher than the normal concentration of pyruvate. Hence the fall in pyruvate concentration caused by ethanol, according to Table 8 to about one-tenth of the K_m value, is expected to lower the rate of gluconeogenesis from all precursors that form pyruvate as an intermediate (if the K_m value of the rat liver enzyme is similar to that of chick liver enzyme, a point on which there is no information).

Factors affecting the inhibition of gluconeogenesis from lactate. Gluconeogenesis from lactate is maximal in the presence of fatty acids plus glucagon and under these conditions the inhibition by ethanol is diminished (Table 3). This implies that in the presence of ethanol the percentage stimulation of gluconeogenesis by fatty acids and glucagon is greater than in its absence. As yet no satisfactory explanation can be offered for the effect of fatty acids. Winer & Theorell (1959, 1960) have shown that certain fatty acids can inhibit alcohol dehydrogenase, but the fatty acid added in the present experiments, namely 2mM-oleate, does not inhibit liver alcohol dehydrogenase significant-

ly (see also Theorell & McKinley-McKee, 1961; Theorell & Sund, 1963).

Apparent discrepancies with findings of other authors. The observation by Williamson (1968) that ethanol can increase gluconeogenesis from alanine under certain conditions (see also Kreisberg, 1967) illustrates the importance of the experimental conditions for the action of ethanol. In the experiments reported in the present paper alanine and ethanol were added once only, whereas in the experiments of J. R. Williamson (personal communication) alanine was added at regular intervals, to maintain an approximately constant alanine concentration. The experimental conditions are therefore not comparable. The main rate-limiting reaction of gluconeogenesis from alanine is the formation of oxaloacetate from pyruvate, as indicated by the fact that the rates of gluconeogenesis from oxaloacetate are higher than those from alanine or at the pyruvate concentrations reached on addition of alanine, and ethanol can be expected to affect this reaction in two ways. As discussed above, it decreases the concentration of pyruvate and can thereby inhibit the pyruvate carboxylase reaction. It can also act as a precursor of acetyl-CoA, which can accelerate the pyruvate carboxylase reaction. Which of the two effects prevails depends on the experimental conditions.

Cause of inhibition of gluconeogenesis by ethanol in the presence of various precursors. It has been discussed above how the inhibition of gluconeogenesis from lactate, alanine, serine and galactose may be attributed to a primary change of the $[NAD^+]/[NADH]$ ratio. The inhibitions of gluconeogenesis from glycerol, dihydroxyacetone and fructose (Table 4) might be expected to be connected with the fact that the decreased $[NAD^+]/[NADH]$ ratio can cause an accumulation of α -glycerophosphate (Nikkila & Ojala, 1963), but observations on the concentrations of this intermediate under various conditions do not support this assumption. Thus on addition of dihydroxyacetone (10mM) and ethanol (10mM) the α -glycerophosphate concentration of the freeze-clamped perfused liver was 4.5mM after 30min., compared with 0.4mM with dihydroxyacetone alone, but with fructose alone the α -glycerophosphate concentration rose to 3.2mM and this value was not increased by ethanol. No definite statement can therefore be made about the cause of the ethanol effect on gluconeogenesis from glycerol, dihydroxyacetone, fructose and proline, although it seems likely that the effects are connected with the alcohol dehydrogenase reaction. Presumably the effect of ethanol on gluconeogenesis from fructose and glyceraldehyde is connected with the increased rate of ethanol oxidation in the presence of fructose

or glyceraldehyde (see Stuhlfauth & Neumaier, 1951; Holzer & Schneider, 1955; Thieden & Lundquist, 1967; Merry & Marks, 1967).

Alcohol-induced hypoglycaemia. The inhibitory effects of ethanol on gluconeogenesis described in the present paper support the concept that the hypoglycaemia observed after alcohol intake in underfed human beings (Brown & Harvey, 1941; Madison, 1968) is caused by an inhibition of gluconeogenesis, the primary effect being the shift in the $[NAD^+]/[NADH]$ ratio. For fuller discussion of this subject see Krebs (1968) and Madison (1968). The concentrations of ethanol that cause major inhibitions are reached in the blood after a moderate intake of alcoholic beverages. British law does not regard motorists as being unduly incapacitated by concentrations up to 18mm-ethanol in the blood. The inhibition of gluconeogenesis from lactate is maximal at 10mm.

This work was supported by U.S. Public Health Service Grant no. AM08715.

REFERENCES

- Brown, T. M. & Harvey, A. M. (1941). *J. Amer. med. Ass.* **117**, 12.
- Dalziel, K. & Dickinson, F. M. (1966). *Biochem. J.* **100**, 34.
- Dickinson, F. M. & Dalziel, K. (1967). *Biochem. J.* **104**, 165.
- Field, J. B., Williams, H. E. & Mortimore, G. E. (1963). *J. Clin. Invest.* **42**, 497.
- Forsander, O. A. (1968). In *Stoffwechsel der isoliert perfundierten Leber*, p. 210. Ed. by Staib, W. & Scholz, R. Berlin: Springer-Verlag.
- Forsander, O. A., Räihä, N., Salaspuro, M. & Mäenpää, P. (1965). *Biochem. J.* **94**, 259.
- Forsander, O., Räihä, N. & Suomalainen, H. (1958). *Hoppe-Seyl. Z.* **312**, 243.
- Freinkel, N., Arky, R. A., Singer, D. L., Cohen, A. I., Bleicher, S. J., Anderson, J. B., Silbert, C. K. & Foster, A. E. (1965). *Diabetes*, **14**, 350.
- Greenberger, N. J., Cohen, R. B. & Isselbacher, K. J. (1965). *Lab. Invest.* **14**, 264.
- Hems, R., Ross, B. D., Berry, M. N. & Krebs, H. A. (1966). *Biochem. J.* **101**, 284.
- Hems, R., Stubbs, M. & Krebs, H. A. (1968). *Biochem. J.* **107**, 807.
- Holzer, H. & Schneider, S. (1955). *Klin. Wschr.* **33**, 1066.
- Isselbacher, K. J. & Greenberger, N. J. (1964a). *New Engl. J. Med.* **270**, 351.
- Isselbacher, K. J. & Greenberger, N. J. (1964b). *New Engl. J. Med.* **270**, 402.
- Isselbacher, K. J. & Krane, S. M. (1961). *J. biol. Chem.* **236**, 2394.
- Kalckar, H. M. & Maxwell, E. S. (1956). *Biochim. biophys. Acta*, **22**, 588.
- Krebs, H. A. (1968). In *Advances in Enzyme Regulation*, vol. 6, p. 468. Ed. by Weber, G. Oxford: Pergamon Press Ltd.
- Krebs, H. A., Bennett, D. A. H., de Gasquet, P., Gascoyne, T. & Yoshida, T. (1963). *Biochem. J.* **86**, 22.
- Krebs, H. A. & Veech, R. L. (1969). In *Bari Round Table Discussion on the Energy Level and Metabolic Control in Mitochondria*. Bari: Adriatica Editrice (in the Press).
- Kreisberg, R. A. (1967). *Diabetes*, **16**, 784.
- Lieber, C. S. (1967a). *Fed. Proc.* **26**, 1443.
- Lieber, C. S. (1967b). In *Biochemical Factors of Alcoholism*, p. 167. Ed. by Maickel, R. P. Oxford: Pergamon Press Ltd.
- Lochner, C. S., Wulff, J. & Madison, L. L. (1967). *Metabolism*, **16**, 1.
- Madison, L. L. (1968). *Advanc. metab. Disorders*, **3**, 85.
- Madison, L. L., Lochner, A. & Wulff, J. (1967). *Diabetes*, **16**, 252.
- Maxwell, E. S. (1957). *J. biol. Chem.* **229**, 139.
- Merry, J. & Marks, V. (1967). *Lancet*, ii, 1328.
- Nikkila, E. A. & Ojala, K. (1963). *Life Sci.* **2**, 717. *Proc. Soc. exp. Biol., N. Y.*, **113**, 814.
- Reboucas, G. & Isselbacher, K. J. (1961). *J. Clin. Invest.* **40**, 1355.
- Robinson, E. A., Kalckar, H. M., Troedsson, H. & Sanford, K. (1966). *J. biol. Chem.* **241**, 2737.
- Ross, B. D., Hems, R., Freedland, R. A. & Krebs, H. A. (1967b). *Biochem. J.* **105**, 869.
- Ross, B. D., Hems, R. & Krebs, H. A. (1967a). *Biochem. J.* **102**, 942.
- Salaspuro, M. P. (1966). *Scand. J. clin. Invest.* **18**, Suppl. no. 92, p. 145.
- Schimassek, H. (1963). *Biochem. Z.* **336**, 468.
- Schimassek, H. (1968). In *Stoffwechsel der isoliert perfundierten Leber*, p. 109. Ed. by Staib, W. & Scholz, R. Berlin: Springer-Verlag.
- Schmidt, E. & Schmidt, F. W. (1960). *Klin. Wschr.* **38**, 957.
- Scrutton, M. C. & Utter, M. F. (1965). *J. biol. Chem.* **240**, 1.
- Smith, M. E. & Newman, H. W. (1969). *J. biol. Chem.* **234**, 1544.
- Stuhlfauth, K. & Neumaier, H. (1951). *Med. Klin.* p. 591.
- Theorell, H. (1967). *Harvey Lect.* no. 61, p. 17.
- Theorell, H. & Bonnichsen, R. K. (1951). *Acta chem. scand.* **5**, 1105.
- Theorell, H. & McKinley-McKee, J. S. (1961). *Acta chem. scand.* **15**, 1797.
- Theorell, H. & Sund, H. (1963). In *The Enzymes*, vol. 7, p. 25. Ed. by Boyer, P. D., Lardy, H. A. & Myrback, K. New York: Academic Press Inc.
- Theorell, H. & Yonetani, T. (1963). *Biochem. Z.* **338**, 537.
- Thieden, H. I. D. & Lundquist, F. (1967). *Biochem. J.* **102**, 177.
- Williamson, D. H., Lund, P. & Krebs, H. A. (1967). *Biochem. J.* **103**, 514.
- Williamson, J. R. (1968). *Fed. Proc.* **27**, 649.
- Winer, A. D. & Theorell, H. (1959). *Acta chem. scand.* **13**, 1038.
- Winer, A. D. & Theorell, H. (1960). *Acta chem. scand.* **14**, 1729.