Orotate Decreases the Inhibitory Effect of Ethanol on Galactose Elimination in the Perfused Rat Liver

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1. The galactose-elimination rate in perfused livers from starved rats was decreased in the presence of ethanol (2–28 mM) to one-third of the control values. Orotate injections partly reversed the effect of ethanol, so that the galactose-elimination rate was about two-thirds of the control values. Orotate alone had no effect on the galactose-elimination rate. 2. Ethanol increased [galactose 1-phosphate] and [UDP-galactose], and decreased [UDP-glucose] and [UTP] both with and without orotate. Orotate increased [UTP], [UDP-glucose] and [UDP-galactose], both with and without ethanol. The increase of [galactose 1-phosphate] in the presence of ethanol was inhibited by orotate. Orotate alone had no appreciable effect on [galactose 1-phosphate]. 3. Both the effect of ethanol and that of orotate on the galactose-elimination rate can be accounted for by assuming inhibition of galactokinase by galactose 1-phosphate with K_i about 0.2 mM, the inhibition being either non-competitive or uncompetitive. 4. The primary effect of ethanol seems to be inhibition of UDP-glucose epimerase (EC 5.1.3.2), followed by accumulation of UDP-galactose, trapping of UDP-glucose and increase of [galactose 1-phosphate]. Orotate decreased the effect of ethanol, probably by increasing [UDP-glucose].

The main pathway of the hepatic conversion of galactose is assumed to involve phosphorylation to galactose 1-phosphate with consumption of ATP (galactokinase, EC 2.7.1.6), conversion of galactose 1-phosphate and UDP-glucose into UDP-galactose and glucose 1-phosphate (uridylyltransferase, EC 2.7.7.12), and epimerization of UDP-galactose to UDP-glucose (UDP-glucose epimerase, EC 5.1.3.2) (Leloir, 1951; Kalckar *et al.*, 1953).

Ethanol inhibits hepatic galactose elimination both *in vitro* (Isselbacher & Krane, 1961) and *in vivo* (Tygstrup & Lundquist, 1962; Tygstrup *et al.*, 1974; Keiding, 1974), probably via inhibition of the UDP-glucose epimerase, which is sensitive to changes in the redox state of the cytosol (Maxwell, 1957).

Experiments on combined galactose and ethanol metabolism in the perfused pig liver (Keiding *et al.*, 1974), however, indicate that the rate-limiting step in the galactose metabolism is the phosphorylation of galactose to galactose 1-phosphate. This reaction is inhibited *in vitro* by the product, galactose 1-phosphate (Cuatrecasas & Segal, 1965; Walker & Khan, 1968). In the pig liver experiments mentioned above, ethanol inhibition of the galactose elimination was associated with the elevation of [galactose 1-phosphate]. Galactokinase therefore may be regulated *in vivo* by product inhibition.

The increase in [galactose 1-phosphate] in the presence of ethanol in the perfused pig liver (Keiding *et al.*, 1974) seems to be related to changes in the

hepatic uridine nucleotide concentration; thus the accumulation of UDP-galactose was associated with a marked depletion of UDP-glucose (and UTP), UDP-glucose being a substrate for the forward transferase reaction and UDP-galactose a product. Ethanol therefore appears to cause an apparent inhibition of the transferase reaction in the sense that in experiments with ethanol a given forward flux of galactose molecules requires a higher galactose 1-phosphate concentration than in experiments without ethanol.

Decker *et al.* (1971) showed that injection of orotate into rats markedly increased the hepatic concentrations of UTP and UDP-glucose. In the present study we therefore examined whether intraperitoneal injection of orotate into rats prevent the decrease in both hepatic UDP-glucose and galactose-elimination rate seen during combined galactose and ethanol metabolism in the perfused rat liver.

Experimental

Animals

Female Wistar rats weighing 203–225 g were starved for 19–22 h before the liver perfusion. Choline orotate (ester between choline and orotic acid; Hepatofalk, Freiburg, Germany) was given to 22 animals as four intraperitoneal injections each of 0.1 mmol in 0.5 ml of water, 25, 16, 7 and 1 h before

the liver perfusion; 35 control rats received no injections.

Liver perfusion

The perfusion technique was that of Hems *et al.* (1966) with minor modifications. The perfusion medium was 1-day-old washed bovine erythrocytes in Krebs–Henseleit buffer (Krebs & Henseleit, 1932) with a haematocrit of 0.26–0.29. Ethanol was removed from the bovine serum albumin powder [fraction V; ethanol-precipitated; Sigma (London) Chemical Co., London S.W.6, U.K.] by distillation of the solution *in vacuo* at 40°C for 5 h (if no distillation was applied, the final ethanol concentration in the medium was 0.6–1.3 mM). The medium was oxygenated by air+CO₂ (95:5). The temperature was 37°C, pH 7.4. Once-through perfusion by means of a roller pump (O. Dick, Copenhagen, Denmark) ensured a constant flow rate ranging from 1.3 to 2.2 ml/min per g wet wt. of liver. The portal pressure, recorded by an open air side tube, ranged from 8 to 17 cm of water.

Galactose (Kabi, Stockholm, Sweden) was added to the medium to a final mean concentration of 1.7 mM (range 1.5–1.9 mM). Ethanol was added to obtain concentrations up to 28 mM.

During an experimental period, beginning 25 min after the start of the portal perfusion and lasting for 10 min, samples of the inflow and outflow medium were taken. A liver tissue sample of about 0.8 g was taken by freeze-clamping at the end of the experimental period.

Analysis

Samples of the medium were precipitated with 0.3 M-HClO₄ for analysis of galactose (Kurz & Wallenfels, 1970), ethanol (Bernt & Gutmann, 1970) and lactate (Hohorst, 1970), and with 1 M-HClO₄ followed by neutralization with 1 M-KOH for analysis of pyruvate (Czok & Lamprecht, 1970); galactose was analysed in ten inflow and outflow samples, ethanol in five sets of samples, and lactate and pyruvate in two outflow samples. Oxygen saturation was measured in two sets by the haemoreflexor method (Zijlstra, 1958; Radiometer, Copenhagen, Denmark). The haematocrit was measured in micro-capillary tubes after centrifugation in a Christ micro-haematocrit centrifuge for 5 min. The haemoglobin concentration was determined spectrophotometrically as cyano-haemoglobin (according to the recommendations of The International Committee for Standardisation in Hematology, 1966).

The liver tissue samples were stored in liquid N₂ for 18–42 h and pulverized frozen tissue samples were precipitated with 0.75 M-HClO₄, neutralized with

0.62 M-KOH (3 M acid and base for ATP, ADP and AMP), and analysed for galactose (Kurz & Wallenfels, 1970), galactose 1-phosphate (Gitzelmann, 1970), UDP-galactose (Keppler & Decker, 1970a), UDP-glucose (Keppler & Decker, 1970b), UTP (Keppler *et al.*, 1970), ATP (Lamprecht & Trautschold, 1970), ADP and AMP (Jaworek *et al.*, 1970). The liver tissue was analysed for glycogen [hydrolysis as described by Pflüger (1909) and enzymic assay of glucose (Bergmeyer *et al.*, 1970)], triglyceride (Eggstein & Kuhlmann, 1970), DNA and RNA (Schmidt & Thannhauser, 1945) and protein (Groves *et al.*, 1968). The concentration of solids in liver tissue was determined after drying to a constant weight at 50°C.

Calculations

The elimination rates of galactose, ethanol and oxygen were estimated as the flow rate multiplied by the inflow–outflow concentration differences; the oxygen concentration was estimated from the saturation and haemoglobin concentration.

At the galactose concentrations used in the present study (1.7 mM) the elimination rate of galactose approximates to the maximum elimination rate, V (Vilstrup & Keiding, 1974). The relation between V and the concentration of galactose 1-phosphate in hepatocyte water (p mM) was described by inhibition of galactokinase by the product galactose 1-phosphate, the inhibition being either non-competitive or uncompetitive:

$$V = (V' \cdot K_i) / (K_i + p) \quad (1)$$

V' is the maximum elimination rate corrected for inhibition ($\mu\text{mol/min}$) and K_i the inhibitor constant (mM). The estimation of V' and K_i was performed by the logarithmic transformation of eqn. (1), which was found to be the best variance-stabilizing transformation in respect to the statistical uncertainties of V and p (Hald, 1952).

Results

Ethanol elimination

The ethanol-elimination rate increased with increasing ethanol concentrations up to about 2 mM, and at higher concentrations it was approximately constant. Orotate pretreatment did not change the elimination rate. The relation between elimination rate and concentration was evaluated by a mathematical model of enzymic elimination of substrates flowing through the intact liver (Bass *et al.*, 1976; Keiding *et al.*, 1976). The estimate of the maximum elimination rate was $14 \pm 1 \mu\text{mol/min}$ per whole liver and that of K_m $0.4 \pm 0.1 \text{ mmol/litre}$ of medium ($\pm$ S.D. of the estimate, $n = 43$).

Fig. 1. Galactose-elimination rate at different ethanol concentrations in perfused livers from orotate-treated (Δ) and control ($\square$) rats

Livers from starved 200g rats were perfused with 1.7mm-galactose. Orotate-treated rats had been given four intraperitoneal injections each of 0.1mmol of choline orotate over 25h before the perfusion. The ethanol concentration is the logarithmic average of the concentration in the inflow (c_i) and outflow (c_o), i.e. $(c_i - c_o) / \ln(c_i/c_o)$, estimated by a mathematical model of enzymic elimination of substrates flowing through the intact liver (Keiding *et al.*, 1976).

The [lactate]/[pyruvate] ratio increased with increasing ethanol concentrations, and above 2mm it was approximately constant; it was not influenced appreciably by orotate. In experiments with no administration of ethanol the ratio was 10 ± 3 (mean $\pm$ S.E.M., $n = 11$), and at ethanol concentrations above 2mm it was 34 ± 2 ($n = 11$), $P < 0.001$.

Galactose metabolism

Fig. 1 shows that ethanol inhibited the galactose-elimination process and that maximum inhibition was obtained at ethanol concentrations above 2mm. Orotate significantly diminished the effect of ethanol; the galactose-elimination rate at ethanol concentrations above 2mm was about twice as high when orotate had been given ($P < 0.001$). In experiments with no ethanol, orotate caused no significant change in the galactose-elimination rate ($P > 0.3$), see Table 1.

The changes in liver metabolites induced by ethanol and by orotate are shown in Table 1, which gives the mean galactose-elimination rate and metabolite concentrations in experiments without ethanol administration (i.e. ethanol concentration below 0.05mm), and with ethanol administration so that ethanol concentrations between 0.05 and 2mm and above 2mm were obtained. Ethanol markedly increased the concentration in hepatocyte water of galactose 1-phosphate and UDP-galactose, and decreased [UDP-glucose] and [UTP]; this was seen both in experiments with and without orotate

Table 1. Effect of ethanol and/or orotate on galactose metabolism in perfused rat livers

Ethanol (mm) No. of expts.	Concentration in hepatocyte water (mm)					t test		
	-Orotate		+Orotate		Ethanol effect (>2mm)	Orotate effect		
	<0.05	0.05-1.99	<0.05	0.05-1.99		-Orotate	+Ethanol	
Galactose-elimination rate (μ mol/min per whole liver)	2.04 $\pm$ 0.03	1.28 $\pm$ 0.10	0.66 $\pm$ 0.03†	1.68 $\pm$ 0.14	2-28 8	***	***	***
Galactose 1-phosphate	0.65 $\pm$ 0.02	1.72 $\pm$ 0.25	3.50 $\pm$ 0.17	1.31 $\pm$ 0.31	2-28 9	***	***	***
UDP-galactose	1.23 $\pm$ 0.05	2.89 $\pm$ 0.25	4.25 $\pm$ 0.12	5.80 $\pm$ 1.40	2-28 9	***	***	***
UDP-glucose	0.85 $\pm$ 0.02	0.54 $\pm$ 0.09	0.22 $\pm$ 0.02	2.10 $\pm$ 0.38	2-28 9	***	***	***
UTP	0.69 $\pm$ 0.05	0.34 $\pm$ 0.11	<0.03	6.81 $\pm$ 1.55	2-28 9	***	***	***
UDP-galactose/UDP-glucose	1.46 $\pm$ 0.08	8.64 $\pm$ 2.77	20.5 $\pm$ 2.1	3.73 $\pm$ 1.69	2-28 9	***	***	***
UDP-galactose+UDP-glucose +UTP	2.76 $\pm$ 0.05	3.78 $\pm$ 0.12	4.47 $\pm$ 0.12	14.7 $\pm$ 1.3	2-28 9	***	n.s.	***
Glycogen as glucose	0.77 $\pm$ 0.45 (n=6)	1.42 $\pm$ 0.65 (n=6)	0.57 $\pm$ 0.07 (n=6)	8.84 $\pm$ 4.94 (n=5)	2-28 9	n.s.	***	*

† $n = 13$.

Fig. 2. Galactose-elimination rate in relation to galactose 1-phosphate concentration in perfused livers from orotate-treated (▲) and control (□) rats

Experimental conditions were as in Fig. 1. Galactose 1-phosphate concentration is given as mmol/l of hepatocyte water, estimated from the measured concentration in liver-tissue biopsies.

pretreatment. Orotate substantially increased UTP, UDP-glucose and UDP-galactose concentrations in experiments both with and without ethanol, so that the concentrations were raised by a factor of 2–10. In experiments with ethanol, orotate decreased galactose 1-phosphate to about two-thirds, and in experiments with no ethanol administration it caused a small but statistically significant increase ($P < 0.05$, t test).

The ethanol effect was maximum at 2 mM, and increasing concentrations did not induce further changes for any of the measurements. At ethanol concentrations below 2 mM the ethanol effect was less pronounced than at higher concentrations except for the concentrations of UTP, UDP-glucose and glycogen, which in experiments with orotate were almost the same at low and high ethanol concentrations (Table 1).

The galactose-elimination rate decreased with increasing concentrations of galactose 1-phosphate, and the relation was the same with and without orotate (Fig. 2). The relation between the galactose-elimination rate and the concentration of galactose 1-phosphate was described by inhibition of galactokinase by the product, galactose 1-phosphate, (see under 'Calculations'); the estimate of K_i was 0.21 ± 0.03 mM ($\pm$ S.D. of the estimate, $n = 45$) and that of V corrected for inhibition was 4.8 ± 0.4 μ mol/min per total liver.

Control data for the effect of orotate

We evaluated whether the orotate pretreatment had any toxic effect on the rats by means of changes in body weight during the period of starvation, liver weight and composition, and oxygen uptake during the perfusion.

The orotate treatment did not affect the animals visibly; the fall in body weight during the starvation period was from 214 ± 2 g (mean $\pm$ S.E.M., $n = 17$) to 196 ± 2 g (22) in the orotate-treated animals and from 212 ± 1 g (18) to 202 ± 1 g (35) in the controls, the decrease being significantly different ($P < 0.001$, t test). Preliminary experiments showed that at this orotate dosage the mortality was zero (LD_{50} was about 0.2 mmol $\times 4$).

The liver weight was increased significantly ($P < 0.001$) by orotate from 5.78 ± 0.06 g (mean $\pm$ S.E.M., $n = 35$) in control experiments to 6.44 ± 0.11 g (22). The orotate effect was accounted for partly by increase in the water content of the livers from 4.08 ± 0.11 g per liver (7) to 4.50 ± 0.11 g (11) ($P < 0.02$) and partly by an increase of the protein content from 1.39 ± 0.07 g (10) to 1.59 ± 0.04 g (13) ($P < 0.025$). Also, the content of RNA increased significantly ($P < 0.001$) from 55 ± 1 mg (18) to 65 ± 2 mg (21); DNA was not changed significantly, being 11.1 ± 0.7 mg (18) with no orotate and 11.3 ± 0.4 (21) with orotate. The hepatic content of triglyceride was not changed significantly by orotate, being 37 ± 6 mg (17) with no orotate and 38 ± 4 mg (18) with orotate; this is consistent with the work of Sidransky *et al.* (1963), who found that orotate induces fatty livers in fed but not in starved rats.

The oxygen uptake was increased by orotate from 20.1 ± 0.6 μ mol/min per liver (mean $\pm$ S.E.M., $n = 35$) in control experiments to 24.0 ± 0.8 ($n = 22$) ($P < 0.001$). The mean adenine nucleotide concentrations, measured in four rats without orotate (two with no ethanol and two with 10 mM-ethanol) were 2.7 mmol of ATP/kg wet wt. of liver, 1.2 mmol of ADP/kg and 0.28 mmol of AMP/kg. In four rats after orotate administration the mean values were 2.2 mmol of ATP/kg, 1.6 mmol of ADP/kg and 0.28 mmol of AMP/kg. There was no significant difference between orotate-treated and control animals.

Ethanol had no significant effect on any of the controls measured.

Discussion

The present experiments show that injection of orotate into rats decreased the fall in hepatic [UDP-glucose] and [UTP] and enhanced the rise in [UDP-galactose] seen during combined galactose and ethanol metabolism. Concomitantly the increase of [galactose 1-phosphate] and inhibition of galactose elimination by ethanol was decreased.

The results are consistent with the concept that the inhibitory effect of ethanol on the hepatic galactose elimination is primarily caused by inhibition of UDP-glucose epimerase (Isselbacher & Krane, 1961), since ethanol markedly increased the substrate of the epimerase reaction, UDP-galactose, and simultaneously enhanced the outflow [lactate]/[pyruvate]

ratio. The decrease by orotate of the ethanol inhibition of galactose elimination most likely was not due to an effect of orotate on the epimerase, since the outflow [lactate]/[pyruvate] ratio did not change. Similarly, orotate alone did not change the [UDP-galactose]/[UDP-glucose] ratio.

However, the effect of ethanol alone, of ethanol plus orotate and of orotate alone are fully accounted for if it is assumed that the galactokinase is rate-limiting (Keiding *et al.*, 1974, 1976) and regulated by inhibition by its product, galactose 1-phosphate (Fig. 2). The inhibition in the perfused rat liver with K_i about 0.2 mM is more pronounced than that seen for rat liver galactokinase *in vitro*, where K_i values range from 1 to 5 mM (Cuatrecasas & Segal, 1965; Walker & Khan, 1968).

It should be noted that the reactions in the present study are not in equilibrium in the sense that the forward and backward reactions are equal; on the contrary there is a net forward flux of galactose through the first three steps of the hepatic galactose metabolism. At a fixed galactose concentration, the concentration of galactose 1-phosphate in hepatocyte water is regulated by its production rate by galactokinase, and by its removal rate by the successive transferase and epimerase steps. As suggested above, the epimerase probably was not influenced by orotate. It therefore might be suggested, first, that the net forward transferase reaction is apparently inhibited by ethanol, in the sense that a given flux of galactose molecules requires higher galactose 1-phosphate concentrations; secondly, the results indicate that this inhibition is partly reversed by orotate, so that the increase in [galactose 1-phosphate] is decreased. This probably is caused by the changes in the concentrations of UDP-glucose as a co-substrate for the forward reaction and of UDP-galactose as a product (Bertoli & Segal, 1966; Segal & Rogers, 1971); the present data, however, do not allow for a quantitative description of the transferase kinetics, partly because the backward reaction rate and the concentration of glucose 1-phosphate were not measured.

The effect of orotate on [UDP-glucose] is possibly the result of the increased [UTP] by simple mass action acting through the UDP-glucose pyrophosphorylase reaction, which has been claimed to be near-equilibrium *in vivo* (Guynn *et al.*, 1974). The increase in [UTP] in the orotate experiments probably is due partly to increased availability of substrate for the biosynthesis and partly to enhanced activities of the uridine nucleotide-synthesizing enzymes (Bresnick *et al.*, 1968; Pausch *et al.*, 1972).

In the intact rat (Keiding, 1974), the effect of ethanol (at 7 mM) was less pronounced than in the present perfused rat liver; the galactose elimination was decreased to about two-thirds of control values, [galactose 1-phosphate] was doubled, and the

estimate of K_i was approximately the same. This might be related to a limited capacity of the perfused livers to maintain the concentration of UDP-glucose, which was decreased to about one-quarter in the perfused liver and only to about two-thirds in the intact liver. Also, the finding that the galactose-elimination rate was not decreased in the presence of ethanol at concentrations below 2 mM in man (Tygstrup & Lundquist, 1962; Tygstrup *et al.*, 1974), in contrast with the findings in the present study, may be explained by differences in the uridine nucleotide metabolism, or it could be related to a different response of the redox state to ethanol (Forsander *et al.*, 1965; Krebs *et al.*, 1969).

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