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The Effect of Ethanol Concentration on Ethanol Oxidation Rate in Rat Liver Slices

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The rate of ethanol oxidation, as measured by the incorporation of radioactive carbon into non-volatile substances by rat liver slices incubated in phosphate buffer, was increased about 60 % when ethanol concentration was raised from 4 to 80 mM.

In the presence of 18 mM pyrazole the ethanol oxidation at low concentrations was nearly abolished, at high ethanol concentration a substantial ethanol oxidation occurred in spite of the high pyrazole concentration which blocks ADH,* as showed by the low lactate/pyruvate ratio in these experiments. The findings suggest that the microsomal ethanol oxidizing system becomes increasingly important and ADH mediated oxidation decreases at high concentrations of ethanol. Moreover the effect of high and low concentrations of ethanol with and without pyrazole on the lactate/pyruvate and on β-hydroxybutyrate/acetoacetate ratios was investigated.

It is known that catalase,[1] hydroxylase or a mixed function oxidase which requires NADPH as cofactor [2–4] can catalyze the conversion of ethanol to acetaldehyde, but until recently only the ADH pathway was supposed to be of physiological significance. The ADH mediated reaction was believed to be the rate limiting reaction in oxidation of ethanol to acetate,[5] so ethanol oxidation was supposed to be constant, provided the concentration of ethanol was above a certain low limit.

Lieber and DeCarli [4] have demonstrated that the microsomal fraction of the liver cell has a considerable ethanol oxidizing capacity, and that this capacity is increased by prolonged ethanol administration. In this work an attempt was made to estimate whether the rate of ethanol oxidation in rat liver slices depends on the concentration of ethanol and to obtain an impression of which enzymatic systems are involved in the oxidation of ethanol at different concentrations.

A preliminary account of this work has been communicated.[6]

* E. C. codes: Alcohol dehydrogenase, E.C. 1.1.1.1. Catalase, E.C. 1.11.1.6. Peroxidase, E.C. 1.11.1.7.

MATERIALS AND METHODS

Liver slices from fed female Wistar rats weighing 180 – 200 g were prepared and incubated in phosphate buffer [7] at pH 7.4 at 37°C as previously described [8] for 1 h. The glucose concentration in all experiments was 12 mM. Oxygen, or in some experiments atmospheric air, was used as the gas phase.

Radioactive assay. Slices weighing 50 – 80 mg were incubated in a Gilson respirometer in 2.25 ml incubation medium containing 4, 40, and 80 mM ethanol and from 27 to 108 nC 1-^{14}C-ethanol (NEN Chemicals, GmbH). At the end of the incubation period 200 μl of the incubation medium were evaporated to complete dryness in partial vacuum after addition of 20 μl 1 M NaOH. After two additions of nonradioactive ethanol and successive evaporation, the material was dissolved in a known amount of water and counted in a liquid scintillation counter. Samples from flasks containing liver slices, but without incubation were treated in the same manner and used as blank values in the calculations of the non-volatile radioactivity (radioactivity remaining in the medium after evaporation of ethanol). Control experiments with 1-^{14}C-acetate treated in the same manner showed a recovery of 95 – 102 %. A few experiments were made using atmospheric air as gas phase. In these experiments only 30 – 40 mg of slices were used but otherwise the procedue was the same.

The carbon dioxide produced during the incubation was absorbed by KOH on filter paper and the production of the $^{14}CO_2$ from 1-^{14}C-ethanol measured by counting the filter paper in a Tri-Carb liquid scintillator after acidification and 10 min shaking of the incubation medium.

At the end of the incubation period the slices were extracted with 10 % (v/v) cold ethanol dried in a desiccator and then counted as above. The results were corrected for the small radioactivity found in slices not incubated but otherwise treated in the same manner.

Enzymatic determinations. Acetate was determined in the incubation medium at the end of the incubation or at 15 min intervals. The reaction was stopped by addition of $HClO_4$. For acetate determination the samples and acetate standards were incubated for 12 h at pH 1 in order to destroy acetoacetate [9] and acetate determined enzymatically.[10] Incubated acetate standards and recovery experiments gave the same results. No trace of acetoacetate was found in these samples when examined after the incubation. Lactate,[11] pyruvate,[12] acetoacetate,[13] and β-hydroxybutyrate [14] were determined at the end of the incubation (1 h) after deproteinization with $HClO_4$.

Acetaldehyde was determined spectrophotometrically by means of an aldehyde dehydrogenase and NAD^+ in 100 mM triethanolamine buffer, pH 9.0. The aldehyde dehydrogenase from liver was kindly prepared by Dr. N. Grunnet from our laboratory. The samples used were not deproteinized but the reaction was stopped by addition of 1/10 vol. 1 M NaOH.

Oxygen uptake was measured in a Gilson respirometer.

RESULTS

Ethanol metabolism. A direct measurement of ethanol oxidation rate in this type of experiments when the ethanol concentration in the incubation medium is as high as 40 and 80 mM is difficult because the quantity of ethanol removed is far too small to allow a reliable determination. An indirect estimation of the ethanol oxidation rate is, however, possible by the determination of the end products of ethanol oxidation.

This calculation from data in Table 1 gives an ethanol utilization rate of 21.6, 30.5 and 34.8 μmol/g wet wt. h when ethanol concentration in the medium is 4, 40, and 80 mM, respectively.

In the presence of 18 mM pyrazole the ethanol oxidation is nearly completely inhibited at low concentrations of ethanol but when the ethanol concentration was raised to 40 and further to 80 mM the utilization rate was

Table 1. Products of ^{14}C-ethanol metabolism in rat liver slices. The rat liver slices were incubated in phosphate buffer containing 11 mM glucose at 37° for 1 h. Additions as indicated. The results are given in μmol/g liver wet wt. h ± S.E.M. with the number of determinations in parenthesis.

Ethanol concentration (gasphase: oxygen)	Non-volatile radioactivity Medium	Slices	$^{14}CO_2$
4 mM	19.3 ± 1.1 (15)	1.2 ± 0.3 (5)	1.1 ± 0.1 (11)
40 mM	27.9 ± 1.6 (7)	1.5 ± 0.3 (5)	1.1 ± 0.2 (11)
80 mM	32.5 ± 1.8 (15)	1.6 ± 0.2 (5)	0.7 ± 0.2 (11)
Pyrazole (18 mM) added			
4 mM	3.6 ± 0.4 (6)	0.27 ± 0.03 (6)	0.2 ± 0.1 (5)
40 mM	14.6 ± 2.0 (6)	1.0 ± 0.2 (6)	1.0 ± 0.2 (4)
80 mM	23.9 ± 2.6 (6)	1.5 ± 0.2 (6)	0.7 ± 0.3 (5)
Ethanol concentration (gasphase: air)			
4 mM	17.5 ± 2.4 (11)		
80 mM	23.9 ± 2.8 (12)		

increased to 16.6 and 26.1 μmol/g wet wt. h. The main incorporation of the radioactivity was found in the nonvolatile fraction in the medium which was increased about 62 % by changing the ethanol concentration from 40 to 80 mM. The $^{14}CO_2$ production was the same whether 4 or 40 mM ethanol was present and slightly less when the ethanol concentration was raised to 80 mM. No effect of different concentrations of ethanol was observed in the radioactivity incorporated into the slices. 18 mM pyrazole depressed the formation of $^{14}CO_2$ and incorporation of the radioactivity in the non-volatile substances at low concentrations of ethanol.

Incorporation of radioactivity in the non-volatile fraction was lower when air was used as gas phase than with oxygen. These results are in agreement with those reported by Videla and Israel,[15] who found a higher rate of ethanol metabolism in liver slices incubated with 95 % O_2 than in slices incubated with 18 % O_2. The relative changes of high and low ethanol concentrations were, however, the same.

Acetate formation. As ethanol is oxidized almost quantitatively to acetate[16–18] by the liver, most of the non-volatile radioactivity in the medium found after incubation must be due to acetate formation.

Using 4 mM ethanol, the acetate formation, when determined enzymatically was 17.6 ± 1.6 μmol/g liver wet wt. h. The formation of the non-volatile radioactivity in the medium was 21.1 ± 1.7 and using 80 mM ethanol the values were 24.2 ± 1.5 and 32.9 ± 2.6. All values are the means of 8 experiments.

If the relative changes were calculated the non-volatile radioactivity was 20 ± 4 % higher than acetate determined enzymatically when ethanol concentration was 4 mM and 36 ± 6 % higher when the concentration was 80 mM. The difference in increase was, however, not significant within 5 % limit.

Fig. 1 shows the time course of acetate formation.

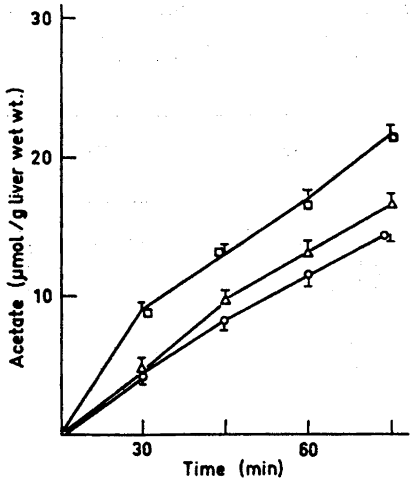

Fig. 1. Acetate formation by rat liver slices in incubation medium containing 4 mM (○), 40 mM (△) and 80 mM (□) ethanol as a function of incubation time. Each point represents the mean ± S.E.M. of 6–10 determinations.

These experiments give slightly lower values for acetate after 1 h incubation period than those reported above. The explanation might be evaporation of acetaldehyde or ethanol during gassing of the flasks which was repeated every 15 min.

Lactate and pyruvate. The lactate/pyruvate ratio (Table 2) was increased to very high values in all the experiments with ethanol when no pyrazole

Table 2. Effect of ethanol on the concentrations of lactate, pyruvate and on lactate/pyruvate ratio. Experimental conditions as in Table 1. The concentrations in nmol/ml of the incubation medium are given as means ± S.E.M. with the number of determinations in parentheses.

Ethanol concentration	Lactate	Pyruvate	Lactate/pyruvate
0 mM	469 ± 24 (16)	33.5 ± 2.2 (16)	15 ± 1 (16)
4 mM	752 ± 53 (14)	5.4 ± 0.5 (15)	172 ± 33 (14)
40 mM	754 ± 52 (14)	4.0 ± 0.4 (14)	232 ± 44 (14)
80 mM	783 ± 75 (15)	6.1 ± 1.4 (13)	147 ± 16 (13)
Pyrazole (18 mM) added			
0 mM	618 ± 56 (6)	38.7 ± 3.2 (6)	17 ± 2 (6)
4 mM	499 ± 27 (4)	23.5 ± 1.0 (5)	21 ± 1 (4)
40 mM	733 ± 64 (5)	19.2 ± 1.4 (4)	36 ± 3 (4)
80 mM	697 ± 48 (5)	13.6 ± 2.0 (5)	55 ± 8 (5)

was present, indicating a considerable reduction of the cytoplasmic NADH/NAD^+ system. This increase was largely abolished by pyrazole.

The lactate concentration was increased by ethanol when no pyrazole was present. Pyrazole alone gave a small increase in lactate concentration, which was abolished by 4 mM ethanol. At higher ethanol concentrations no significant difference in lactate concentrations was observed in the presence of pyrazole.

β-Hydroxybutyrate and acetoacetate. The β-hydroxybutyrate/acetoacetate ratio is increased to the same extent after administration of 4, 40, and 80 mM ethanol (see Table 3). All the changes caused by ethanol were largely abolished by 18 mM pyrazole, but some increase persisted at high ethanol concentration. Pyrazole alone caused a significant decrease (see Discussion) in this ratio.

Table 3. Effect of ethanol on β-hydroxybutyrate and acetoacetate production and on the β-hydroxybutyrate/acetoacetate ratio. Experimental conditions as in Table 1. Results are given in μmol/g wet wt. h ± S.E.M. with the number of determinations in parentheses. The β-hydroxybutyrate/acetoacetate ratio is calculated from the concentrations at the end of the incubation period; the production is corrected for the acetoacetate and β-hydroxybutyrate present in the slices at the start of the experiments (0 and 0.45 ± 0.05 μmol/g wet wt.).[8]

Ethanol concentration	Acetoacetate	β-Hydroxybutyrate	β-Hydroxybutyrate/acetoacetate
0 mM	1.27 ± 0.07 (6)	0.87 ± 0.09 (6)	1.01 ± 0.06 (6)
4 mM	1.57 ± 0.07 (6)	2.66 ± 0.23 (6)	1.97 ± 0.09 (6)
40 mM	1.41 ± 0.21 (6)	2.00 ± 0.28 (6)	1.86 ± 0.19 (6)
80 mM	1.01 ± 0.13 (6)	1.63 ± 0.16 (6)	2.15 ± 0.18 (6)
Pyrazole (18 mM) added			
0 mM	1.76 ± 0.08 (10)	0.71 ± 0.07 (10)	0.66 ± 0.02 (10)
4 mM	1.80 ± 0.10 (10)	0.93 ± 0.17 (10)	0.76 ± 0.06 (10)
40 mM	1.67 ± 0.10 (10)	0.82 ± 0.07 (10)	0.79 ± 0.07 (10)
80 mM	1.56 ± 0.08 (10)	0.86 ± 0.13 (9)	0.85 ± 0.10 (9)

Acetaldehyde formation and oxygen consumption rate. The formation of acetaldehyde during the incubation with 4 mM ethanol was 0.78 ± 0.23 μmol/g wet wt. h and 0.83 ± 0.22 μmol/g wet wt. h with 80 mM ethanol in the incubation medium. The results are the means of 6 experiments ± S.E.M.

The oxygen consumption was independent of the ethanol concentration in the range examined (Table 4).

Table 4. Effect of different concentrations of ethanol on oxygen uptake. The rate of oxygen uptake by rat liver slices incubated in phosphate buffer was determined by using a Gilson respirometer. The results are given as μmol/g wet wt. min ± S.E.M. with the number of determinations in parentheses.

Ethanol conc. (mM)	No addition	Pyrazole, 18 mM
0	1.10 ± 0.04 (6)	1.11 ± 0.10 (6)
4	1.18 ± 0.04 (6)	1.00 ± 0.06 (6)
80	1.06 ± 0.03 (6)	0.87 ± 0.05 (6)

DISCUSSION

The constant ethanol oxidation rate found in previous investigations [19–22] has given support to the assumption that the ADH pathway is the only pathway for ethanol metabolism. The works of Gordon,[23] Lieber and DeCarli,[4] and the present experiments show, however, that in some experimental conditions the ethanol oxidation rate depends on the ethanol concentration. At high pyrazole concentration, which should nearly completely inhibit the ADH reaction, the effect of ethanol concentration on the oxidation rate is even more apparent. The reason for the discrepancy between these findings and the previous ones seems to be the low ethanol concentration used in the earlier investigations.

The K_m of the NADPH dependent microsomal ethanol oxidation system with respect to ethanol is about one order of magnitude higher than the K_m for the ADH system.[4] This means that the contribution of this system will only be apparent at high ethanol concentrations. The fall in L/P ratios after addition of pyrazole confirms the assumption that ADH is nearly completely inhibited but the fall also indicates that ethanol oxidation takes place in a way not requiring NAD^+. Some rise in L/P ratio is observed when the ethanol concentration is increased, but the values are still much lower than in control experiments without pyrazole. This increase in L/P ratio may be a reflection of the increased oxidation of ethanol to acetaldehyde brought about by the microsomal system, as the oxidation of acetaldehyde to acetate proceeds probably by the NAD^+ requiring aldehyde dehydrogenase.

When NADH is formed in the cytoplasmic compartment during ethanol oxidation the reducing equivalents must be transferred to mitrochondria prior to the oxidation. This transfer will be reflected in the mitochondrial redox couple β-hydroxybutyrate/acetoacetate. The increase caused by ethanol is abolished to a considerable extent by pyrazole, but nevertheless the elevation persisting in L/P in the cytoplasmic compartment is accompanied by a slight but significant increase in the β-hydroxybutyrate/acetoacetate ratio.

The experimental findings suggest that the microsomal system becomes increasingly important and the ADH mediated oxidation decreases at high concentrations of ethanol. A simple calculation shows that about 80 % of the ethanol metabolism is caused by the ADH system, when the ethanol concentration is 4 mM, but only about 25 % at 80 mM ethanol in the incubation medium. If one can extrapolate from experiments with slices to *in vivo* conditions the microsomal system would take care of most of the ethanol oxidation at high alcohol concentrations.

As any mechanism of microsomal ethanol oxidation presumably should take place without the production of utilizable energy in the form of ATP, the organism should show a specific dynamic effect of ethanol on oxygen uptake. Such an effect has in fact been observed in alcoholics.[24] No such effect in rat liver slices was found as under the conditions employed the oxygen uptake was quite independent on the concentration of ethanol used (Table 4).

In the liver the chief product of ethanol metabolism is acetate [16–18] and if the small amount of acetaldehyde present is neglected, the amount of acetate formed plus the amount of acetate oxidized to CO_2 together with the amount

of acetate incorporated in other substances will be equal to the amount of ethanol oxidized. When acetate was measured directly instead of the non-volatile radioactivity in the medium a somewhat lower value was found. An explanation for this difference could be either the formation of acetaldehyde or the incorporation of the radioactivity in some other substances than acetate. The acetaldehyde concentration in our experiments is too small to account for this difference. The incorporation of radioactivity in other metabolites, *e.g.* citrate, is therefore the most likely explanation for the discrepancy.

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