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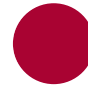

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Published in:
Alcoholism: Clinical and Experimental Research

Publication date:
1986

Document version
Publisher's PDF, also known as Version of record

Citation for published version (APA):
Quistorff, B., Lundquist, F., & Iversen, H. (1986). The deuterium isotope effect on ethanol metabolism in perfused rat liver: effect of reversed perfusion on ethanol and oxygen uptake. *Alcoholism: Clinical and Experimental Research*, *10*(6), 69-72.

Download date: 26. okt.. 2020

0145-6008/86/1006-069S$2.00/0
ALCOHOLISM: CLINICAL AND EXPERIMENTAL RESEARCH

Vol. 10, No. 6
Supplement 1986

The Deuterium Isotope Effect on Ethanol Metabolism in Perfused Rat Liver: Effect of Reversed Perfusion on Ethanol and Oxygen Uptake

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Livers from rats fasted for 24 hr were subjected to nonrecirculating perfusion with Krebs-Ringer bicarbonate solution containing 10 mM ethanol. The deuterium isotope effect was measured using (1-*R*)-[1-^{14}C,1-^{2}H]ethanol. A value of 2.57 ± 0.09 (SD) was obtained independent of the direction of perfusion. Oxygen uptake and ethanol metabolism in contrast were significantly increased when reverse perfusion (i.e., from vena cava to vena portae) was used. The magnitude of the isotope effect indicates that contribution from microsomal ethanol-oxidizing system if this is the only supplementary system is 9.8% under the experimental conditions. At high ethanol concentrations, the contribution would approach 18%. Equal activities of microsomal ethanol-oxidizing system and catalase under the experimental conditions would mean that both contribute 7.3% of the total ethanol metabolism. At high ethanol concentrations (80 mM), however, catalase will be 6.8% and microsomal ethanol-oxidizing system is calculated to 13.3%. Preliminary experiments with rats pretreated with phenobarbital showed no change in the isotope effect or in the rate of ethanol metabolism, but a 40–50% increase in oxygen consumption. The acetaldehyde concentration in the effluent medium was below 1 μM.

THE ACCURATE determination of the deuterium isotope effect on alcohol dehydrogenase,[1] catalase,[2] and the microsomal ethanol-oxidizing system (MEOS)[3] should make it possible to determine the relative contribution of these systems to the metabolism of ethanol in the liver. The isotope effect is defined as the factor by which the velocity is reduced when the hydrogen atom removed during the oxidation to acetaldehyde is replaced by a deuterium atom in the ethanol used. It appears that in all three enzyme systems the hydrogen atom in question is in the 1-pro-R position of ethanol.

Damgaard introduced the use of ^{14}C incorporated into the same ethanol molecule as deuterium as a tool for the study of isotope effects. With this substrate ^{14}C activity of the acetaldehyde produced is a measure of the deuterium removed. The ^{14}C activity was determined relative to the tritium activity of acetaldehyde produced from added [2-^{3}H]ethanol to obtain the isotope effect. In biological systems, acetaldehyde cannot be used for this purpose owing to the low concentration, but acetate is very suitable, being present in blood at a concentration of 0.5–1.0 mM. The conversion of [^{14}C]acetaldehyde to [^{14}C]acetate takes place at the same rate as that of [^{3}H]acetaldehyde to [^{3}H]acetate. In this way the overall isotope effects of ethanol metabolism in isolated liver cells from fasted rats and pigs were found to be 2.69 and 2.80, respectively. An unpleasant complication proved to be the presence of acetaldehyde.[4] The reverse reaction, i.e., reduction of acetaldehyde to ethanol, was found to take place to a large extent, depending on the concentration of acetaldehyde. This causes a rapid decline in the measured apparent isotope effect because the ethanol formed in the back reaction will have lost the deuterium while retaining ^{14}C, so the radioactivity of the products acetaldehyde and acetate will gradually approach that of ordinary protium [^{14}C]ethanol; in other words the apparent isotope effect will decrease toward 1. At high acetaldehyde concentrations, the rate of reduction may be as high as 10 times the net ethanol oxidation rate. We interpreted this finding as the rapid back and forth reaction involving the enzyme-coenzyme complex,[4] the net ethanol oxidation velocity being determined by the dissociation of NADH from the complex. In order to avoid this complication it is essential to ensure that the acetaldehyde concentration is small (i.e., less than 1–2 μM).

In this investigation the isotope effect on ethanol oxidation in perfused liver from rats fasted for 24 hr was measured as a step toward measurements in intact animals.

MATERIALS AND METHODS

Female Wistar rats weighing 200 ± 10 g fasted for 24 hr were used except in the experiments with phenobarbital-induced animals where four male rats were included. These animals were given sodium phenobarbital in the drinking water (0.8 g/liter) for 4–7 days. They consumed 15–25 mg of barbiturate/day. The animals were anesthetized with pentobarbital, 400 μl of 50 mg/ml, and a nonrecirculating perfusion was established, using the perfusion setup described in Ref. 5, which provides for fast change of flow direction through the liver or change of perfusion medium. Oxygen concentration in the perfusion fluid was recorded by oxygen electrodes placed in small (0.24 ml) magnetically stirred, thermostated Perspex chambers situated immediately before and after the

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Received for publication March 3, 1986; accepted for publication March 11, 1986.

This investigation was supported by grants from the Danish Medical Research Council.

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liver catheters. Likewise, the pressure difference over the liver was continuously recorded. The flow rate was measured by calibration of the pump and controlled by frequent measurements of the weight of the effluent. The perfusion medium was Krebs-Henseleit bicarbonate solution saturated with a 95/5 (v/v) mixture of oxygen and carbon dioxide, pH 7.4, with or without 10 mM ethanol. The temperature at the outflow was about 35°C. Oxygen tension at the inlet was about 550 Torr. Flow was adjusted to 23–30 ml/min so that the oxygen tension in the effluent was above 70 Torr.

There was always a certain exudation, $< 7\%$, of perfusate through the liver capsule, increasing somewhat with time of the ethanol perfusion. However, this fluid had passed through the liver, as the isotope effect measured in this exudate was the same as that measured in the effluent. Therefore the flow rate determined by the pump could be used in the calculations.

The standard procedure was the following. The experiment was started by flushing the liver with the Krebs-Henseleit solution until every trace of blood was absent from the effluent, the liver was uniformly colored, without visible unperfused areas, and a steady oxygen consumption obtained. Then the perfusion was continued 10 min with the Krebs-Henseleit solution followed by a 10-min period in which Krebs-Henseleit containing nonradioactive ethanol was used. During these perfusions, oxygen consumption was monitored both with normal and reversed flow. The perfusion medium was then switched to Krebs-Henseleit containing radioactive ethanol at the same concentration (10 mM). After 7 min, the direction of flow was changed and a second 7-min period followed. Samples of effluent were collected every min for weighing and analyses. In every second experiment, reverse perfusion was used in the first period with radioactive ethanol.

The activity of ^3H-labeled ethanol was about three times that of the ^{14}C in order to ensure an accurate determination of both isotopes by scintillation counting. The double-labeled ethanol was prepared according to Damgaard,[1] 2-^3H-labeled ethanol was made from [6-^3H]glucose by fermentation with bakers' yeast with the modification that sodium azide was added to prevent oxidation of the ethanol produced. [^3H] Acetate, [^{14}C]ethanol, and [6-^3H]glucose were from Amersham, United Kingdom. Isotope-containing ethanol preparations were found to contain labeled acetate and acetaldehyde, which are formed spontaneously. It is therefore important to purify the preparations by distillation/diffusion after addition of semicarbazide, to remove acetaldehyde, and after addition of base to pH 9 to remove acetate. The preparation should be used within 1–2 days after purification. The purity of the double-labeled ethanol was checked according to Damgaard[1] by measurement of the isotope effect on commercial horse liver alcohol dehydrogenase.

Analyses

Acetaldehyde was determined in the effluent and also in the liver, which was frozen by freeze clamping at the end of the experiment by a modification of Iversen and Damgaard.[6] Ethanol was measured enzymatically. The isotope effect was determined by counting ^3H and ^{14}C in acetate isolated by diffusion.[7] The 10-ml samples of effluent brought to pH 9.5 were reduced in volume by distillation to about 2 ml before diffusion in Conway units.[7] After evaporation of the diffusate to dryness followed by removal of traces of radioactive ethanol through chasing three times, each with 0.3 ml of nonradioactive ethanol, the residue was dissolved in water and ^3H and ^{14}C activity measured by scintillation counting. The isotope effect is calculated as the ratio of ^3H to ^{14}C in the acetate divided by that in the perfusion medium.

RESULTS

Preliminary experiments were made with a somewhat less elaborate technique. Samples of eluate (2 ml) were evaporated to dryness and all radioactive ethanol removed by repeated chasing with nonradioactive ethanol directly in counting vials. However, if some of the acetate produced from radioactive ethanol is further converted, other radioactive products than acetate will be present, and these substances, e.g., citrate and ketone bodies, will have lost some tritium through reactions involving the methyl group of acetate. For instance one-third of the tritium is lost as water in the condensation of acetyl-CoA with oxaloacetate, and further losses will take place at other reactions of the tricarboxylic acid cycle. When only the nonvolatile material is measured, the $^3H/^{14}C$ ratio will be less than in acetate and the apparent isotope effect therefore also smaller than the true figure. This source of error is avoided when the isotope ratio is measured in isolated acetate.

Exchange of tritium in the methyl group of acetate with hydrogen from water through labilization of the bond after activation to acetyl-CoA followed by hydrolysis to acetate would reduce the apparent isotope effect. This process, however, does not occur to a measurable extent in hepatocytes.[4] As seen from Table 1, the difference between the isotope effects measured after simple evaporation and on isolated acetate is small, but statistically significant.

In search of an explanation for the difference in isotope effect measured on isolated hepatocytes and perfused liver, experiments were made in which the direction of flow through the organ was reversed in order to reveal a possible zonation of the ethanol- and acetaldehyde-metabolizing

Table 1. Isotope Effect on Ethanol Metabolism in Liver from Normal Fasted, Female Rats

Perfusion*	Isotope effect	
	Apparent†	True‡
Experiment 22. Flow = 22.7 ml/min		
Normal	2.50 (2.38–2.56)	2.75 (2.58–2.92)
Reverse	2.54 (2.48–2.57)	2.66 (2.62–2.71)
Experiment 23. Flow = 21.2 ml/min		
Reverse	2.58 (2.52–2.64)	2.51 (2.38–2.59)
Normal	2.49 (2.43–2.52)	2.64 (2.59–2.67)
Experiment 24. Flow = 24.1 ml/min		
Normal	2.49 (2.45–2.53)	2.53 (2.49–2.59)
Reverse	2.50 (2.48–2.51)	2.62 (2.58–2.66)
Experiment 25. Flow = 26.1 ml/min		
Reverse	2.54 (2.53–2.55)	2.59 (2.55–2.64)
Normal	2.44 (2.37–2.52)	2.42 (2.35–2.48)
Experiment 26. Flow = 26.4 ml/min		
Normal	2.48 (2.43–2.52)	2.49 (2.41–2.57)
Reverse	2.56 (2.54–2.58)	2.53 (2.45–2.57)
Experiment 27. Flow = 24.4 ml/min		
Reverse	2.56 (2.53–2.61)	2.54 (2.46–2.59)
Normal	2.53 (2.46–2.58)	2.51 (2.47–2.56)
Average $p = 0.02$	2.52 ± 0.04	2.57 ± 0.09

* Normal direction of perfusion (porta to cava); Reverse direction of perfusion.

† Apparent isotope effect is obtained by measuring the radioactivity ratio $^3H/^{14}C$ in the residue after removing water and ethanol from samples.

‡ True isotope effect is obtained after isolation of acetate by diffusion. Figures in parentheses indicate the range of individual samples (three or four) from the same experiment.

enzymes in the liver. No difference in apparent isotope effect was found, but it was constantly observed that reversed flow caused an increased ethanol metabolism (about 30%, see Table 3). Increased oxygen consumption was also observed during reverse perfusion. This effect of flow direction on ethanol oxidation and oxygen consumption was reversible, i.e., changes could be repeated within one experiment several times upon switching of flow direction. The change did, however, decrease with time of perfusion after approximately 30 min.

The concentration of acetaldehyde measured in several samples during each perfusion period was in all cases below 0.9 μM. The acetaldehyde concentration in the liver biopsies ranged from 0.7–3 nmol/g of tissue, a figure which may be too high owing to artifactual formation of acetaldehyde from ethanol.

A series of experiments were made with livers from rats given phenobarbital in the drinking water for about 8 days. This treatment has been found to increase formation of a microsomal enzyme complex, which oxidizes ethanol though at a lower specific rate than that produced by prolonged ethanol treatment.[8] We did, however, not observe any statistically significant increase in alcohol metabolism per 100 g of body weight in these animals.

The livers from these rats after perfusion with Krebs-Ringer solution showed a characteristic pattern of perivenous brown spots, presumably caused by increases in the concentration of cytochrome P_{450} as suggested by Ji et al.[9]

The results are presented in Tables 2 and 3. Evidently the isotope effect was exactly the same as for normal animals, 2.57, and no significant change in the rate of ethanol oxidation was observed. The oxygen uptake was, however, increased about 50% in all phenobarbital-induced animals (Table 3). Again, reverse perfusion during ethanol metabolism caused increased oxygen uptake both in normal and in barbiturate-treated rats. However, a small but significant decrease in oxygen consumption was observed during normal perfusion when ethanol was present in the medium.

Table 2. Isotope Effect on Ethanol Metabolism in Rats Receiving Phenobarbital for 4–6 Days

Perfusion*	Isotope effect	
	Apparent	True†
Experiment 28. Flow = 24.5 ml/min		
Normal	2.33	2.51
Reverse	2.37	2.58
Experiment 29. Flow = 23.5 ml/min		
Reverse	2.31	2.62
Normal	2.21	2.50
Experiment 30. Flow = 20.7 ml/min		
Normal	2.70	2.48
Reverse	2.54	2.60
Experiment 31. Flow = 20.5 ml/min		
Reverse	2.65	2.38
Normal	2.59	2.63
Experiment 32. Flow = 27.3 ml/min		
Normal	2.48	2.68
Reverse	2.51	2.54
Experiment 33. Flow = 26.6 ml/min		
Reverse	2.62	2.67
Normal	2.55	2.64
Average		
Normal	2.47 ± 0.18	2.57 ± 0.09
Reverse	2.50 ± 0.14	2.57 ± 0.10

* Normal direction of perfusion; reverse direction of perfusion.
† See footnotes to Table 1.

Table 3. Ethanol Metabolism and Oxygen Consumption

Perfusion	Normal rats ($n = 6$)	p	Phenobarbital rats ($n = 6$)	p
	μmol/min ± SD		μmol/min ± SD	
Ethanol metabolism				
Normal	3.32 ± 0.37	<0.01	3.97 ± 0.96	NS*
Reverse	4.59 ± 1.0		4.30 ± 1.27	
Oxygen consumption				
No ethanol: normal	13.1 ± 1.4	<0.001	18.9 ± 3.7	<0.2
10 mM ethanol: normal	11.8 ± 1.6	<0.001	17.4 ± 1.2	<0.01
10 mM ethanol: reverse	14.0 ± 1.9		19.5 ± 2.4	

Ethanol metabolism was measured by tritium-labeled ethanol. Radioactivity was determined in the acid reaction products (acetate) and corrected for 15% oxidation of the acetate formed from ethanol. Oxygen consumption was calculated from the difference in oxygen concentration between affluent and effluent medium.
* NS, not significant.

DISCUSSION

The use of isotope effects as a method to determine the contributions of different enzyme systems to ethanol metabolism is still under development. Damgaard[2] introduced the use of $^{2}H/^{14}C$ double-labeled ethanol for this purpose. Through this device, the fate of deuterium-labeled ethanol can be followed by measuring ^{14}C radioactivity in the reaction products. However, it was realized from the beginning that the method measures only the forward, but not the net reaction rate. The reverse alcohol dehydrogenase (ADH) reaction turned out to be much faster than anticipated, depending strongly on the acetaldehyde concentration, with the consequence that acetaldehyde may compromise the measurements as mentioned previously. In man, however, the acetaldehyde concentration in blood during alcohol metabolism is very low even in most alcoholics, so it may be hoped that in vivo determination of isotope effects will be feasible in man and other species in which acetaldehyde concentration during alcoholemia remains low.

The only group apart from our own which has employed the isotope effect method is that of Lieber and collaborators, who made a remarkable in vivo study in an animal as small as the deermouse.[10] Their results are very encouraging for further studies on the application of the method.

The magnitude of the isotope effect in perfused liver from fasted rats is not very different from that observed in isolated cells[4] or in some preliminary in vivo experiments.[11] From the figure of 2.57 for the overall isotope effect, the limits for the contributions of MEOS and catalase can be calculated, but definite values for the relative contribution of all three systems require additional measurements in the same system, either after inhibition of one of the enzymes or preferably by using two ethanol concentrations, taking advantage of the high K_m for MEOS

of about 10 mM. Thus, experiments at high (30–50 mM) and low (5–10 mM) ethanol concentration should provide sufficient information to determine all three contributions. The accuracy obtainable may, however, be small, depending on the magnitude of the relative contributions. Assuming that only ADH and MEOS give significant contributions, the value of 2.57 means that ADH provides 90.3% and MEOS 9.7% of the activity, assuming isotope effects of 3.0 and 1.15 for ADH and MEOS, respectively.

As the ethanol concentrations used in these experiments (10 mM) is very near the K_m for MEOS, the maximal contribution of MEOS (observed at very high ethanol concentrations) can be calculated to be 16–18%, depending on the K_m assumed for ADH. A similar calculation in the case when catalase is the only accessory ethanol-metabolizing enzyme yields a contribution by catalase of 27–29% of the total ethanol oxidation. K_m for peroxidation by catalase is low compared to the ethanol concentration. The contributions of MEOS and catalase may also be calculated if only the ratio of the two activities is known or assumed. If, for instance, they are equal they will each contribute about 7.3% of the total activity at an ethanol concentration of 10 mM.

The phenobarbital experiments gave no statistically significant change of ethanol metabolism, and the isotope effect remained the same as in the control group. There was, however, a nearly 50% increase in the oxygen consumption compared to the untreated animals, in contrast to the findings in Thurman and Scholz.[12] The cause of this is unknown, but a possible explanation might be that the barbiturate through inhibition of NADH oxidation in the respiratory chain might induce a futile oxidation of NADH, i.e., without ATP formation via a cytochrome P_{450}-dependent oxidase.

The novel observation that reverse perfusion provoked a higher ethanol (and oxygen uptake) in both groups of animals is also at present unexplained. However, a number of metabolic processes is now known to be zonated within the microcirculatory unit of the liver, e.g., gluconeogenesis and urea synthesis.[5,13] Thus, a change of oxygen gradient as produced in these experiments may well result in a different oxygen consumption. It should be noted, of course, that this widely used hemoglobin-free liver perfusion is unphysiological in several respects, e.g., the absence of plasma proteins, hormones, and erythrocytes in the medium, the somewhat subnormal temperature, and the very high perfusion rates. The difference observed in alcohol oxidation with normal and reversed perfusion is unlikely to be caused by a zonation of ADH, since there is only a minor, <5% gradient of alcohol across the liver, due to the high flow rate. More likely the difference is caused by a changed capacity for NADH reoxidation brought about by the oxygen gradient itself. Previous experiments with redox scanning have indeed indicated a significantly increased redox gradient along the sinusoids during alcohol oxidation.[14]

Prolonged treatment with alcohol in a liquid diet induces a more efficient cytochrome P_{450} system for ethanol oxidation.[8] The same species of cytochrome P_{450} is, however, induced by treatment with acetone, which seems to be the natural inducer.[15] Experiments with acetone administration[16] as well as perfusion experiments under more physiological conditions are in progress.

ACKNOWLEDGMENTS

The expert technical assistance of Lillian Lund Hansen and Lissi Immerdal is gratefully acknowledged.

REFERENCES

1. Damgaard SE: Primary deuterium and tritium isotope effects upon V/K in the liver alcohol dehydrogenase reaction with ethanol. Biochemistry 20:5662–5669, 1981

2. Damgaard SE: Isotope effect in peroxidation of deuterium-labelled ethanol by liver catalase. Biochem J 191:613–618, 1980

3. Damgaard SE: The D(V/K) isotope effect of the cytochrome P-450-mediated oxidation of ethanol and its biological applications. Eur J Biochem 125:593–603, 1982

4. Lundquist F, Iversen HL: Kinetics and consequences of the exchange reaction between ethanol and acetaldehyde in hepatocytes. Acta Pharmacol Toxicol (Copenh) 55(Suppl. I) Poster 2:1984

5. Quistorff B: Gluconeogenesis in periportal and perivenous hepatocytes of rat liver, isolated by a new high-yield digitonin/collagenase perfusion technique. Biochem J 229:221–226, 1985

6. Iversen HL, Damgaard SE: Determination of acetaldehyde in human blood using thiourea to inhibit ethanol interference. Clin Chim Acta 135:151–158, 1983

7. Lundquist F, Fugmann U, Rasmussen H: A specific method for the determination of free acetate in blood and tissues. Biochem J 80:393–397, 1961

8. Joly J-G, Hetu C, Mungikar AM: Relative specificity of the ethanol induced rat liver cytochrome P-450 for ethanol oxidation activity in vitro, in Lieber CS (ed): Biological Approach to Alcoholism. Washington DC, United States Department of Health and Human Serviec, pp 304–315, 1983

9. Ji S, Lemasters JJ, Thurman RG: A non-invasive method to study metabolic events within sublobular regions of hemoglobin-free perfused liver. FEBS Lett 113:37–41, 1980

10. Tagaki T, Alderman J, Lieber CS: In vivo roles of alcohol dehydrogenase (ADH), catalase and the microsomal ethanol-oxidizing system (MEOS) in deermice. Alcohol 2:9–12, 1985

11. Lundquist F, Quistorff B, Iversen HL: Deuterium isotope effects in alcohol metabolism. Perfused liver and intact rats. Acta Pharmacol Toxicol (Copenh) 57(Suppl. I) Abstr. 22:1985

12. Thurman RG, Scholz R: Mixed function oxidation in perfused rat liver. The effect of amminopyrine on oxygen uptake. Eur J Biochem 10:459–467, 1969

13. Quistorff B, Dich J, Grunnet N: Periportal and perivenous hepatocytes retain their zonal characteristics in primary culture. Biochem Biophys Res Commun (in press)

14. Quistorff B, Chance B: Redox scanning in the study of metabolic zonation of liver, in Thurman RG, Kaufmann F, Jungermann K (eds): Regulation of Hepatic Metabolism. New York, Plenum Publishing, pp 185–207, 1986

15. Johansson I, Eliasson E, Norsten C, Ingelman-Sundberg M: Hydroxylation of acetone by ethanol- and acetone-inducible cytochrome P_{450} in liver microsomes and reconstituted membranes. FEBS Lett 196:59–64, 1986

16. Lundquist F, Quistorff B, Iversen H: Isotope effects and ethanol metabolism in perfused rat liver. Effect of reverse perfusion. Alcohol Alcohol 21:ISBRA abstr. 65, 1986