Biochemical Pharmacology, Vol. 29, pp. 1029–1034.

0006-2952/80/0401-1029 $02.00/0

CONCENTRATION-DEPENDENT ETHANOL METABOLISM IN PERFUSED LIVER

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(*Received* 6 *June* 1979; *accepted* 16 *October* 1979)

Abstract—Ethanol at initial concentrations of 9.7, 32.5 and 52 mM was added to a recirculating liver perfusion system in the absence of added substrate. A concentration-dependent increase in ethanol metabolism was observed in perfused liver. The β-hydroxybutyrate/acetoacetate ratio (B/A) increased on addition of a low ethanol concentration (9.7 mM). The ratio, however, declined at the higher concentration (52 mM). The decline has been reported previously by others in *in vivo* studies in the rat. Addition of 10 mM L-alanine to the perfusate increased ethanol metabolism, oxygen consumption and urea formation. The concentration-dependent increase in ethanol metabolism and the decline in B/A ratio did not occur in the presence of alanine. Ethanol metabolism in the presence and absence of alanine was completely blocked by 4-methylpyrazole. The changes in B/A ratio were likewise blocked. It is postulated that the concentration-dependent increase in ethanol metabolism is dependent on the alcohol dehydrogenase pathway. The concentration-dependent increase in ethanol metabolism observed under *in vitro* conditions was previously reported not to occur *in vivo*. Since the additon of alanine to the perfusate blocked the concentration-dependent ethanol metabolism, it would appear that the primary difference between *in vivo* and *in vitro* observations is the absence of adequate energy substrates under *in vitro* conditions. Possible mechanisms for the elevated ethanol metabolism under *in vitro* conditions are discussed.

Recently, we have shown that concentration-dependent increases in ethanol metabolism do not occur *in vivo* in the naive rat [1, 2]. Our studies are in agreement with the majority of older studies (reviewed by Jacobsen [3] and Hawkins and Kalant [4]) and with the recent findings of Guynn and Pieklik [5]. On the other hand, recent *in vivo* studies by Feinman *et al.* [6] and Wendell and Thurman [7] do claim a concentration-dependent ethanol metabolism. Thus, in this regard the controversy remains unresolved.

Regardless of the controversy concerning *in vivo* studies, *in vitro* systems do show a concentration-dependent rate of ethanol metabolism. Such concentration-dependent increases in ethanol metabolism have been observed in perfused liver [8–10], liver slices [11, 12] and in isolated hepatocyte preparations [13–15]. The mechanism for the *in vitro* effect, however, is not clear. Because liver alcohol dehydrogenase (ADH) is saturated at very low ethanol concentrations (0.5 to 2.0 mM), non-ADH pathways, such as a microsomal ethanol oxidizing system (MEOS) and catalase, have been invoked to explain the effect.

In the present work, we have examined the concentration-dependent effect of ethanol on ethanol metabolism in the recirculating perfused liver system in the presence and absence of L-alanine. L-Alanine was added to avoid the possibility of intracellular depletion of substrates during the lengthy perfusion procedure. Effects on urea metabolism and on cytoplasmic and mitochondrial redox states were also examined.

MATERIALS AND METHODS

Animals. Male Wistar rats, weighing 200–250 g (obtained from Biobreeding Laboratories, Ottawa, Ontario), were used in all experiments. They were maintained on Purina chow and H_2O *ad lib.*

Liver perfusion. The rats were anesthetized with pentobarbital and then heparinized (2000 units). The portal vein was cannulated with a PE 205 tube, and oxygenated Krebs–Henseleit bicarbonate buffer [16] at 37° was infused at 20 ml/min without interruption of the blood flow. The vena cava was cannulated, and the liver was then transferred to the perfusion apparatus while still maintaining perfusion. The perfusion apparatus had the following features: (a) perfusion temperature was maintained at a constant 37° by a temperature equilibration coil located directly upstream to the liver so that the perfusion temperature was independent of flow rate; (b) the perfusion fluid was passed through a filter, debubbler and perfusion pressure monitor before entrance to the liver; (c) a silastic tubing oxygenator (see Berry *et al.* [17]) was used; and (d) an oxygen tension monitor permitted measurement of inflow and outflow oxygen tensions of the liver perfusion fluid.

The constriction of the ouflow cannula was adjusted so that a back pressure of 5–10 cm of water was maintained, as recommended by Scholz and Bücher [18]. At the beginning of the experiment, the flow rate was adjusted so that the effluent oxygen tension from the liver was between 152 and 190 mm of mercury. The flow rate varied between 30 and 45 ml/min. The inflow oxygen tension varied between 676 and 692 mm of mercury. The total volume of the

perfusate was 70 ml. For examining the concentration-dependent metabolism of ethanol, the following protocol was used. The perfusion fluid was Krebs–Henseleit bicarbonate buffer containing 2 g/100 ml of bovine serum albumin (fraction V, Sigma Chemicals Co., St. Louis, MO). The perfusion fluid was filtered through a Nalgene filter (0.2 μm pore size) prior to use. The pH of the fluid after equilibration with 95% oxygen and 5% CO_2 in the oxygenator was 7.4. There was an initial equilibration period of 30 min at the end of which a 5-ml sample of the perfusate was taken for determination of lactate, pyruvate, β-hydroxybutyrate and acetoacetate. Samples for urea measurement were taken at 4-min intervals throughout the equilibration period. At the end of the 30-min period, the fluid in the perfusion system was replaced by fresh fluid containing ethanol. Samples for ethanol and urea analyses were taken at 4-min intervals. At the end of 30 min, 5 ml of the perfusate were removed for analyses of β-hydroxybutyrate, acetoacetate, pyruvate and lactate. Fresh fluid was then put into the system again and the next concentration of ethanol was used. Three starting concentrations of ethanol were used—either 9.7, 32.5 or 52 mM. These concentrations were added in a random order to the perfusion system. Livers perfused with and without alanine were done on alternate days. Correction for evaporative loss was made by running the perfusion system without a liver present. Ethanol loss was approximately 10 per cent hr, and was independent of either flow rate or ethanol concentration.

Analytical. Lactate, pyruvate, β-hydroxybutyrate, acetoacetate and ethanol were determined by standard enzymatic procedures as described previously [19]. Urea was determined by the enzymatic method of Chaney and Marbach [20].

Statistical analysis. The results were analyzed by Student's *t*-test. Comparison of the means with and without alanine was calculated by the unpaired *t*-test because separate animals were used, whereas comparison of variables at different alcohol concentrations within the same liver was done by means of the paired *t*-test.

RESULTS

The effects of ethanol concentration on ethanol uptake in the presence and absence of L-alanine are shown in Fig. 1. In the absence of alanine in the perfusion fluid, it was observed that, as the concentration of ethanol was increased, the uptake of ethanol was also increased. Addition of alanine to the perfusion fluid increased ethanol metabolism at the two lower ethanol concentrations but not at the highest concentration. Alanine, however, eliminated the concentration-dependent increase in ethanol metabolism observed in the absence of alanine. The addition of 4-methylpyrazole (4 mM) to the perfusate blocked ethanol metabolism at all ethanol concen-

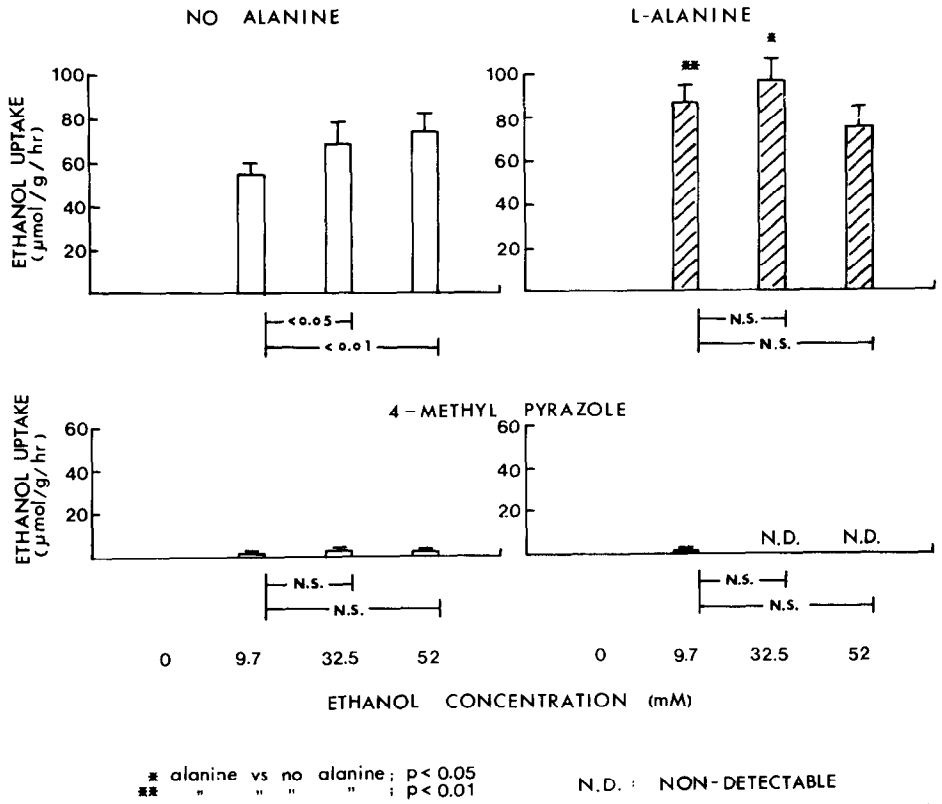

Fig. 1. Effects of ethanol concentration on ethanol metabolism in the presence and absence of alanine or 4-methylpyrazole. Values are expressed as means ± S.E.M. Statistical comparisons between different ethanol concentrations are by paired *t*-tests. Comparisons between alanine and no alanine are by unpaired *t*-tests. N = six animals in each set.

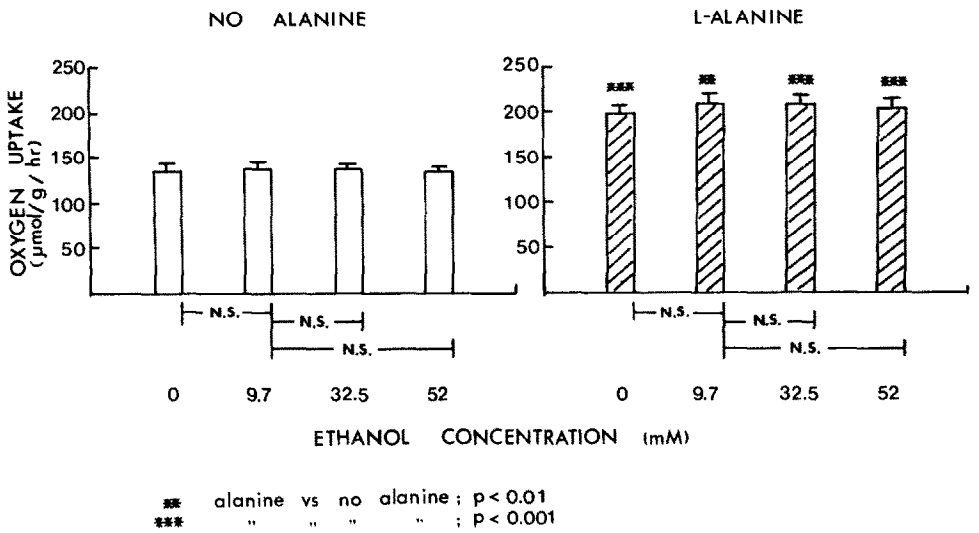

Fig. 2. Effects of ethanol concentration on oxygen uptake in the presence and absence of alanine. Other details are the same as for Fig. 1. N.S. = not significant.

trations, both in the presence and absence of alanine (Fig. 1).

As shown in Fig. 2, oxygen uptake was not affected by any of the concentrations of ethanol used, either in the presence or absence of alanine, although oxygen uptake was increased at all concentrations of ethanol in the presence of alanine.

Similarly, urea output was increased markedly in comparison to the no-alanine groups, but there was no significant concentration dependent effect on urea output either in the presence or absence of alanine (Fig. 3).

In both the presence and the absence of alanine, the lactate/pyruvate (L/P) ratio increased on addition of ethanol. The additon of 4-methylpyrazole blocked the increase in the L/P ratio at all concentrations of ethanol, in the presence as well as the absence of alanine (Fig. 4).

In the absence of alanine, the β-hydroxybutyrate/acetoacetate (B/A) ratio increased significantly on additon of the low concentrations of ethanol (9.7 and 32.5 mM). The ratio, however, declined as the concentration of ethanol was increased (9.7 mM vs 52 mM ethanol). This decline has been reported previously by others [5] in *in vivo* studies in the rat. In the presence of alanine, the B/A ratio also increased on addition of a low concentration of ethanol, but there was no decline in this ratio at the higher ethanol concentration. The changes in B/A ratios were blocked by 4-methyl pyrazole (Fig. 5).

DISCUSSION

In the absence of alanine, our studies show the concentration-dependent increase in ethanol metabolism observed previously in perfused liver, liver slices and isolated hepatocytes [8–15]. The increase

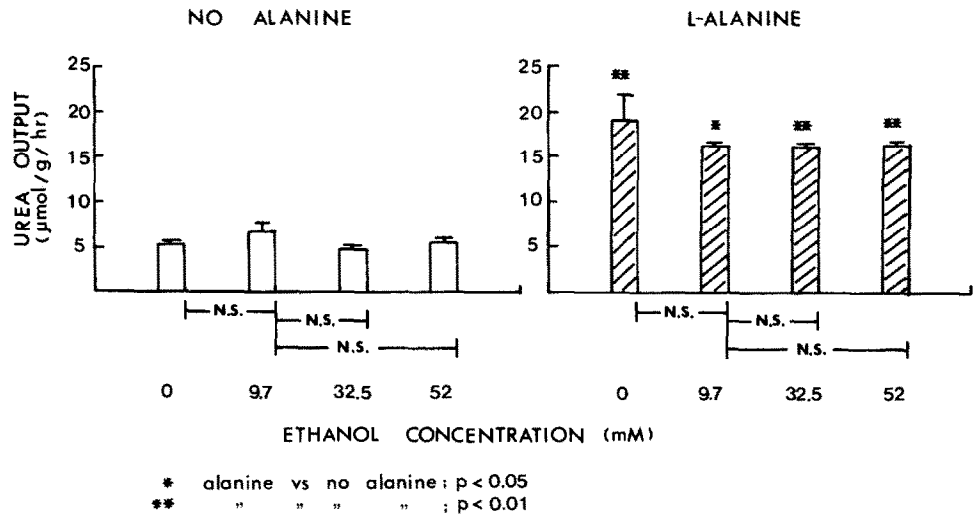

Fig. 3. Effects of ethanol concentration on urea output. Other details are the same as for Fig. 1. N.S. = not significant.

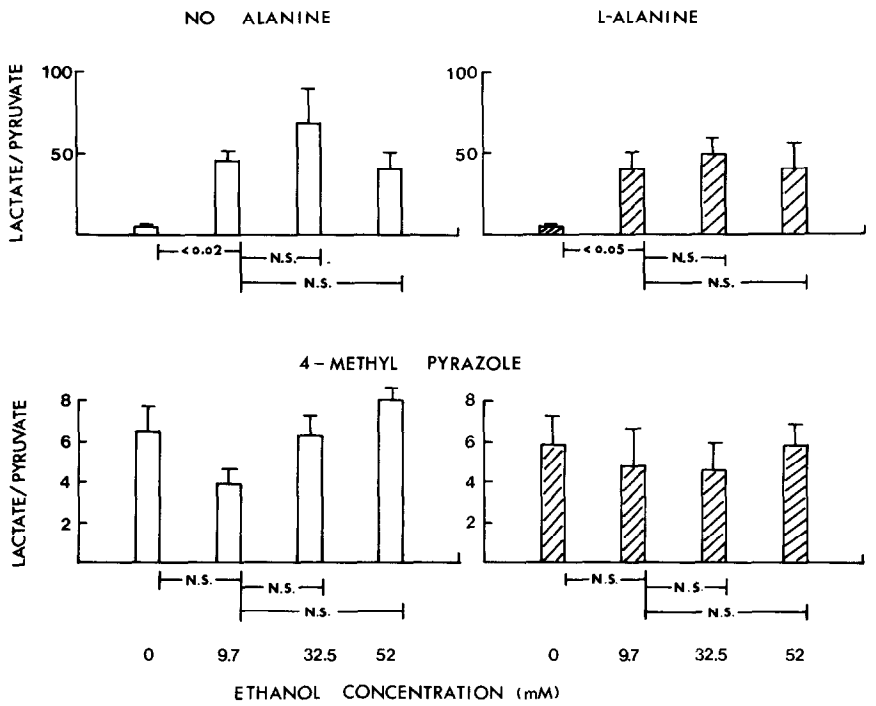

Fig. 4. Effects of ethanol concentration on lactate/pyruvate ratio in the presence and absence of alanine or 4-methylpyrazole. Note the difference in scale on the vertical axis in the 4-methylpyrazole sets. Other details are the same as for Fig. 1. N.S. = not significant.

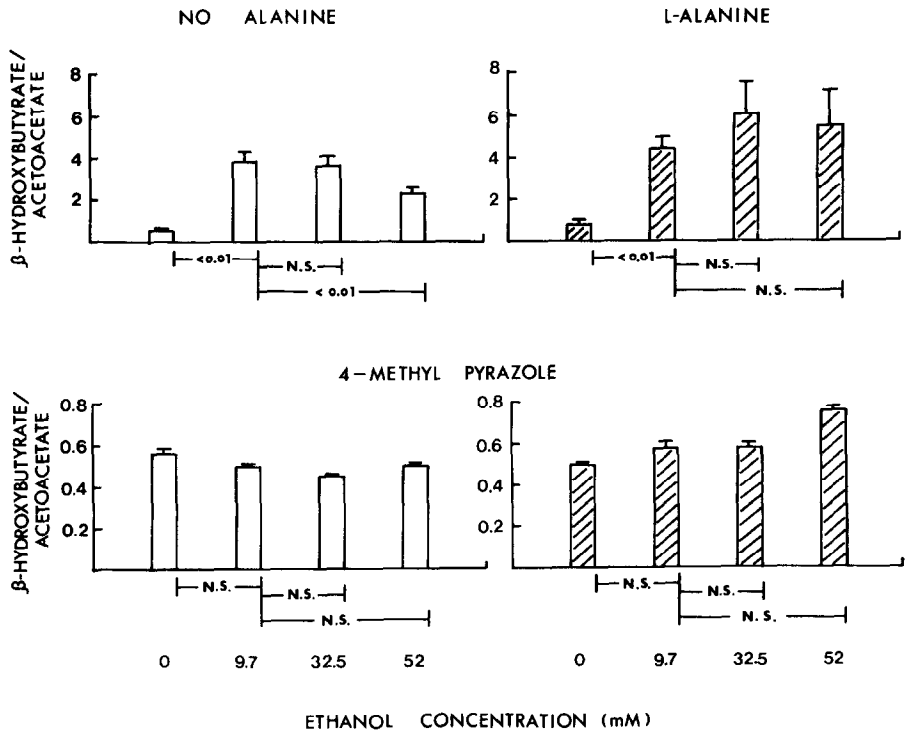

Fig. 5. Effects of ethanol concentration on the β-hydroxybuyrate/acetoacetate ratio in the presence and absence of alanine or 4-methylpyrazole. Note the difference in scale on the vertical axis in the 4-methylpyrazole sets. Other details are the same as for Fig. 1. N.S. = not significant.

in ethanol metabolism has been attributed by various investigators to the activity of a non-ADH pathway, such as a catalase system [10] or a microsomal ethanol oxidizing system [12–15].

One reason the increase has been attributed to non-ADH pathways is that pyrazole-insensitive ethanol disappearance increased with ethanol concentration. In the present study, the pyrazole-insensitive component was not detectable. Two factors could contribute to the discrepancy. One is the evaporative loss of ethanol. Ethanol loss is, of course, pyrazole-insensitive and increases proportionally to the ethanol concentration. If correction for loss is inadequate, there will be an apparent increase in ethanol metabolism. Conversely, an over-correction would diminish the estimate for the non-ADH pathways. A second factor is the completeness of inhibition of ADH by pyrazole and 4-methylpyrazole. Examination of published values suggests that, as ethanol concentration is increased, ADH-dependent metabolism may decrease [10], not change [11], or increase [15]. It is quite possible that the different effects observed by different investigators result from changes in the degree of ADH inhibition by the pyrazole analogues. Pyrazoles are competitive inhibitors of ethanol oxidation by rat liver ADH [21]. Raising the ethanol concentration could, therefore, reduce the inhibition by pyrazoles. The effect would depend on the concentration of pyrazole used, relative to the ethanol concentration. If too low a pyrazole concentration were used, the estimate for ADH-dependent metabolism would be reduced and the estimate for ADH-independent ethanol disappearance would be increased as the ethanol concentration was increased. In the present study, a high concentration of 4-methylpyrazole was used (4 mM) and no ethanol metabolism was detectable in its presence.

The present observation that 4-methylpyrazole completely blocks the concentration-dependent increase in ethanol metabolism indicates that the concentration-dependent effect depends on the ADH pathway. It could be argued, however, that 4-methylpyrazole has inhibited the MEOS and catalase activities as well as the ADH activity. However, since 4-methylpyrazole inhibited MEOS by only 34 per cent in studies with isolated microsomes [22], this explanation is not entirely adequate. There are a number of possible mechanisms by which an increase in ethanol oxidation could occur via the ADH pathway. A generalized increase in the reoxidation of reducing equivalents by the mitochondria is ruled out as there was no increase in oxygen uptake at the higher ethanol concentrations.

Another possibility is that the effect depends on the acetaldehyde loss that increases with ethanol concentration in the perfused liver [23]. Ethanol and acetaldehyde are in equilibrium in the ADH-mediated reaction; therefore, raising the ethanol concentration will also raise the acetaldehyde concentration in the cytoplasm of the liver cells [24, 25]. The cell membrane is permeable to acetaldehyde. Increasing amounts of acetaldehyde would be carried away in the perfusion fluid as the acetaldehyde concentration increases. Once in the perfusion fluid, the volatile acetaldehyde would be lost from the system. In the liver cells, the conversion of ethanol to acetaldehyde and acetaldehyde to acetate is limited by the availability of NAD. The NAD that would have been used for the oxidation of the lost acetaldehyde could than be used for additional ethanol oxidation. The result would be an increase in the disappearance of ethanol proportional to the ethanol concentration.

In the presence of alanine, there is increased O_2 consumption and, therefore, increased ethanol metabolism due to increased re-oxidation of reducing equivalents. The increased re-oxidation rate should decrease acetaldehyde levels and hence reduce the rate of loss at higher ethanol concentrations.

A portion of the acetaldehyde is converted to acetate inside the mitochondria, bypassing the requirement for transfer of reducing equivalents through the shuttle systems. Loss of acetaldehyde from the cells might have the effect of reducing the B/A ratio in the mitochondria. Such an effect might explain why the ratio declined in the absence of alanine but not in its presence. Such a decline in B/A ratio also occurs *in vivo* [5, 26].

Another possible mechanism for the concentration-dependent increase in ethanol metabolism is that ethanol at high concentrations could further inhibit the citric acid cycle, thus freeing NAD for ethanol oxidation. Ethanol might have this effect by inhibiting one of the enzymatic steps.

If such a mechanism indeed exists, how alanine might eliminate the concentration-dependent effect is not clear at present. More studies on the effects of alanine and other substrates on concentration-dependent ethanol metabolism are required.

The additon of only alanine as a substrate, although being an artificial situation, is probably closer to *in vivo* conditions than that of perfusion with just Ringer's solution. In studies in rat liver perfused with whole blood using 18% oxygen [27] and in rat liver perfused with 20% whole blood [28], no concentration-dependent ethanol metabolism was observed. These observations raise the possibility that the concentration-dependent effect *in vitro* is an artifact resulting from inadequate energy substrates.

Acknowledgements—We wish to express our gratitude to Drs. Y. Israel, H. Kalant and K. Lindros for their advice and interest, and for their valuable suggestions and criticism of this manuscript.

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