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ETHANOL METABOLISM IN THE ISOLATED, PERFUSED RAT LIVER*

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Abstract—The rate of ethanol oxidation in the continuously perfused, isolated rat liver was studied by measuring the rate of disappearance of the alcohol from the perfusate. Corrections were made for losses due to aspiration. The initial rate of ethanol oxidation was about what was expected from studies *in vivo*, but the rate decreased with time until at the end of 3 hr it was less than half that observed initially. The suspicion that the declining rate of ethanol oxidation may have been due to lowering NAD : NADH ratios was strengthened by the observation that the decline was impeded, or diminished rates of ethanol oxidation were restored, when pyruvate and fructose, substances known to promote the conversion of NADH to NAD, were added to the perfusion fluid. Studies were performed which showed that the initial rate of ethanol oxidation does not decline *in vivo*.

A RECENT publication from this laboratory[1] showed that the rate of ^{14}C-methanol oxidation in the isolated, perfused rat liver compared favorably with that estimated to occur in the liver *in situ*. On the other hand, the rate of 1-^{14}C-ethanol oxidation to $^{14}CO_2$ in the perfused liver was observed to be much slower than predicted from studies *in vivo*. This was explained as due to a rate limitation imposed by the relatively slow oxidation of acetate in the perfused liver. This aspect of the study agreed with the experiments of Lundquist *et al.*[2] who showed that human livers *in situ* were unable to oxidize all of the acetate resulting from a high intake of alcohol and that extra-hepatic tissues assumed much of this function. Thus, while the first step in the oxidation of ethanol, that involving alcohol dehydrogenase (ADH) and NAD, may be the rate limiting step in the complete oxidation of ethanol in the whole animal, it is not the rate limiting step in the isolated, perfused liver. The original objective of the current study was to determine if the first step in the oxidation of ethanol proceeds in the isolated, perfused liver at a rate commensurate with that predictable from studies performed *in vivo*.

Soon after these studies began, it became apparent that whereas the initial rate of ethanol oxidation, as measured by the rate of disappearance of the alcohol from the perfusion fluid, was about as rapid as would have been predicted from studies *in vivo*, it declined rapidly with time. This recalled studies by Marshall and Owens[3] and Forney *et al.*[4] where it was concluded that the mouse oxidized ethanol more rapidly during the first hour after its administration than at hourly intervals thereafter. In the

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current studies, experiments were devised to determine whether the decline in the rate of ethanol oxidation seen in the perfused liver also occurs in the intact rat.

MATERIALS AND METHODS

Chemicals. Sodium pyruvate (A grade), β-D-fructose and 1-^{14}C-ethanol were purchased from Cal-Biochem Inc., Sigma Chemical Co. and New England Nuclear Corp. respectively. The specific activity of 1-^{14}C-ethanol was determined as described previously.[5]

Measurement of the rate of ethanol oxidation in the isolated, perfused rat liver. Livers from male, Sprague–Dawley strain rats (350–450 g) were continuously perfused with 120 ml of heparinized rat blood (75% blood, 25% saline) employing an atmosphere of 95% O_2–5% CO_2, as described previously.[1] The perfusion apparatus was encased in a chamber held at 37°. An equilibration period of 30 min was allowed between the installation of the liver in the apparatus and the addition of ethanol or other materials, which were added directly to the perfusion fluid reservoir. Two min after the introduction of ethanol (20%, w/v), and at six 30-min intervals thereafter, 2·2 ml of blood was taken for duplicate analyses of ethanol. Ethanol lost by evaporation was trapped in magnesium perchlorate–calcium chloride tubes,[1] which were changed at the same times that blood samples were collected. All ethanol determinations were made using the method of Kozelka and Hine.[6] The amount of ethanol lost by evaporation was subtracted from the amount of ethanol disappearing from the blood during each corresponding 30-min interval, the difference representing the amount of ethanol disappearing from the blood as a result of oxidation. Water lost from the perfusion fluid by evaporation was replaced periodically. Livers were weighed at the end of the perfusion period and the rate of ethanol oxidation was expressed as mg of ethanol oxidized per g of liver per hr. Fructose and sodium pyruvate were added in 1-ml vol. of distilled water.

Measurement of the rate of oxidation of ethanol in vivo. Rats with the same specifications of those used in the perfusion studies were injected i.p. with a 20% (w/v) solution of 1-^{14}C-ethanol (2·7 g/kg) and placed in a metabolism chamber. Ethanol contained in the air leaving the chambers was collected in magnesium perchlorate–calcium chloride tubes and expired $^{14}CO_2$ was collected in bubbler tubes containing 3 N NaOH solution as described by Tephly *et al.*[5] in their studies of methanol oxidation. Rats were stunned at 0, 1, 2, 3, 5, 7 and 9 hr after injection and homogenized in a Waring commercial blender (Model CB-5) containing 150 ml each of cold (5–10°) 10% sodium tungstate solution, 1 N H_2SO_4 and water. To prevent overheating of the homogenate and to protect the bearing of the blending blade, homogenization was continued for no longer than 1 min at any one time and the container was placed in ice for 3 or 4 min between the several 1 min intervals required for thorough blending. The magnesium perchlorate–calcium chloride mixture containing expired ethanol was dissolved in about 1 l. of cold water and mixed with the homogenate. Urine that had accumulated during each experimental period was also added and the mixture was diluted to 4 l. Three 40-ml vol. of this mixture were analyzed for their ethanol contents by the method of Kozelka and Hine.[6] Recovery of ethanol from rats homogenized immediately after injection of the alcohol was 100 ± 4%. Untreated rats gave blanks of zero. While ethanol determinations on whole rats were not continued beyond the 9-hr interval after alcohol administration, $^{14}CO_2$ collections were made for as long as 15 hr.

RESULTS

Ethanol oxidation in the isolated, perfused rat liver. The rate of ethanol oxidation in the perfused liver, as measured by the disappearance of ethanol from the perfusion fluid after correcting for losses due to aspiration is seen to decline with time (Fig. 1). The data used in Fig. 1 have been replotted in Fig. 2 to show the rate of decline of ethanol metabolism per g of liver per hr at successive 30-min intervals. From an initial rate of about 10 mg/g of liver/hr during the first 30-min interval, the rate declined to about 4 mg/g of liver/hr during the interval between 150 and 180 min. In consideration of the possibility that the rather high initial concentration of ethanol (0·375%) might have

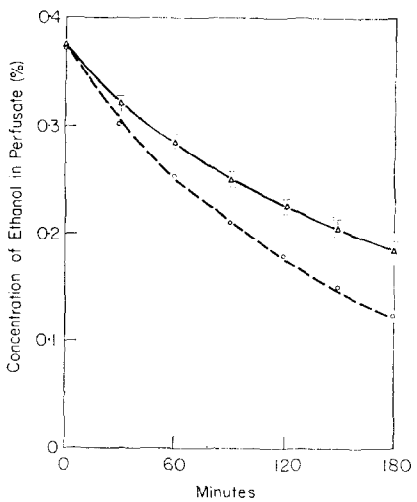

FIG. 1. Oxidation of ethanol in the isolated, perfused liver; disappearance of ethanol from the perfusion fluid. ○———○, Actual concentration of ethanol; △———△ concentration corrected for loss of ethanol due to aspiration. Each value represents the mean of three experiments. Vertical bars denote ± S. E.

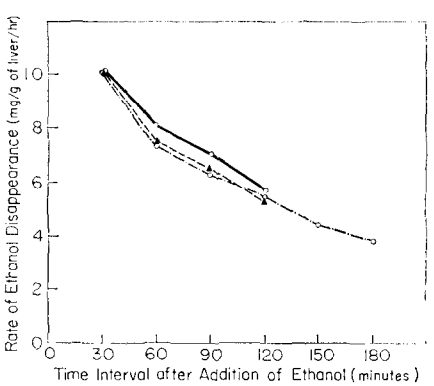

FIG. 2. Rate of ethanol oxidation in the isolated, perfused liver. ○— · — · —○, Ethanol added at zero time; initial concentration, 0·375%. ○———○, Ethanol added at 90 min; initial concentration, 0·375%. ▲— — —▲, Ethanol added at zero time; initial concentration, 0·20%. Each point represents the mean of three experiments. No statistically significant differences were found between the points at each time interval.

had some untoward effect on the overall viability of the liver, the rate of ethanol oxidation was studied at the lower initial concentration of 0·20%. It may be seen in Fig. 2 that both the rates of metabolism during the first 30-min period and the rates of decline throughout subsequent periods were not changed by lowering the initial ethanol concentration from 0·375 to 0·20%. The possibility was also considered that the decline in ethanol oxidation might reflect a gradual reduction of overall viability of the liver during perfusion. To test this possibility, the liver was perfused for an additional 90 min before ethanol was added. If the perfused liver had been "dying" during this period, as a result of influences not directly related to alcohol metabolism, ethanol oxidation should have taken place at a rate about equal to that seen after 90 min in the previous studies where ethanol was added at zero time, namely, at about 7 mg of ethanol oxidized/g of liver/hr. In Fig. 2 it may be seen that the rates of ethanol oxidation during the first 30-min interval were similar regardless of whether ethanol was added to the perfusion fluid at 0 or at 90 min. The declines in rates were also similar.

Effects of pyruvate and fructose on the declining rate of ethanol oxidation in the perfused liver. The declining rate of ethanol oxidation in the perfused liver might be explained on the basis of a progressive rate limitation imposed by falling NAD levels. This would occur if the NADH, which forms as a product of the interaction of ethanol, NAD and ADH, fails to regenerate at an adequate rate. Several investigators[7–10]

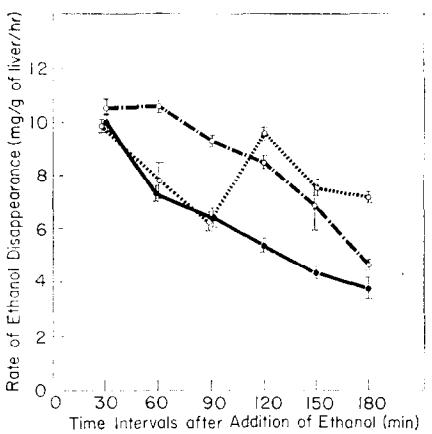

FIG. 3. Effect of sodium pyruvate on the rate of ethanol oxidation in the isolated, perfused rat liver. The initial concentration of ethanol was 0·375%. ●——●, Ethanol alone; ○— · — · —○, Ethanol plus sodium pyruvate, 220 mg added at zero time and 55 mg at 30 and 60 min. ○ · · · ○, Ethanol plus sodium pyruvate, 220 mg added at 90 min and 55 mg at 120 and 150 min. Each point represents the mean of three experiments. Vertical bars denote ± S. E.

have demonstrated that pyruvate increases ethanol oxidation and this effect is thought to result from the oxidation of NADH to NAD when pyruvate is reduced to lactate. Hepatic NAD:NADH ratios are lowered during ethanol metabolism in the intact rat and pyruvate raised both the NAD:NADH ratio and the rate of alcohol metabolism.[10–12] The effect of pyruvate on ethanol oxidation is shown in Fig. 3. When 220 mg of sodium pyruvate was added to the perfusion fluid 90 min after the addition of

ethanol, the rate of ethanol disappearance was raised from 6·2 to 9·5 mg/g of liver/hr, which closely approximates the rate of oxidation during the initial 30-min period. Addition of 55 mg of sodium pyruvate at 120 and 150 min did not maintain the restored rate of oxidation. When pyruvate was added at zero time, the initial rate of ethanol oxidation was maintained for the succeeding 30 min, after which time, the rate declined despite the addition of 55 mg of sodium pyruvate at 30 and 60 min.

Fructose accelerates ethanol oxidation,[13,14] probably through the restoration of NAD that results when ADH and NADH react with the glyceraldehyde formed from fructose-1-phosphate.[15,16] The dismutation reaction involving the reduction of fructose to sorbitol is also thought to contribute to the fructose effect.[15,17] In Fig. 4 it may be

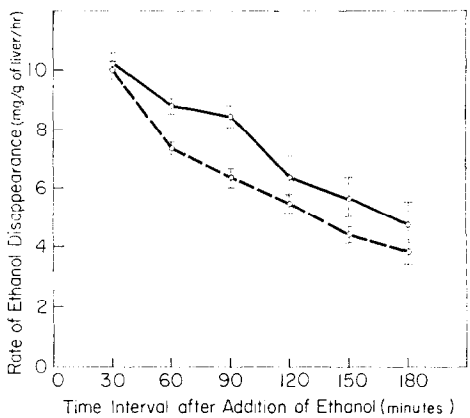

FIG. 4. Effect of fructose on the rate of ethanol oxidation in the isolated, perfused rat liver. The initial concentration of ethanol was 0·375%. O– – – O, Ethanol alone; O——O, Ethanol plus fructose, 240 mg added 15 min before zero time and 120 mg added at 60 min. Each point represents the mean of four experiments. Vertical bars denote ± S. E.

seen that the addition of 240 and 120 mg of fructose, 15 min before and 60 min after the introduction of ethanol, partially impeded the decline in the rate of oxidation for at least 90 min.

Ethanol oxidation in the intact rat. The kinetics of ethanol oxidation in the intact rat were studied by measuring ethanol disappearance from the whole animal and by following the oxidation of 1-^{14}C-ethanol to $^{14}CO_2$. A level of 1-^{14}C-ethanol administration (2·7 g/kg) was selected to provide blood concentrations of ethanol within the limits employed in the perfusion studies (0·2 and 0·375%). The results are presented in Fig. 5. Between 0–7 hr after administration ethanol is seen to disappear from the whole animal at the linear rate of about 325 mg/kg/hr. Thus, there is no decline in the initial rate of ethanol oxidation *in vivo* comparable to that seen in the perfused liver. Initially, the rate of $^{14}CO_2$ formation lags behind the rate of ethanol disappearance but between 3–7 hr the rates are essentially equal. The lag period may conceivably represent the time required for the acetate produced in the liver to reach concentrations in the extra-hepatic tissues which are favorable for its rapid oxidation to CO_2. At the end of 9 hr 94·4% of the administered 1-^{14}C-ethanol had been oxidized, as measured by disappearance, and 90·6% of the total had appeared as $^{14}CO_2$. At the end of 15 hr

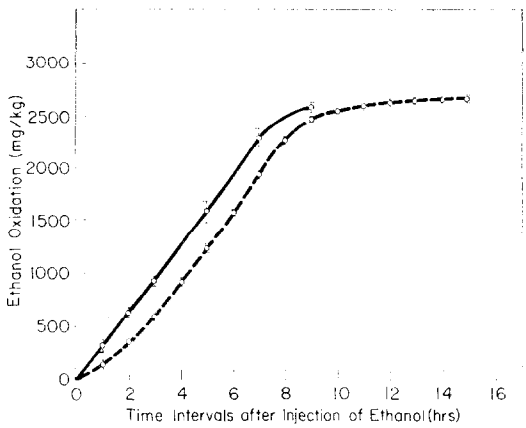

FIG. 5. Rate of ethanol oxidation in the intact rat after the injection of 1-^{14}C-ethanol (2·7 g/kg, i.p.) as measured by disappearance of ethanol (○———○) and by the collection of $^{14}CO_2$ (○– – –○). Each point represents the mean of three experiments. Vertical bars denote ± S. E.

97·3% of the original ethanol had been converted to $^{14}CO_2$. This shows that very little ethanol is lost by pulmonary and renal excretion, which is in disagreement with Lundquist *et al.*[18] who estimated that when the concentration of ethanol in the blood of rats is 0·2%, between $\frac{1}{3}$ and $\frac{1}{2}$ of the total elimination of ethanol is due to losses through the lungs. To provide more direct proof that the lungs and kidneys contributed little to the disappearance of ethanol, five rats with specifications similar to those used in the previous studies were injected i.p. with ethanol (2·7 g/kg) and placed in metabolism chambers. Respired ethanol was collected using magnesium perchlorate–calcium chloride tubes as described previously.[5] Urine was collected simultaneously. During the first 15 hr after administration of the ethanol 3·34 ± 0·8% and 0·64 ± 0·4% of the total dose were excreted via the pulmonary and renal routes respectively.

DISCUSSION

The livers weighed about 4 per cent of the weights of the rats used in this study. If the assumption is made that the first step of ethanol oxidation takes place almost entirely in the liver, a fair assumption, then the liver *in situ* metabolized ethanol at the rate of 325 mg/40 g of liver/hr or 8 mg/g of liver/hr. The isolated, perfused liver oxidized ethanol at the rate of about 10 mg/g of liver/hr during the first 30-min interval. Because the rate of oxidation declines very rapidly during the early period of perfusion, the initial rate of oxidation must have been considerably higher than 10 mg/g of liver/hr. Thus, during the early few minutes of perfusion, the liver is capable of oxidizing ethanol at a more rapid rate than the liver *in situ*. Recently, Gordon[19] published a study of the oxidation of ethanol by the perfused liver. It can be estimated from her data that ethanol oxidation during the first 30 min proceeded at the rate of about 20 mg/g of liver/hr. However, as the perfusion was continued, the rates she observed compared quite favorably with those seen in the current study. For example, during the interval between 60 and 90 min a rate of ethanol oxidation of about 7 mg/g of liver/hr was seen, whereas the rate given by Gordon[19] for the same time interval was about 6 mg/g of liver/hr.

Gordon[19] observed the rate of ethanol oxidation to decline exponentially with time. The rate of exponential decline was shown to be biphasic, with the semi-logarithmic plot showing one slope until a concentration of 0·04% of ethanol was reached in the perfusate and a quite different slope thereafter. It was concluded that the declining rate of ethanol oxidation is dependent on the ethanol concentration and that a change in the oxidative mechanism occurs at a concentration of about 0·04%. The current studies offer a different explanation for the declining rate of ethanol oxidation seen in the perfused liver. The first phase of the declining rate of oxidation is most probably due to a lowering of the NAD : NADH ratio and does not depend on the ethanol concentration. This conclusion is supported by the similarity in the rates of oxidation during the first 30-min interval and the rates of decline in subsequent intervals regardless of whether the initial concentration of ethanol was 0·375 or 0·20% (Fig. 2). The second phase of the declining rate of oxidation reported by Gordon[19] occurred at about 0·04%, which is about the concentration where first order kinetics should go into effect for the ADH-NAD system.

In a second series of experiments which employed an atmosphere containing 18 rather than 95% of oxygen, Gordon[19] found a marked reduction in the rate of ethanol oxidation. Furthermore, there was no apparent decline in the rate of oxidation with time. This can now be interpreted to mean, that at this greatly reduced rate of ethanol oxidation, the rate limiting step is not at the NAD level of oxidation, but somewhere along the oxidative chain leading to molecular oxygen. The statement is made that, when an 18% oxygen atmosphere is employed, the observed rate of ethanol oxidation of 36 mg/100 ml of perfusate/10 g of liver/hr, which can be estimated to be about 3·6 mg/g of liver/hr, is "within the range published for the intact rat". This statement is in error. A rate of ethanol oxidation of 3·6 mg/g of liver/hr is equivalent to 144 mg of ethanol oxidized/kg of intact rat/hr (i.e. 40 g of liver/kg rat $\times$ 3·6 = 144 mg/kg rat/hr), which is less than half the rate observed in the current study employing whole rats. The single publication[20] referred to by Gordon which actually specifies rates of ethanol oxidation in the intact rat, gives values ranging between 329–535 mg of ethanol oxidized kg of rat/hr. Thus, when the oxygen content of the breathed air (20%) and the atmosphere used in the perfusion apparatus (18%) were about equal, the liver of the intact rat oxidized ethanol at a rate more than twice that observed in the isolated, perfused liver. This suggests that, at atmospheric oxygen tension, the perfused liver is not oxygenated as efficiently as the liver *in situ*, conceivably because the perfusate is not oxygenated as efficiently as the blood *in vivo*.

Räihä and Oura[12] concluded from their studies with intact rats that during ethanol oxidation the pyridine nucleotide system is maintained at a new steady state at which the level of NADH is increased as compared to the level during normal respiration. The lowered NAD:NADH ratio, which reflects the new steady state of the NADH reoxidizing mechanisms, was not reached progressively, but was attained within 30 min (the shortest interval employed) after the administration of ethanol, and remained constant thereafter during the 4-hr study. This is in accord with the observation that the disappearance of ethanol by oxidation in the whole rat is linear when ethanol concentrations are high enough to saturate the ADH–NAD system (greater than 0·04 % of ethanol). If the declining rate of ethanol oxidation in the perfused liver results from a progressive fall in the NAD:NADH ratio, as is suggested by the responses observed with pyruvate and fructose, then the NADH reoxidizing mechanisms are

not being maintained at a steady state as they are in the intact animal. Why they are not raises speculation as to the role that extra-hepatic tissues may play in maintaining hepatic NAD:NADH ratios in intact animals when ethanol and other substrates are oxidized.

While the data *in vivo* (Fig. 5) do not indicate an accelerated rate of ethanol oxidation during the early period after administration, as is claimed by Marshall and Owens[3] and by Forney *et al.*[4] to occur in the mouse, it would be difficult to show such an effect unless it was of much greater magnitude than has been suggested. When the rate of metabolism is measured by determining the rate of disappearance of the substrate, small errors are greatly magnified during the early periods of the study when very little substrate has disappeared with respect to the initial concentration. Thus in the study by Forney *et al.*[4] during the first 30 min after the administration of 4·0 g of ethanol, the interval for which the accelerated rate of oxidation is claimed, only 11% of the original dose of ethanol was oxidized. An analytical error of 2 per cent at this stage of the experiment would result in about a 20 per cent error in the estimated rate of oxidation. At 3 hr after the injection of ethanol, about 50% of the ethanol had disappeared; a 2 per cent analytical error at this time would only result in a 4 per cent error in the estimated rate of oxidation. Thus the statistical analysis employed by these investigators, which compared the rate of disappearance of ethanol during the first 30 min with rates determined at subsequent time intervals, is not valid. Kinard[21] reinvestigated the question and concluded that there is no statistical difference in the rates of ethanol oxidation in the mouse during ½-, 1- and 4-hr intervals after administration.

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