Biochemical Pharmacology, Vol. 25, pp. 17–20. Pergamon Press, 1976. Printed in Great Britain.

SUBCELLULAR SITE OF ACETALDEHYDE OXIDATION IN RAT LIVER*

ROGER J. M. CORRALL, PAMELA HAVRE, JOSEPH MARGOLIS, MING KONG and BERNARD R. LANDAU
Department of Medicine, Case Western Reserve University, School of Medicine, Cleveland, Ohio 44106, U.S.A.

(*Received* 27 *December* 1974; *accepted* 11 *April* 1975)

Abstract—Slices of rat liver were incubated with (*R*)ethanol-1-^{3}H and (*S*)ethanol-1-^{3}H at a concentration of 1 mg/ml. During the course of the incubation, the ethanol in the flask containing the (*S*) isomer, but not the (*R*) isomer, was enriched with ^{3}H. For the same quantity of ^{3}H metabolized from the (*R*)ethanol-1-^{3}H as from the (*S*)ethanol-1-^{3}H, much less ^{3}H from the (*S*) than from the (*R*) isomer was incorporated into the lactate formed during the incubation. This indicates that the (*R*) hydrogen has a much greater access than the (*S*) hydrogen to the pool of NADH in the cytosol utilized in the reduction of pyruvate to lactate. It is concluded that the formation of NADH from acetaldehyde occurs under these conditions, primarily in a compartment other than the cytosol. It is presumed that this compartment is mitochondrial.

Until recently, acetaldehyde oxidation by the hepatocyte was generally believed to occur in the cytosol so that two equivalents of NADH were formed in the cytosol per mole of ethanol utilized. Reducing equivalents formed were assumed to be rapidly transported from the cytosol to the mitochondria via the malate–aspartate cycle to account for the rapid reduction of pyridine nucleotides occurring in the mitochondria of liver oxidizing ethanol [1]. More recently, the major portion of aldehyde dehydrogenase activity in rat liver has been localized to its mitochondria [2–4]. Most recently, Parrilla *et al.* [5] showed that inhibition of the extra mitochondrial reactions associated with the malate–aspartate cycle did not alter the reduction of mitochondrial pyridine nucleotides occurring in rat hepatocytes incubated with low concentrations of acetaldehyde but did to some extent with higher concentrations of acetaldehyde. They [5, 6] concluded that acetaldehyde at concentrations below 0·2 to 0·4 mM was oxidized predominantly in the mitochondria.

The present study was intended to localize the site of formation of NADH during acetaldehyde oxidation in the intact liver cell without recourse to inhibitors. The approach depends upon the stereospecificity of hydrogen removal in the oxidation of ethanol to acetaldehyde [7, 8]. The oxidation of 1 mole of (*R*)ethanol-1-^{3}H to acetaldehyde catalyzed by alcohol dehydrogenase will yield one equivalent of ^{3}H-labeled NADH, while the oxidation of (*S*)ethanol-1-^{3}H will yield one equivalent of unlabeled NADH. One equivalent of unlabeled NADH will be produced on oxidation of the unlabeled acetaldehyde formed from the (*R*)ethanol-1-^{3}H and one equivalent of ^{3}H-labeled NADH produced from the labeled acetaldehyde formed from (*S*)ethanol-1-^{3}H. Thus, for the same quantities of the (*R*) and (*S*) ethanols oxidized to acetate, the same quantities of labeled NADH will be formed. The ^{3}H should be on the same side of the pyridine ring of the nucleotide, whether from the (*R*) or (*S*) ethanol, since alcohol dehydrogenase and acetaldehyde dehydrogenase are A-type enzymes [9].

Therefore, if acetaldehyde oxidation, like ethanol oxidation, occurs in the cytosol, the cytosolic pool of NADH should be identically labeled with ^{3}H. The specific activity of the NADH pool formed from (*S*)ethanol-1-^{3}H relative to that from (*R*)ethanol-1-^{3}H should then reflect the extent of formation of NADH from acetaldehyde in the cytosol. These relative specific activities can be determined from the relative specific activities of the ^{3}H-labeled lactate formed during the metabolism of the (*R*) as compared to the (*S*)ethanol-1-^{3}H, since lactic dehydrogenase is localized to the cytosol and is also an A-type enzyme.

EXPERIMENTAL PROCEDURE

Materials. (*R*)ethanol-1-^{3}H, (*S*)ethanol-1-^{3}H and acetaldehyde-1-^{3}H were prepared and purified as previously described. Ethanol-1-^{14}C was purchased from New England Nuclear Corp., Boston, Mass., and Amersham/Searle Corp., Arlington Park, Ill., and also purified as previously described [8].

Animals. Sprague–Dawley white female rats weighing 260–300 g were fed *ad lib.* until the time of killing by decapitation.

Incubation. Five experiments were performed. In each experiment, six flasks with content were incubated. Each of the 500-ml Erlenmeyer flasks contained 30 ml medium. The medium [10] contained in m-moles/liter: K^+, 110; Mg^{2+}, 20; Ca^{2+}, 10; HCO_3^-, 40; Cl^-, 130; and glucose and ethanol, each at a concentration of 1 mg/ml. Ethanol-1-^{14}C (0·2 to 1·5 μCi) was added to all the flasks, (*R*)ethanol-1-^{3}H (1–5 μCi) to three of the flasks, and (*S*)ethanol-1-^{3}H (1–5 μCi) to the remaining three flasks. Flasks containing the (*R*) and (*S*) ethanols were paired. One pair, serving as controls, was incubated without addition of liver slices. Liver slices from two rats were randomly distributed into the second pair of flasks and liver slices from two other rats were distributed into

* This work was supported by Grant AM-14507 from the U.S. Public Health Service, and grants from the Distilled Spirits Council of the United States, Inc., and the National Science Foundation.

the third pair. Between 3·6 and 4·4 g of slices was added to each flask. Paired flasks differed in weight of slices by no more than 0·1 g. The contents of the flasks were gassed with 95% O_2–5% CO_2 for 10 min and then stoppered and incubated at 37° with shaking for 90 min. To terminate incubation, 3 ml of 2 N H_2SO_4 was injected into each flask through a rubber inlet in the stopper. CO_2 evolved was collected in a vial which was suspended from each stopper and into which 3 ml of 1 N CO_2-free NaOH was introduced by injection through the inlet. In three of the experiments, 32 mg acetaldehyde in aqueous solution was injected through the inlet into each of the flasks after addition of the sulfuric acid.

Analyses. The sealed flasks were stored 12 hr to complete absorption of the $^{14}CO_2$ into the sodium hydroxide. The acidified medium with slices from each flask was then cooled in ice and homogenized. The homogenate in a closed bottle was centrifuged at 4°. The supernatant was neutralized, 1 ml of 0·3 N $Ba(OH)_2$ and 1 ml of 5% $Zn(SO)_4$ were added and the mixture was recentrifuged at 4°. The concentrations of ethanol in an aliquot of the supernatant and the initial medium were determined enzymatically [11]. From these determinations, the volume of medium incubated, and the volumes of the supernatant (corrected to the volume of the homogenate), the quantity of ethanol taken up during the incubation was determined. There was no disappearance of ethanol in flasks without added slices. A 10-ml aliquot of each supernatant was made alkaline with NaOH and distilled. The first ml of distillate was used to determine the amount of ^3H that remained in ethanol at the completion of incubation, the next 5 ml of distillate was discarded, and the next ml (the seventh ml) was used to determine incorporation of ^3H into water.

To the first ml of distillate and to an aliquot of the initial medium, carrier unlabeled ethanol was added and the *p*-nitrobenzoate derivative of ethanol was prepared from these aqueous solutions [12]. In the first three experiments, ethanol was then regenerated from each *p*-nitrobenzoate by refluxing it in 1 N NaOH and then distilling the resulting solution. An aliquot of the first portion of each of these distillates was added to scintillation fluid (Aquasol purchased from the New England Nuclear Corp.) and assayed for ^3H and ^{14}C. Internal standards were used in these and in all other assays of radioactivity. Ethanols assayed after formation of the *p*-nitrobenzoate from the distillate of the supernatants obtained in the incubations without slices had $^3H/^{14}C$ ratios identical to the ratios in the ethanols initially present in the incubation medium, providing evidence for the adequacy of the procedure.

The ratio of ^3H to ^{14}C in dis./min in the ethanol of each flask at the completion of incubation was calculated as follows. Aliquots of the initial solution were assayed for ^3H and ^{14}C and from these data the total ^3H and ^{14}C in dis./min added as ethanol was calculated. The ^3H present in the ethanol at the completion of incubation was calculated from the $^3H/^{14}C$ ratio in the ethanol at the completion of incubation, the ^{14}C in the ethanol at the beginning of incubation, and the fraction of ethanol that was not utilized as determined from the uptake of ethanol. For this calculation it is assumed that there is no isotope discrimination between ^{14}C and ^{12}C so that the percentage uptake of ethanol and of the ^{14}C of the ethanol-1-^{14}C is the same. In the last two experiments, the *p*-nitrobenzoates were oxidized to $^{14}CO_2$ and ^3H_2O in an oxidizer (model 306, Packard Instrument Co., Downers Grove, Ill.). Uptake of ^{14}C and ^3H could then be calculated directly from the aliquot taken, the quantity of nitrobenzoate oxidized, and its activity. The uptake of ^{14}C of ethanol-1-^{14}C was in good agreement with the uptake of ethanol determined enzymatically [11].

An aliquot of the seventh ml of the distillate was assayed for ^3H and ^{14}C content. All ^{14}C in this distillate was assumed to be in ethanol, and all ^3H other than that in ethanol was assumed to be in water. The ^3H in ethanol was estimated from the $^3H/^{14}C$ ratio in ethanol in the distillate, as just detailed, and the ^{14}C in the distillate. In the seventh ml of distillate, about 25 per cent of the total ^3H was thus found to be due to ethanol with the remainder ascribed to ^3H_2O. Negligible ^3H was found in ^3H_2O in the incubations without slices. When ^3H_2O was distilled in a control experiment, the specific activity of the initial and final distillate was the same. The total incorporation of ^3H into water has, therefore, been calculated from the ^3H in the aliquot of the distillate and the total volume of the supernatant.

To another aliquot of each supernatant, carrier lactate was added. The aliquot was acidified with H_2SO_4 and the lactate extracted with ether. The lactate in the ether was isolated as its sodium salt and purified on a Celite column [13]. Lactate from the column was converted to its phenacyl derivative [14], which was assayed for ^3H and ^{14}C. The incorporation of ^3H and ^{14}C into lactate was calculated from this ratio, the weight of the phenacyl lactate assayed, the quantity of lactate added as carrier, and the volume of the aliquot of the supernatant. Again in the last two experiments, the phenacyl lactates were oxidized and the $^{14}CO_2$ and ^3H_2O counted.

In the three experiments in which carrier acetaldehyde was added, an aliquot of the supernatant from each flask was distilled *in vacuo* at room temperature into dimedone reagent [8] contained in a glass-jointed tube immersed in ice water. After the collection of a few ml of distillate, the tube was stoppered and warmed to complete the formation of acetaldimedone, which was collected and assayed for ^{14}C and ^3H. The quantities of ^3H and ^{14}C in acetaldehyde in the incubation medium plus slices at the completion of incubation were calculated from the 32 mg acetaldehyde added as carrier and the weight of acetaldimedone assayed for radioactivity. Negligible ^3H and ^{14}C radioactivity was in the acetaldimedone isolated from the incubations without slices, except in one experiment where there was a quantity of ^{14}C, despite purification of the ethanol-1-^{14}C used. The ethanol-1-^{14}C was a new batch which, when purchased, had several per cent of its activity precipitated with dimedone reagent. The quantity of acetaldehyde present in the slices and incubation medium of each flask at the completion of incubation for the two experiments where there was no contamination was calculated from the total ^{14}C in acetaldehyde at the completion of incubation and the molar specific acti-

vity of the acetaldehyde-1-^{14}C formed during the incubation, assumed to be the same as that of the ethanol-1-^{14}C incubated.

A control experiment showed that no significant quantity of acetaldehyde was lost during the course of the incubation. Acetaldehyde-1-^{3}H (5 mg) was injected into medium with slices immediately after acidification and without incubation. It was then incubated for 90 min, carrier acetaldehyde (32 mg) was added and the above procedure was followed. Twenty-six mg acetaldimedone was recovered. Its specific activity was such that the theoretical yield of acetaldimedone from 37 mg acetaldehyde (257 mg) would have contained 97 per cent of the dis./min added to the flask.

The $^{14}CO_2$ absorbed into the sodium hydroxide was precipitated as $Ba^{14}CO_3$. The $Ba^{14}CO_3$ was weighed and then treated with H_2SO_4 and the $^{14}CO_2$ evolved collected in ethylenediamine in methylcellusolve [15] and assayed for ^{14}C. Incorporation into $^{14}CO_2$ was calculated from the specific activity of the $^{14}CO_2$ and the quantity of CO_2 estimated to be present in the medium and gas phase of each flask.

RESULTS

Results of the five experiments are presented in Table 1. Uptake of ethanol, measured enzymatically, was about 50 per cent of the added ethanol with very similar uptakes in the paired incubations. The per cent uptake of ^{3}H from (*R*)ethanol-1-^{3}H was similar to the per cent uptake of ethanol determined enzymatically, i.e. the $^{3}H/^{14}C$ ratio in the ethanol remaining at the completion of incubation was similar to, although somewhat higher than, the ratio in the ethanol at the beginning of the incubation. In contrast, the uptake of ^{3}H from (*S*)ethanol-1-^{3}H was only about half of the uptake of ethanol, i.e. enrichment of ^{3}H in ethanol occurred, so that the $^{3}H/^{14}C$ ratio in ethanol at the end of incubation was higher than that in ethanol at the beginning of incubation.

In support of a similar metabolism of ethanol-1-^{14}C in the flasks containing the (*S*) and (*R*) isomers are the similar yields of ^{14}C in $^{14}CO_2$. About four times as much of the uptake of ^{3}H from (*R*)ethanol-1-^{3}H as from (*S*)ethanol-1-^{3}H was recovered in lactate. However, of the uptakes of ^{3}H, a similar amount, about 60 per cent, was recovered in water from both isomers. The per cent of the ^{3}H uptake recovered in acetaldehyde was very small with the (*R*)ethanol-1-^{3}H. It was more with the (*S*)ethanol-1-^{3}H, but of the uptake of ^{3}H it was still a small percentage. In Table 2 are recorded the $^{3}H/^{14}C$ ratios in ethanol and acetaldehyde for the two experiments with the (*S*) isomer. The ratios in ethanol, as already noted, were higher at the completion than initiation of incubation. The ratios in acetaldehyde were the same as or greater than those in the ethanol remaining in the medium at the completion of incubation. Acetaldehyde in the reaction mixture at the completion of incubation in these experiments contained 0·1 to 0·4 per cent of the added ^{14}C; assuming that the specific activity of the acetaldehyde had the same molar specific activity as that of the ethanol-1-^{14}C, this calculates to a total acetaldehyde content of 30–120 μg.

Table 2. $^{3}H/^{14}C$ Ratio in ethanol at the beginning and completion and in acetaldehyde at the completion of incubation with (*S*)ethanol-1-^{3}H and ethanol-1-^{14}C

Expt.	Ethanol Initial	Ethanol Final	Acetaldehyde Final
1	2·53	3·27	3·28
	2·53	3·37	3·62
2	2·14	3·30	3·44
	2·14	2·80	2·81

DISCUSSION

Our major conclusion is that the (*S*) hydrogen of ethanol has much less access to the NADH pool used in pyruvate reduction than does the (*R*) hydrogen, when the ethanol is metabolized by rat liver slices at concentrations between 1·0 and about 0·5 mg/ml, the initial and final concentrations in the medium. This is concluded from the fact that for the same quantity of ^{3}H in ethanol-1-^{3}H utilized, much less ^{3}H from the (*S*) ^{3}H-labeled than from the (*R*) ^{3}H-labeled hydrogen is incorporated into lactate. Since lactate dehydrogenase is well documented to be in the cytosol, the specific activity of lactate must reflect the specific activity of this NADH pool.

Several explanations for the above observation are possible. Acetaldehyde could be oxidized by a process not involving NADH formation or by a dehydrogenase of the B-type. There are several aldehyde dehydrogenases in liver, including one with NADPH as a cofactor [3, 4, 16], and xanthine oxidase has, for example, been reported to catalyze the oxidation of acetaldehyde [17]. However, specificity, affinity and activity data would indicate that it is the NADH-dependent aldehyde dehydrogenases that are functionally important and the acetaldehyde dehydrogenase from bovine liver that has been examined has proven to be of the A-type [18].

The decreased incorporation of ^{3}H into lactate from the (*S*) compared to the (*R*) ethanol cannot be explained by an isotopic effect, since only a small amount of ^{3}H from the (*S*) ethanol that was utilized was recovered in acetaldehyde, while large, similar percentages of ^{3}H were recovered in water from the (*S*) and the (*R*) ethanol. Conceivably, separate NADH pools in the cytoplasm could exist, one having greater access to the hydrogen removed from ethanol than acetaldehyde, but there is a considerable amount of

Table 1. Metabolism by rat liver slice of (*R*)ethanol-1-^{3}H and (*S*)ethanol-1-^{3}H each in the presence of ethanol-1-^{14}C*

Ethanol isomer	% Ethanol uptake	% ^{3}H uptake	% ^{14}C uptake to CO_2	Lactate	% ^{3}H uptake to Water	Acetaldehyde
R	52·9 ± 2·2	44·2 ± 3·6	11·4 ± 2·0	11·7 ± 2·7	59·4 ± 4·5	0·07 ± 0·01
S	50·8 ± 2·3	27·4 ± 1·9	9·5 ± 1·6	2·7 ± 0·2	62·0 ± 3·3	1·6 ± 0·6

* Mean ± S. E.

data to support the presence of a single pool of NADH in the cytosol [19].

The most likely explanation is that the conversion of ethanol to acetaldehyde occurs in the cytosol, but the acetaldehyde oxidation occurs primarily in another compartment, presumably the mitochondrial compartment. The data indicate that under the conditions of our study at most one-quarter of the oxidation of acetaldehyde occurred in the cytosol.

In this discussion we have assumed ethanol oxidation to be catalyzed solely by alcohol dehydrogenase. There is evidence that catalase and a microsomal oxidizing enzyme system may contribute, at least to a degree, to the conversion of ethanol to acetaldehyde. In these oxidations the (*R*) hydrogen of ethanol is removed, but NADH is not formed [8, 20]. To the extent that these reactions occur rather than oxidation via alcohol dehydrogenase, the extent of the compartmentation determined with the (*R*) and (*S*) isomers would be underestimated.

As noted, the data of Parrilla *et al.* [5] and Lindros *et al.* [6] indicate that the oxidation of acetaldehyde occurs almost entirely in the mitochondrial compartment at aldehyde concentrations below 0·2 and 0·4 mM, concentrations normally encountered in ethanol metabolism. The 30 120 μg acetaldehyde estimated to be in the medium plus liver slices at the termination of incubation, even if present solely in the approximately 3 ml water in the slices, would give an acetaldehyde concentration of 0·2 to 0·8 mM.

The enrichment of ethanol with ^{3}H relative to ^{14}C during the incubation with the (*S*) isomer presumably reflects a primary isotopic effect during the dehydrogenation of acetaldehyde-1-^{3}H, since, as shown in Table 2, the acetaldehyde is enriched with ^{3}H relative to ^{14}C. Since the dehydrogenation of ethanol to form acetaldehyde, catalyzed by alcohol dehydrogenase, is readily reversible, resynthesis of ethanol from acetaldehyde would explain the enrichment of ^{3}H in the ethanol. We (P. Havre and B. Landau, unpublished observations) have performed experiments identical to those described in this study but with ethanol unlabeled and sorbitol-2-^{3}H added to the medium at a concentration of 0·1 mg/ml. Between 10 and 20 per cent of the ^{3}H taken up was recovered in ethanol, supporting the reversibility of the alcohol dehydrogenase-catalyzed dehydrogenation under the conditions employed.

Rognstad and Clark [21], using a theoretical approach similar to ours, compared the specific yield of ^{3}H in water and glucose formed by liver cells from (*R*) and (*S*)ethanol-1-^{3}H, with the specific yields from substrates oxidized by dehydrogenases with known cytoplasmic and mitochondrial locations. They also concluded that ethanol is oxidized predominantly in the mitochondria. Lactate was present in their incubation media at a concentration of 0·72 mg/ml (in one experiment pyruvate was substituted at a concentration of 1·32 mg/ml), and the (*R*) and (*S*) ethanols at the beginning of the 1-hr incubations were at concentrations between 0·001 and 0·01 mg/ml.

Acknowledgement– The authors gratefully acknowledge Dr. Williamson's providing us with a copy of his manuscript [5] prior to its publication.

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