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Studies on the Interaction between Ethanol and Serotonin Metabolism in Rat, Using Deuterated Ethanol and 4-Methylpyrazole

Margareta Some, Stefan Svensson,† Jan-Olov Höög† and Anders Helander*§*

Departments of *Clinical Neuroscience and †Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden

ABSTRACT. The metabolic interaction between ethanol and serotonin (5-hydroxytryptamine) via alcohol dehydrogenase (ADH; EC 1.1.1.1) was studied in tissue homogenates of Sprague–Dawley rats by following the transfer of deuterium from deuterated ethanol over endogenous NADH to 5-hydroxytryptophol (5HTOL). Homogenates of whole brain, lung, spleen, kidney, liver, stomach, jejunum, ileum, colon, and caecum were incubated in the presence of [$^2H_2$]ethanol and 5-hydroxyindole-3-acetaldehyde (5HIAL), and the [^{2}H]5HTOL formed was identified and quantified using gas chromatography–mass spectrometry. ADH activity was most abundant in liver, kidney, and within the gastrointestinal tract. The highest incorporation of deuterium was obtained in homogenates of kidney, lung, and colon, whereas in brain, which contains very low ADH activity, no incorporation could be demonstrated. Addition of extra NAD^+ (2.4 mM) increased the formation of [^{2}H]5HTOL 2.6-fold in liver homogenates, but only 1.2-fold in kidney homogenates. 4-Methylpyrazole, a potent inhibitor of class I ADH, inhibited the 5HIAL reduction in homogenates of lung, kidney, jejunum, ileum, and colon, and caused a marked drop in 5HTOL oxidation in all tissues except stomach and spleen. These results demonstrate that in the rat a metabolic interaction between ethanol and serotonin via the ADH pathway may take place in several tissues besides the liver, which is the main tissue for ethanol detoxification. BIOCHEM PHARMACOL **59**;4:385–391, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. alcohol dehydrogenase; 5-hydroxytryptamine; 5-hydroxytryptophol; ethanol; 5-hydroxyindole-3-acetic acid; rat

Ethanol and biogenic amines share some catabolic enzymes, and during ethanol oxidation the conversion of serotonin (5-hydroxytryptamine) shifts away from oxidation of the intermediate 5HIAL‖ producing 5HIAA toward the reductive pathway forming 5HTOL [1, 2]. This has been attributed to competitive inhibition of ALDH (EC 1.2.1.3) by ethanol-derived acetaldehyde, and an increased rate of reduction of 5HIAL by ADH (EC 1.1.1.1) as a result of the raised NADH/NAD^+ ratio [3, 4]. In addition, the ADH-catalysed re-oxidation of 5HTOL is inhibited by ethanol, and this may also contribute to the shift in serotonin metabolism [5]. In humans, the urinary 5HTOL/5HIAA ratio remains increased for several hours after blood and urine ethanol concentrations have declined to endogenous levels [6]. On the basis of this time delay, this ratio can serve as a sensitive biochemical marker of recent drinking [7, 8].

Concentrations of 5HTOL and its sulphate and glucuronide conjugates are normally only about 1–5% of the 5HIAA concentration in human brain [9] and cerebrospinal fluid [10], but less than 1% in urine [2]. In the rat, however, the baseline concentration of 5HTOL is considerably higher [11, 12]. Nevertheless, a shift in serotonin metabolism could also be demonstrated in rats administrated an oral dose of ethanol, although the increase in the urinary 5HTOL/5HIAA ratio was much less dramatic (~twofold) [12] compared with that found in man (≥100-fold) [6] after a corresponding ethanol dose. Moreover, in rat, significantly increased 5HTOL/5HIAA ratios were seen in liver, ileum, and spleen, indicating that the interaction between ethanol and serotonin metabolism may also take place outside the liver [12]. However, because of a low serotonin turnover in spleen, this change was possibly attributable to the situation in the circulating blood.

This study was undertaken to further elucidate the mechanisms behind, and the location of, the shift in serotonin metabolism observed during ethanol oxidation. The presence of an interaction via the ADH pathway can be studied by following the transfer of deuterium from deuterium-labelled ethanol over NADH to the end product [13], in this case 5HTOL, as described in Fig. 1. Rat tissue homogenates were incubated with [$^2H_2$]ethanol and

§ Corresponding author: Anders Helander, Ph.D., Alcohol Laboratory, L7:03, Karolinska Hospital, SE-171 76 Stockholm, Sweden. Tel. +46 8 51771530; FAX +46 8 51771532; E-mail: anders.helander@bekl.csso.sll.se

‖ *Abbreviations:* ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; 5HIAA, 5-hydroxyindole-3-acetic acid; 5HIAL, 5-hydroxyindole-3-acetaldehyde; 5HTOL, 5-hydroxytryptophol; and 4MP, 4-methylpyrazole.

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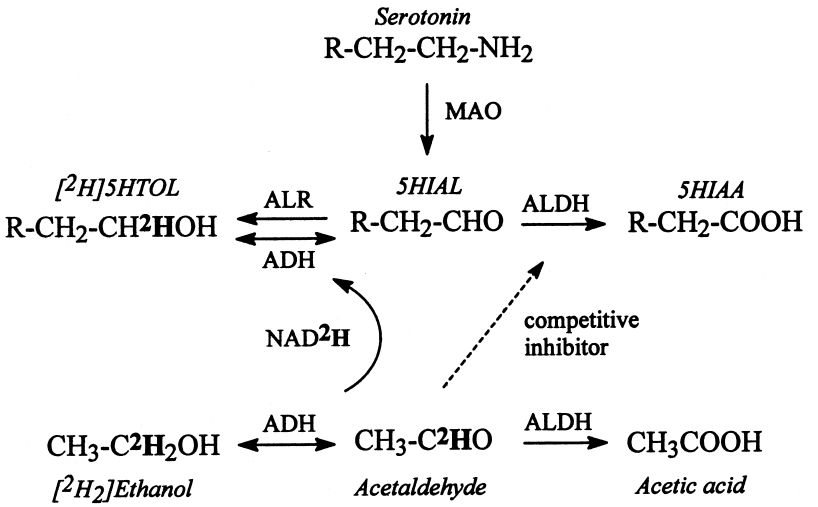

FIG. 1. The metabolic interaction between deuterium-labelled ethanol and serotonin (5HT). R, 5-hydroxyindole; MAO, monoamine oxidase; ALR, aldehyde reductase.

5HIAL, and the [^{2}H]5HTOL formed was quantified by GC–MS. Complementary experiments were carried out in the presence of 4MP, a potent competitive inhibitor of class I ADH.

MATERIALS AND METHODS

Chemicals

5HIAA, 5HTOL, 5-hydroxyindole, 4MP, NAD^+, NADH, and sulphatase (type H1, containing β-glucuronidase activity) were obtained from Sigma, diethyl ether and ethyl acetate from Merck, pentafluoropropionic anhydride from Supelco, and trifluoroethanol from Aldrich. [1,1-$^2H_2$]Ethanol (99.6% ^{2}H) was purchased from Alfred Hempel. The deuterated internal standards ([$^2H_2$]5HIAA and [$^2H_4$]5HTOL) were synthesised as previously described [14, 15]. The 5HIAL–bisulphite complex was prepared enzymatically by use of rat liver monoamine oxidase (MAO; EC 1.4.3.4) [16]. All other chemicals used were of analytical grade.

Assay Conditions

Female Sprague–Dawley rats (~150 g) receiving a standard laboratory diet and tap water *ad lib.* were used. Animals were killed by decapitation and whole brain, lung, spleen, kidney, liver, stomach, jejunum, ileum, colon, and caecum were removed and rapidly placed on ice. The tissue samples were minced and thoroughly washed in physiological saline and finally homogenised (10%, w/v) in 0.1 M potassium phosphate buffer (pH 7.4). Aliquots of 2.0 mL were incubated with 2.5 μM 5HIAL at 37° for 60 min (at that time most of the 5HIAL had been metabolised) in the presence of 20 mM [$^2H_2$]ethanol or 0.25 mM 4MP. The ethanol concentration chosen falls within the normal range observed after drinking, whereas the concentration of 5HIAL occurring *in vivo* may be even lower. Incubations with liver and kidney homogenates were also carried out with 2.4 mM NAD^+, and with 0.1 or 2.4 mM NADH. Control samples contained 5HIAL only, but were otherwise treated as above. The reactions were terminated by addition of 0.5 mL of 8 M perchloric acid. The samples were then spiked with internal standards and, after centrifugation at 3000 *g* for 10 min, the pH of the supernatant was adjusted to 5.5–6.5. The study was approved by the local ethical committee.

Determination of 5HTOL, [^{2}H]5HTOL, and 5HIAA

Concentrations of 5HTOL, [^{2}H]5HTOL, and 5HIAA were determined after enzymatic hydrolysis with sulphatase containing β-glucuronidase activity [12]. A Hewlett Packard 5972 GC–MS system was used for identification and quantification by selected ion monitoring at *m/z* 451 (5HTOL), *m/z* 452 ([^{2}H]5HTOL), *m/z* 454 ([$^2H_4$]5HTOL), *m/z* 438 (5HIAA), and *m/z* 440 ([$^2H_2$]5HIAA). Water solutions of 5HTOL and 5HIAA, prepared as described for the tissue samples, were used as standards. The calibration curve for 5HTOL was also used for quantification of [^{2}H]5HTOL. The position of isotopic labelling in the [^{2}H]5HTOL formed was assigned by the following observations. During fragmentation of the 5HTOL derivative, cleavage occurs at two different locations of the alcoholic side chain, resulting in the contribution of one deuterium to the fragment forming *m/z* 452, whereas the fragment of *m/z* 438 is non-deuterated. The *m/z* 452 fragment is formed by a β-elimination process which involves loss of one proton at the carbon next to the indole nucleus (C-2), whereas only C-2 and one proton remain in the *m/z* 438 fragment formed by β-cleavage [17]. Since deuterium is only observed in the fragmentation by β-elimination, it is positioned on C-1 as shown in Fig. 2. With non-deuterated 5HTOL, the contribution of *m/z* 452 to the sum of *m/z* 451 and *m/z* 452 was 25%, whereas it was 93% with enzymatically prepared deuterated 5HTOL. For the calculation of deuterium incorporation in 5HTOL with the tissue homogenates, these values were set to 0% and 100%, respectively.

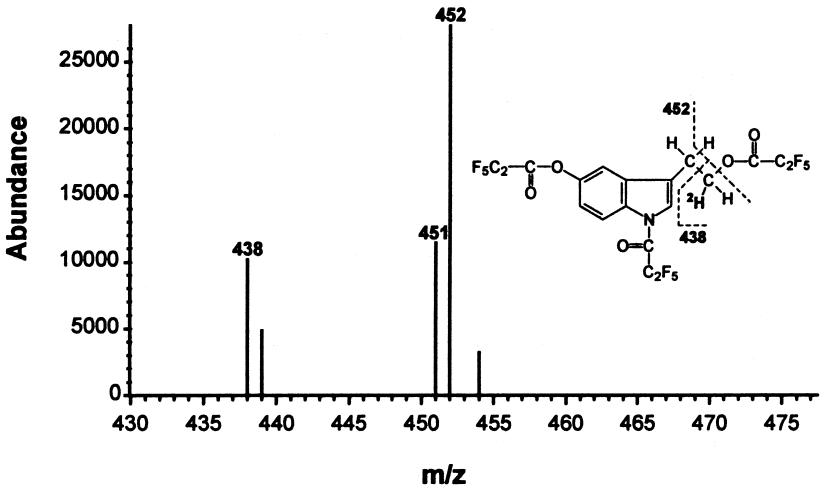

FIG. 2. Electron impact mass spectrum of derivatised [^{2}H]5HTOL. During fragmentation of the 5HTOL derivative, cleavage occurs at two different locations of the alcoholic side chain, resulting in the contribution of one deuterium to the fragment forming *m/z* 452, whereas the fragment of *m/z* 438 is non-deuterated.

The concentration of total 5HTOL was determined as the sum of 5HTOL and [^{2}H]5HTOL.

Cytosolic NAD$^+$-dependent Oxidation of 5HTOL

Tissue homogenates were centrifuged at 48,000 *g* for 30 min, and the supernatants were applied on PD-10 columns (Amersham Pharmacia Biotech) for desalting. The NAD$^+$-dependent 5HTOL-oxidising activity was monitored with a Hitachi 3000 spectrophotometer by following the production of NADH at 340 nm (ε = 6220 cm^{-1} M^{-1}). Enzyme activity was measured at 25° in 0.1 M glycine buffer (pH 10.0), with 2.4 mM NAD$^+$ and 250 μM 5HTOL, and with or without 1.0 mM 4MP present. Total protein concentration was determined with the Bio-Rad protein assay using BSA as standard. Specific activities are given as units/mg (μmol min^{-1} mg total protein^{-1}). Reduction of NAD$^+$ independent of 5HTOL was found to be negligible in these experiments (results not shown).

Statistics

Analysis of variance was performed by Kruskal–Wallis one-way ANOVA. When significance was reached within a group ($P < 0.05$), the Wilcoxon rank–sum test with exact P values was used for pairwise comparisons.

RESULTS

Incorporation of deuterium into 5HTOL, calculated as the ratio of [^{2}H]5HTOL to total 5HTOL, was observed after incubation of rat tissue homogenates with 5HIAL and [$^2H_2$]ethanol (Table 1). The highest incorporation was observed in kidney homogenates followed by lung and colon, whereas a very low formation of [^{2}H]5HTOL was demonstrated in spleen, and no incorporation could be detected in brain. When incubations with liver and kidney homogenates were carried out with 2.4 mM NAD$^+$, the relative amount of [^{2}H]5HTOL increased to 83.7 ± 17.6% and 80.9 ± 0.9% (mean ± SD, N = 3), respectively. In the presence of 0.1 or 2.4 mM NADH, the incorporation of deuterium was reduced to 6.7 ± 1.0% and 2.9 ± 0.4%, respectively, with liver homogenates, and to 21.8 ± 4.0% and 12.0 ± 0.2% with kidney homogenates.

In control homogenates, the highest formation of total 5HTOL was observed in caecum (21.7 ± 9.9 nmol/g) and colon (18.8 ± 7.8 nmol/g), whereas it was lowest in ileum (0.71 ± 0.29 nmol/g) (Fig. 3a). When incubations were performed in the presence of [$^2H_2$]ethanol, a significant increase in the total amount of 5HTOL formed was observed in kidney (~300%, $P = 0.0002$) and liver (~55%, $P = 0.0205$), whereas 4MP induced a significant decrease with homogenates of lung ($P = 0.0022$), kidney ($P = 0.0007$), jejunum ($P = 0.0022$), ileum ($P = 0.0303$), and colon ($P = 0.0013$). The control levels for 5HIAA ranged from 9.4–31.8 nmol/g, with the highest

TABLE 1. Incorporation of deuterium into 5HTOL in rat tissue homogenates incubated with [$^2H_2$]ethanol and 5HIAL

Tissue	N*	[^{2}H]5HTOL† (%; mean ± SD)
Kidney	8	68.0 ± 5.2
Lung	6	46.2 ± 2.7
Colon	6	43.2 ± 2.3
Caecum	8	40.9 ± 4.3
Stomach	5	35.2 ± 8.8
Liver	7	32.0 ± 8.3
Jejunum	6	27.8 ± 26.5
Ileum	5	15.7 ± 6.4
Spleen	8	4.8 ± 2.9
Brain	7	ND‡

*N = number of rats.
†Calculated as the ratio of [^{2}H]5HTOL to the sum of [^{2}H]5HTOL and 5HTOL.
‡Not detectable (detection limit = 1%).

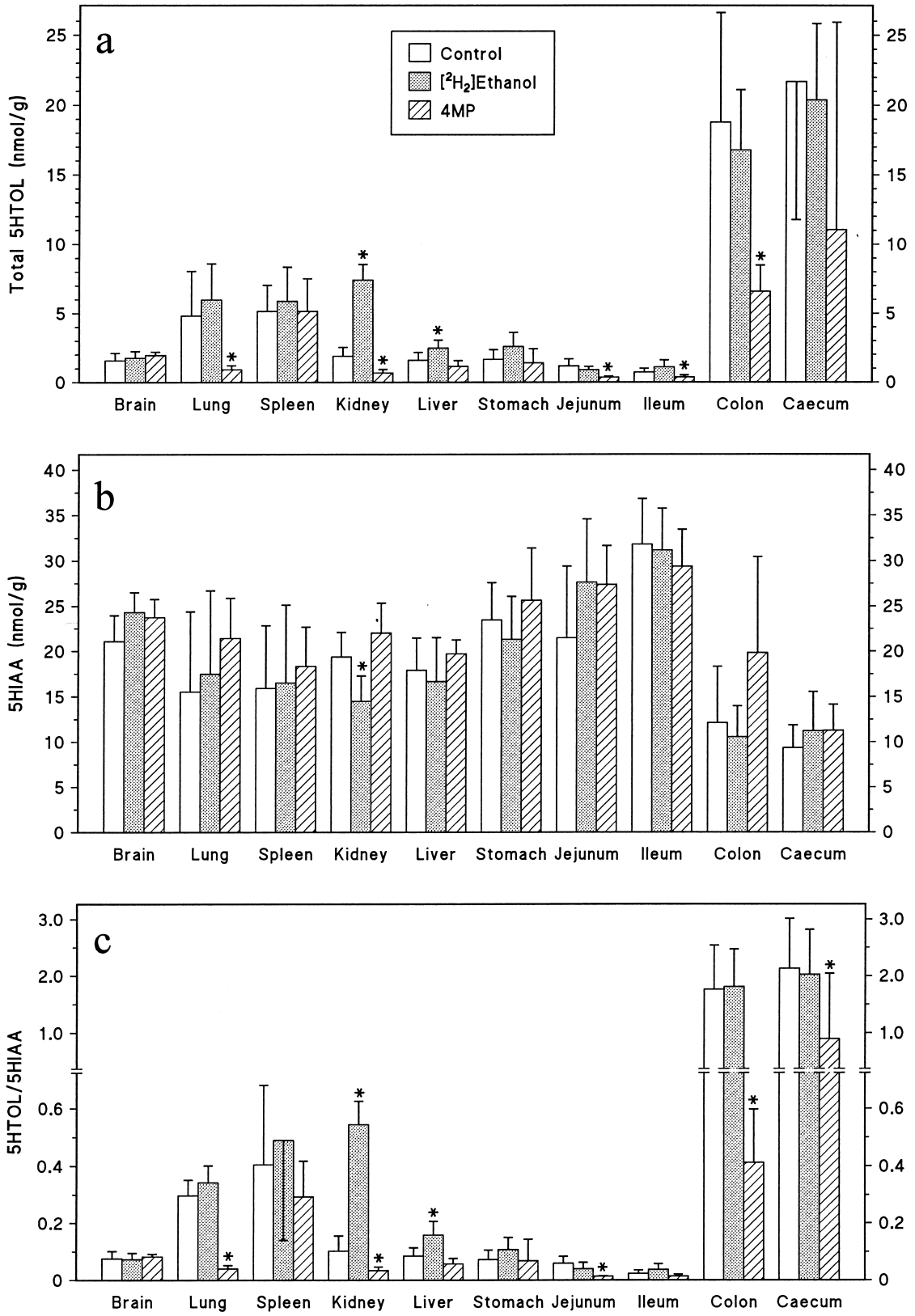

FIG. 3. Formation of 5HTOL and 5HIAA, and the 5HTOL/5HIAA ratio, after rat tissue homogenates were incubated with 5HIAL alone (control) and in the presence of deuterated ethanol ([$^2H_2$]ethanol) or the class I ADH inhibitor 4-methylpyrazole (4MP). The data represent a) total 5HTOL (sum of 5HTOL and [^{2}H]5HTOL), b) 5HIAA, and c) the ratio of total 5HTOL to 5HIAA. Results are mean values ± SD (N = 5–10). * $P < 0.05$ versus control (Wilcoxon rank–sum test).

TABLE 2. NAD^+-dependent 5HTOL-oxidising activity in supernatants of rat tissue homogenates

Tissue (N = 3)*	Specific activity (mU/mg; mean values)	Inhibition by 1 mM 4MP (%; mean values)
Liver	13.2	98
Colon	2.9	100
Kidney	1.8	100
Jejunum	1.6	100
Caecum	1.5	100
Stomach	0.5	20
Ileum	0.4	100
Lung	0.3	80
Spleen	0.2	ND†
Brain	ND‡	—

*N = number of rats.
†Not detectable (detection limit = 7%).
‡Not detectable (detection limit = 0.06 mU/mg).

formation observed in ileum and the lowest in caecum (Fig. 3b). [$^2H_2$]Ethanol induced a significant decrease (~25%, $P = 0.0028$) in the amount of 5HIAA formed in incubations with kidney homogenates. 4MP had no significant influence on the formation of 5HIAA. The molar ratio of total 5HTOL to 5HIAA is presented in Fig. 3c. With control homogenates, the highest ratio was demonstrated in caecum (2.1 ± 0.9) and colon (1.8 ± 0.8), whereas the lowest ratio was found in ileum (0.02 ± 0.01). Addition of [$^2H_2$]ethanol significantly increased the 5HTOL/5HIAA ratio in incubations with kidney (~433%, $P = 0.0002$) and liver (~88%, $P = 0.014$) homogenates compared with controls, whereas 4MP caused a significant decrease in this ratio in lung ($P = 0.0043$), kidney ($P = 0.0016$), jejunum ($P = 0.0022$), colon ($P = 0.0087$), and caecum ($P = 0.0293$). With homogenates of brain, the ratio was unchanged in the presence of either [$^2H_2$]ethanol or 4MP.

The NAD^+-dependent 5HTOL-oxidising activity in supernatants of rat tissue homogenates is given in Table 2. The highest activity was found in liver, whereas no activity could be demonstrated in brain. In incubations with liver, colon, kidney, jejunum, caecum, and ileum homogenates, 4MP caused a complete (98–100%) block of NADH formation. With homogenates of stomach, the inhibition was only 20% and no effect of 4MP was observed in spleen.

When incubations with liver and kidney homogenates were carried out in the presence of 2.4 mM NAD^+, the addition of [$^2H_2$]ethanol induced a marked increase in the 5HTOL/5HIAA ratio (~350% in liver and ~525% in kidney, N = 3), whereas 4MP decreased this ratio compared with controls (Fig. 4). When similar experiments were carried out with 0.1 or 2.4 mM NADH, the ratios became much higher (Fig. 4). With kidney homogenates, a further increase in 5HTOL/5HIAA was seen after addition of [$^2H_2$]ethanol, whereas 4MP reduced this ratio considerably. In liver, the increase in the 5HTOL/5HIAA ratio was higher with 2.4 mM NADH compared with 0.1 mM, and neither [$^2H_2$]ethanol nor 4MP had any apparent effect.

DISCUSSION

The mechanism behind, and the location of, the shift in serotonin metabolism observed during ethanol oxidation was studied by following the transfer of deuterium from [$^2H_2$]ethanol over NADH to 5HTOL via the ADH pathway. The reduction of 5HIAL to 5HTOL is efficiently catalysed by class I γγ ADH [18]. The 5HTOL-oxidising activity was most abundant in rat liver, whereas lower activities occurred in kidney (~14% of that in liver) and within the gastrointestinal tract (3–22% of that in liver). Within spleen, it was only about 1.5% of that in liver and no activity could be demonstrated in brain. This enzyme activity profile correlates well with the tissue distribution of class I ADH in rat [19]. 4MP, a competitive class I ADH inhibitor that inhibits ethanol elimination in rats [20], almost completely blocked the enzyme activity in all tissues except stomach and spleen, thereby further confirming that class I ADH also oxidises 5HTOL in these tissues. However, rat stomach contains ADHs of other classes, e.g. class IV, that are less sensitive to inhibition by 4MP [21]. Whether 5HIAL and 5HTOL are also substrates for class IV ADH has not yet been determined.

The highest incorporation of deuterium into 5HTOL was observed in kidney, lung, and within the gastrointestinal tract, thus confirming a metabolic interaction between ethanol and serotonin. The kidney and lung have a considerable capacity to synthesise serotonin from circulating tryptophan or 5-hydroxytryptophan, and also to metabolise serotonin further by oxidative deamination [22–26]. The enterochromaffin cells of the intestinal mucosa represent a main source of serotonin in mammals [27], and ADH activity has been reported within the gastrointestinal tract [19, 28]. However, a number of bacteria of the gut microflora possess ADH activity [29] and, even though the tissue

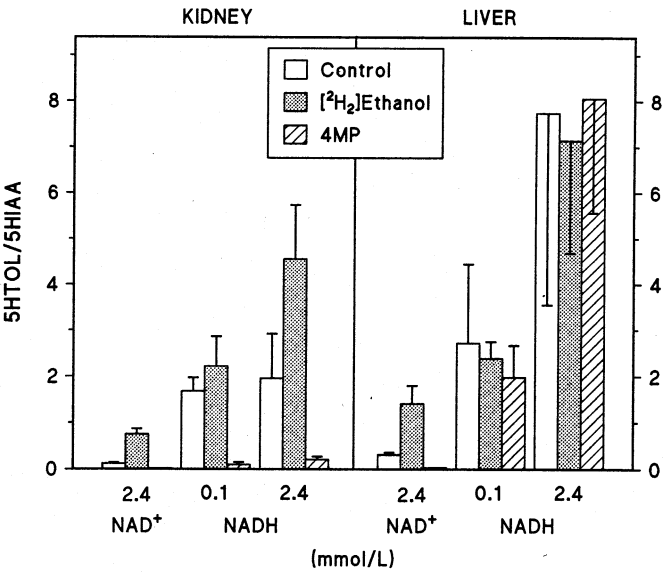

FIG. 4. The 5HTOL/5HIAA ratios determined in kidney and liver homogenates incubated with exogenous NADH. Results are mean values ± SD of 3 experiments.

samples were washed extensively prior to homogenisation, it cannot be excluded that the relatively high formation of 5HTOL in colon and caecum was, to some extent, catalysed by bacterial enzymes. Considering the high ADH activity in rat liver demonstrated in this and previous studies [30], the incorporation of deuterium into 5HTOL was lower than expected. However, when liver homogenates were incubated in the presence of extra NAD^+, the relative amount of [^{2}H]5HTOL increased sharply. Apparently, the concentration of coenzyme in the liver homogenates was not sufficient for these reactions to proceed at a maximal rate. In contrast, extra NAD^+ caused a much less pronounced increase in [^{2}H]5HTOL formation with kidney homogenates. When similar incubations were performed with an excess of NADH, the relative amount of [^{2}H]5HTOL dropped considerably, due to the competition for available deuterium. Here, it should be noted that tissue homogenates are complex systems, and the amounts of NAD^+ and NADH are probably different from the *in vivo* situation, where coenzymes are continuously regenerated. Thus, although the present results confirm that an interaction between ethanol and serotonin metabolism may take place in several tissues besides the liver, it is difficult to assess the relative importance of each tissue in this metabolic interaction.

The interaction between ethanol and serotonin metabolism can also be established as an increased 5HTOL/5HIAA ratio, because of the inverse relationship between 5HTOL and 5HIAA formation [1, 6]. In this study, however, a significant shift in the proportion of serotonin metabolites caused by ethanol was only demonstrated in incubations with kidney and liver homogenates. Likewise, a significant increase in 5HTOL formation was observed in kidney and liver, whereas a concomitant decrease in 5HIAA formation was only found in kidney. In the presence of 4MP, there was a general, albeit insignificant, tendency towards an increased formation of 5HIAA. This is attributed to the inhibition of ADH activity, which will favour oxidation of 5HIAL by ALDH (which is not inhibited by 4MP) into 5HIAA. The ADH activity in spleen was very low and not affected by 4MP. Previous studies found a high serotonin content in spleen [12, 31], but this was probably due to the fact that platelets, which contain high concentrations of serotonin, are stored within this organ. The demonstration in this study of a low incorporation of deuterium into 5HTOL in spleen homogenates supports previous suggestions of a low serotonin turnover.

No ADH activity was observed in rat brain with either 5HIAL or 5HTOL as substrates. This is in concordance with the fact that brain does not possess class I ADH [19, 32]. The concentration of 5HTOL in whole brain from control rats was ~200 pmol/g (results not shown), which is about 3-fold higher than previously reported [11]. Despite the lack of reduction of 5HIAL via the NADH-dependent ADH pathway as demonstrated in this study, addition of 5HIAL increased the 5HTOL concentration 5- to 6-fold. It should be noted that in brain the enzymatic conversion of 5HIAL to 5HTOL may also be mediated by NADPH-dependent aldehyde reductase (ALR; EC 1.1.1.2) [3, 33]. However, this metabolic pathway is only marginally affected by the transfer of deuterium [34], which agrees with the lack of deuterium incorporation into 5HTOL observed in brain. Accordingly, ALR activity, if present, did not affect the formation of deuterated 5HTOL in peripheral tissues. One *in vivo* study in rats using brain microdialysis observed an almost twofold increase in 5HTOL concentration following ethanol administration [35]. Still, this observation does not necessarily imply an increased 5HTOL formation within the brain, because free (unconjugated) 5HTOL is lipophilic and could possibly be distributed across the blood–brain barrier.

In conclusion, the present study demonstrates that ethanol oxidation increases the reduction of 5HIAL via NADH-dependent ADH to form 5HTOL in rat tissue homogenates. Transfer of deuterium from [$^2H_2$]ethanol over NADH to 5HTOL occurred in several tissues besides the liver, which is the main tissue for ethanol detoxification, thus demonstrating an extrahepatic interaction between ethanol and serotonin metabolism via ADH. These observations may have implications for the understanding of this metabolic interaction in man, because previous observations indicated that the site of this interaction is, to some degree, confined to tissues other than the liver, for example, the gastrointestinal tract [6].

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