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Acetaldehyde Mediation in the Mechanism of Ethanol-Induced Changes in Norepinephrine Metabolism

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SUMMARY

The role of acetaldehyde in reducing the oxidative pathway and increasing the reductive route for norepinephrine (NE) metabolism was evaluated. Experiments were performed in which ^{14}C-NE was injected into rats and its metabolic fate *in vivo* was monitored in the urine. Ethanol administration failed to alter the biochemical disposition of this amine, whereas, acetaldehyde produced profound aberrations in NE metabolism. The excretion of ^{14}C-vanillylmandelic acid and dihydroxymandelic acid was decreased as a result of treatment with acetaldehyde, disulfiram, or calcium carbimide. A concomitant increase in labeled 3-methoxy-4-hydroxyphenylglycol was also observed with these treatments. Administration of ethanol to animals previously treated with disulfiram or calcium carbimide potentiated this effect and resulted in elevated acetaldehyde blood levels as measured by a specific gas chromatographic procedure. These findings confirm the hypothesis that competitive inhibition of aldehyde dehydrogenase by acetaldehyde is the quantitatively important mechansim in the genesis of altered neuroamine metabolism by ethanol.

INTRODUCTION

Ethanol has been reported to produce a pronounced decrease in the endogenous output of 5-hydroxyindoleacetic acid (1) and 3-methoxy-4-hydroxymandelic acid in the urine of man (2) and dogs (3). Studies using ^{14}C-serotonin (4–6) and ^{14}C-norepinephrine (7, 8) also revealed a decreased excretion of 5-hydroxyindoleacetic acid and 3-methoxy-4-hydroxymandelic acid, respectively, after ethanol treatment. These changes in the oxidative pathways for the biogenic amines were accompanied by an increased excretion of labeled 5-hydroxytryptophol (6) and endogenous (2) or labeled (7, 8) 3-methoxy-4-hydroxyphenylglycol.

Similar alterations in the metabolism of serotonin have been shown to occur in liver homogenates from ethanol-treated rats to which substrate was added (9), but could not be shown to occur in rat brain after intracaudate injection of ^{14}C-serotonin to ethanol-treated rats (10). Studies utilizing

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tissue slices showed no effect of ethanol on the formation of 5-hydroxyindoleacetic acid or 5-hydroxytryptophol in brain slices, whereas a marked change occurred in serotonin metabolism when liver slices were used (11).

The precise mechanisms involved in the genesis of metabolic alterations in biogenic amines evoked by alcohol are still unsettled, although various suggestions have been proposed to explain these findings. These effects have been attributed to a blockade of release of biogenic amines (3), an inhibition of monoamine oxidase (1), a depletion of NAD with a resultant increased NADH:NAD ratio due to the oxidation of ethanol (4, 7), and, finally, a competitive interaction between acetaldehyde derived from ethanol and the intermediate biogenic aldehydes for the active site on aldehyde dehydrogenase (4, 7). Recently, evidence for the latter hypothesis has received experimental support *in vitro* (12, 13). It was shown that acetaldehyde alone, even in the presence of excess NAD, caused a decrease in the formation of ^{14}C-5-hydroxyindoleacetic acid, with a simultaneous increase in the neutral fraction in rat liver homogenates (12). The same workers demonstrated a competitive interaction between acetaldehyde and 5-hydroxyindoleacetaldehyde, using a brain mitochondrial preparation. The K_i for acetaldehyde in brain was found to be 2.6×10^{-6} M, in agreement with results for bovine aldehyde dehydrogenase (14). In order to appraise the role of acetaldehyde in the mechanisms of alteration of biogenic amine metabolism *in vivo*, the effects of ethanol and acetaldehyde on the metabolic disposition of norepinephrine in rats were investigated.

MATERIALS AND METHODS

Gas Chromatography

Blood levels of acetaldehyde and ethanol were analyzed simultaneously by the gas chromatographic method of Duritz and Truitt (15). The original method was modified to avoid the problem of spontaneous production of acetaldehyde when ethanol is present (16). It was found that obtaining a protein-free filtrate of whole blood would eliminate these artifactual increases in acetaldehyde (16).

For blood analyses, 76 male Wistar rats were used. The animals were decapitated, and their blood was collected in heparinized tubes. No significant difference was found between blood samples obtained by this procedure and samples taken by cardiac puncture. Two milliliters of whole blood were added to 1 ml of ice-cold 0.9% NaCl and 0.5 ml of 5% $ZnSO_4$ contained in a rubber-stoppered plastic centrifuge tube. After the blood had been added, 0.5 ml of 3 N $Ba(OH)_2$ was added through the cap with a syringe. The precipitated samples were centrifuged at 4° at 1500 × *g* for 10 min.

For determinations of acetaldehyde in protein-free filtrates, duplicate 0.4-ml samples of the clear filtrate above the packed, precipitated red cells were transferred to stoppered 5-ml vials which contained 0.4 g of dry NaCl. Vials were then placed in a water bath at 55° and equilibrated for 15 min before analysis of the head space gas. Inclusion of NaCl in the vials more than doubles the sensitivity of the method and gives excellent replication for submicrogram quantities of acetaldehyde.

Recovery was measured by adding known amounts of acetaldehyde and ethanol to the blood and processing these samples as described above. The mean recovery (± standard error) for acetaldehyde with rat blood was 60.3 ± 4.3%, and for ethanol it was 104.2 ± 5.9%, in protein-free filtrates compared with standards prepared in 0.9% NaCl. In all experiments reported in this paper, protein-free filtrates were used and corrected to blood levels.

Administration of ^{14}C-Norepinephrine and Sample Collection

dl-Norepinephrine (carbinol-^{14}C) bitartrate with a specific activity of 31.0 mCi/mmole was purchased from Amersham/Searle Corporation. Vials of 50 μCi were diluted to 2.5 ml, yielding a concentration of 1 μCi/50 μl. Aliquots were stored at −20°.

Thirty-six male Wistar rats, weighing 250–310 g, were fasted overnight before receiving 50 μl (1.0 μCi) of ^{14}C-norepinephrine, via the tail vein, at zero time. The animals were immediately placed in individ-

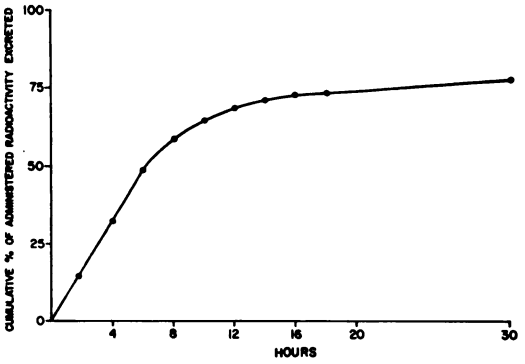

FIG. 1. *Cumulative excretion of ^{14}C in urine of rats following intravenous injection of 1.0 μCi of ^{14}C-norepinephrine*

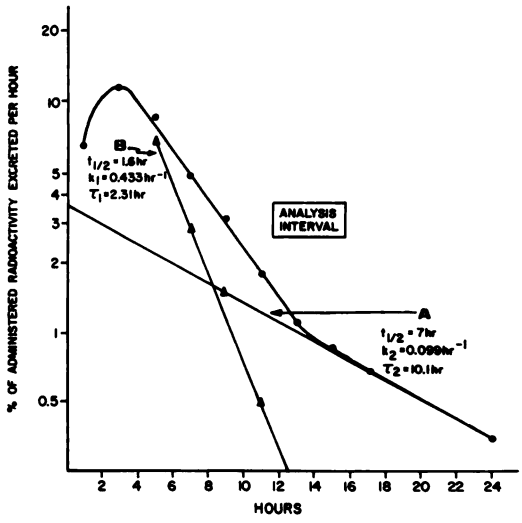

FIG. 2. *Rate of urinary excretion of ^{14}C following administration of ^{14}C-norepinephrine intravenously*

The total radioactivity excreted during the collection interval is expressed as a percentage of the administered dose excreted per hour. The means of six experiments have been plotted at the midpoint of the collection intervals (●——●). Curve *A* represents a tangent to the slow exponential process extrapolated back to zero time (——). Curve *B* represents the difference between curve *A* and the actual data (▲——▲).

ual metabolism cages for the collection of urine samples. Urine was collected at 2-hr intervals for 18 hr and then continuously for another 12 hr. All animals were given an oral dose of 10 ml of a 2% glucose solution at the beginning of each collection period. A 5-ml dose was also given to each animal 30 min prior to the injection of radioactivity. Urine was collected in polyethylene bottles which contained 25 mg of ascorbic acid, 50 mg of EDTA, and 0.5 ml of a "pool" of norepinephrine and its five major metabolites, each at a concentration of 100 μg/ml. For each collection period, the metabolism cage was thoroughly rinsed into the collecting vessel and the urine sample was diluted to 50 ml. This dilution resulted in a final concentration of "carrier" compounds of 1 μg/ml. From this sample, two 0.5-ml aliquots were withdrawn for total urine counts. For the analysis of metabolites, the 12–14-hr and 14–16-hr collections were pooled.

Isolation and Separation of Norepinephrine and Metabolites

The method used for the separation and identification of NE[2] and its five major metabolites was previously developed in one of our laboratories (7). All samples were analyzed by liquid scintillation spectrometry using a toluene–Triton X-100 (2:1) scintillation mixture.

Determination of ^{14}C-norepinephrine and normetanephrine. For the determination of total urine NE and NM in acid-hydrolyzed urine, four 5-ml aliquots of the pooled 12–16-hr urine samples were used for duplicate analyses.

Determination of deaminated ^{14}C-metabolites. For the determination of all deaminated metabolites, 65 ml of urine were hydrolyzed enzymatically by adjusting to pH 5.5; then 1 ml of Glusulase (Endo Laboratories) containing 100,000 units of β-glucuronidase and 50,000 units of sulfatase was added to each sample. The samples were then incubated in a water bath at 37° for 32 hr. For duplicate analysis, four 15-ml aliquots of hydrolyzed urine were used.

[2] The abbreviations used are: NE, norepinephrine; NM, normetanephrine; DHMA, 3,4-dihydroxymandelic acid; DHPG, 3,4-dihydroxyphenylglycol; VMA, 3-methoxy-4-hydroxymandelic acid (vanillylmandelic acid); MHPG, 3-methoxy-4-hydroxyphenylglycol.

Drug Treatment Schedules

One group of rats received ethanol (20%), 4 g/kg orally, at the beginning of the analysis interval, 12 hr after the administration of ^{14}C-norepinephrine. Another group was given three consecutive intraperitoneal injections of acetaldehyde (10%), 300 mg/kg. These animals were dosed at 12, 12½, and 13 hr. Other animals were first treated with an aldehyde dehydrogenase inhibitor, either disulfiram or calcium carbimide. Disulfiram was given as a 2% suspension by gavage at a dose of 200 mg/kg 20 hr and 4 hr prior to the analysis period, which represented 8 hr before and 8 hr after the administration of ^{14}C-norepinephrine. Some of the animals that received disulfiram were also given 4 g/kg of ethanol at the 12-hr time interval. Similarly, citrated calcium carbimide (Temposil) was administrated intraperitoneally at a dose of 50 mg/kg as a 1% suspension at 6 hr and again at 2 hr prior to the analysis period. Control animals were either treated with intraperitoneal injections of 0.9% NaCl, 3 ml/kg, or given this fluid orally, 16 ml/kg. All control values were combined, since the two routes of administration did not affect the results.

RESULTS

Kinetics of Excretion Radioactivity

After the injection of ^{14}C-NE, radioactivity was detected in the urine during the first 2-hr sample period (Fig. 1). Approximately two-thirds of the administered isotope (65%) appears in the urine within 10 hr

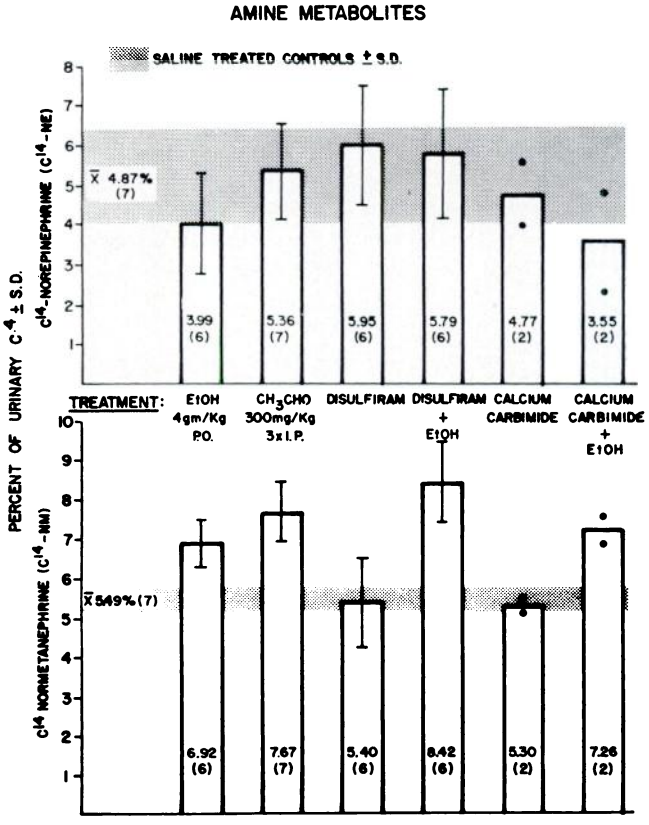

FIG. 3. *Effect of various drug treatments on proportion of norepinephrine and normetanephrine recovered in urine 12-16 hr after injection of 1.0 μCi of ^{14}C-NE intravenously.*

Values in bars represent mean percentages, and numbers in parentheses denote the number of animals used. The points in the calcium carbimide groups represent the actual values obtained in two experiments.

after injection. Another 12% appears in the urine during the succeeding 20 hr. This exponential excretion pattern is typical of that obtained when both optical isomers of NE are injected (17). It was desirable to analyze the urinary radioactivity representing the portion of ^{14}C-NE which mixed with the endogenous bound stores of this neurotransmitter (18, 19). A kinetic analysis of the excretion pattern of radioactivity is depicted in Fig. 2.

The radioactivity excreted during the initial period represents NE which was circulated, redistributed, and rapidly metabolized, and presumably comprises the bulk of the *d*-isomer (20). The radioactivity excreted during the slower phase represents ^{14}C-NE which became mixed with the endogenous pool of this catecholamine and was continually being released from tissues and metabolized (17, 18). From Fig. 2, it can be seen that curve *B* was constructed by subtracting curve *A* (slow exponential process) from the entire curve. Curve *B* represents the differential curve for the rapid process, which is essentially complete at about 12 hr. For this reason, the urine excreted between 12 and 16 hr after injection of the isotope was analyzed for the metabolites of norepinephrine.

Urinary Metabolites of ^{14}C-Norepinephrine

The metabolic pattern of ^{14}C-NE excretion during the analysis interval (12–16 hr) by control rats is represented by the shaded areas in Figs. 3–5. NE and NM account for a total of about 10% of the ^{14}C excreted during this time period. The deaminated catechol metabolites make up another 24% of the urinary radioactivity. Both DHMA and DHPG occur in about equal proportions. The *O*-methylated deaminated metabolites represent 48% of the radioactivity excreted; VMA, 21%; and MHPG, 27%. In the rat, the major metabolite of endogenous norepinephrine is MHPG (21), in contrast to man, in whom the major urinary metabolite is VMA (22).

Effect of Drug Treatments on Excretion of Amines

Figure 3 shows that the different drug treatments had little effect on the amount of ^{14}C-NE excreted during this time period. All the bar graphs overlap the control value ± standard duration. The effects of the various drug treatments on the excretion of ^{14}-C normetanephrine are illustrated ln the lower panel of the figure. Ethanol caused a significant increase in the amount of NM excreted ($p<0.05$). After administration of ethanol, NM excretion was increased 26% above the control level. The administration of acetaldehyde to the rats caused an even greater rise in the proportion of radioactivity excreted as NM (48% rise; $p<0.01$).

Treatment of rats with either of the two aldehyde dehydrogenase inhibitors, disulfiram or calcium carbimide, failed to alter the values obtained for NM excretion. However, when ethanol was administered to animals previously treated with these agents, a significant increase in NM excretion was observed. Treatment with disulfiram prior to the administration of ethanol produced a 62% rise in NM excretion ($p<0.01$). In a similar manner, treatment with calcium carbimide followed by the administration of ethanol increased the NM level by 37% above the control. The effects of these treatments on NM excretion parallel those on the blood levels of acetaldehyde (see Tables 1 and 2).

Deaminated Catechol Metabolites

Figure 4 illustrates the effects of these compounds on the excretion of the deaminated catechol metabolites DHMA and DHPG. Ethanol decreased the proportion of dihydroxymandelic acid from 12.16% to 8.71% of the total urinary radioactivity. Injection of acetaldehyde caused a slightly greater decrease, to 8.17% of the radioactivity excreted. Treatment with disulfiram or disulfiram plus ethanol depressed DHMA excretion to 41% or 63% of the control values, respectively. Calcium carbimide treatment lowered the level of this acid to 5.25% and, in the presence of ethanol, reduced it further, to 4.57% of the urinary radioactivity. These agents had little effect on the excretion of DHPG, which constituted around 12% of the ^{14}C excreted in the urine with all treatments.

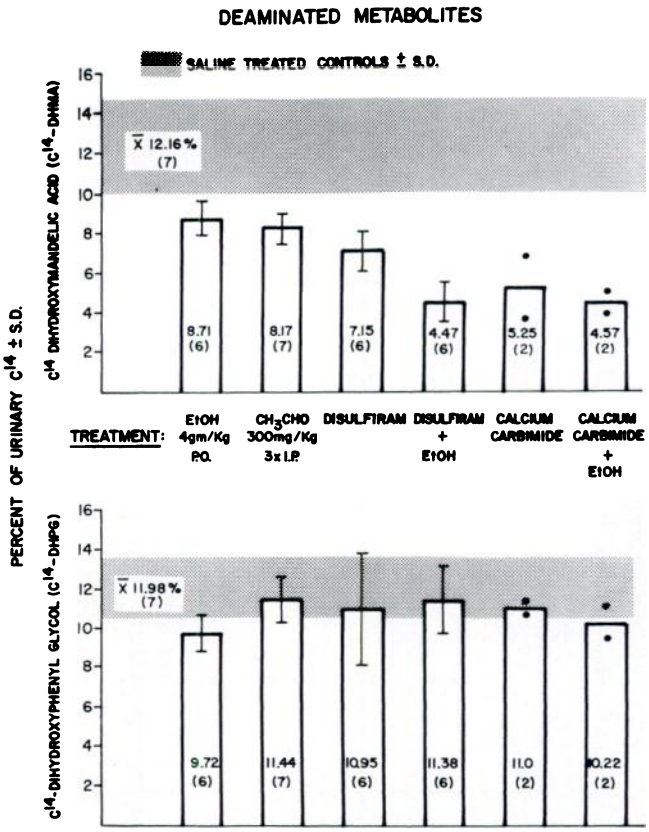

FIG. 4. *Effect of various drug treatments on proportion of deaminated catechol metabolites of NE (DHMA and DHPG) recovered in urine 12–16 hr after intravenous injection of 1.0 μCi of* ^{14}*C-NE*

Values in bars represent mean percentages, and numbers in parentheses denote the number of animals used. The points in the calcium carbimide groups represent the actual values obtained in two experiments.

O-Methylated Deaminated Metabolites

The effects of the drugs on the *O*-methylated deaminated metabolites are shown in Fig. 5. Ethanol in the rat failed to alter significantly the proportions of the two major urinary metabolites. However, administration of acetaldehyde lowered the amount of VMA from 21.0% to 11.8% of the total urinary activity ($p<0.001$). This 9.2% decrease in the acid metabolite was accompanied by a concomitant and equivalent rise in the amount of MHPG excreted (9.8% rise; $p<0.01$). Disulfiram caused VMA excretion to fall from 22.0% to 10.3% ($p<0.01$), while the glycol increased, from control levels of 25.7%, to 33.6% ($p<0.01$) of the ^{14}C excreted. Administration of ethanol to disulfiram-treated rats markedly potentiated this shift in metabolism. Under these conditions VMA fell to 6.25% ($p<0.001$) and MHPG rose to 36.5% ($p<0.001$) of the radioactivity excreted. Calcium carbimide by itself caused a similar shift from the oxidative metabolite (VMA) to the reductive product (MHPG). When ethanol was administered to animals given calcium carbimide, the alteration was more pronounced. It can be seen that administration of acetaldehyde, disulfiram, calcium carbimide, or either of the last two agents in the presence of ethanol caused a significant reduction in ^{14}C-VMA and a concomitant increase in ^{14}C-MHPG. Ethanol alone did not produce this shift.

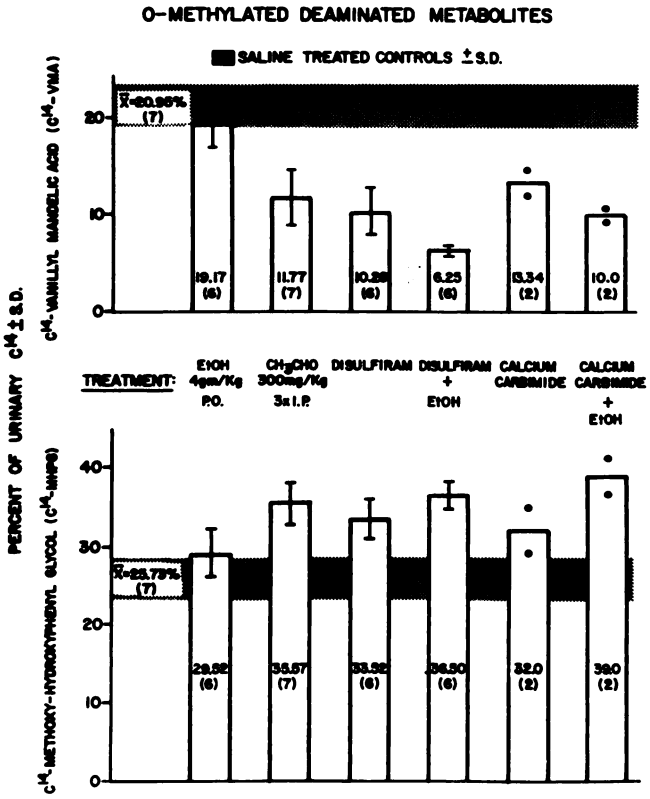

FIG. 5. *Effect of various drug treatments on proportion of O-methylated deaminated metabolites of NE (VMA and MHPG) recovered in urine 12–16 hr after intravenous injection of* ^{14}C*-NE*

Values in bars represent mean percentages, and numbers in parentheses denote the number of animals used. The points in the calcium carbimide groups represent the actual values obtained in two experiments.

Correlation with Blood Levels of Acetaldehyde

This shift in metabolism of norepinephrine from an oxidative route to a reductive one is well correlated with the amount of acetaldehyde present. In the rat, ethanol (4 g/kg) produces blood levels of acetaldehyde of 1.17 μg/ml and 2.65 μg/ml at 1 and 2 hr, respectively, after alcohol administration (Table 1). Experiments were also performed in which rats were given three doses of acetaldehyde (300 mg/kg intraperitoneally) at 30-min intervals and their blood levels were monitored (Table 2). The levels of acetaldehyde in these animals were at least 5–10 times higher than in ethanol-treated rats, and were comparable to those obtained when ethanol was given to disulfiram-treated rats. This regimen of acetaldehyde administration resulted in blood levels ranging from 9.71 to 19.69 μg/ml. Acetaldehyde blood levels after treatment with both ethanol and disulfiram ranged from 14.63 to 19.64 μg/ml (Table 1).

An important observation also shown in Table 2 is the production of ethanol *in vivo* when large amounts of acetaldehyde are injected. The ethanol levels parallel those of acetaldehyde at various times. The formation of ethanol from acetaldehyde demonstrates the reversibility of alcohol dehydrogenase in the intact organism. Furthermore, there was a significant increase in ethanol levels in disulfiram-treated rats compared to control animals ($p<0.0001$, Table 1). The elevation might have been due to the increased level of acetaldehyde, which could compete for the reverse reaction on the active site of alcohol dehydrogenase. This

TABLE 1

Acetaldehyde and ethanol blood levels in control and disulfiram-treated rats given ethanol

Ethanol, 4 g/kg, was administered orally to rats, and their blood levels of ethanol and acetaldehyde were analyzed by gas chromatography at 1 and 2 hr after ethanol administration to control or disulfiram- (200 mg/kg, orally, at 20 and 4 hr prior to ethanol) treated animals. Protein-free filtrates of whole blood were used for the determinations. Values have been corrected for recovery.

Treatment	No. of animals	Ethanol	Acetaldehyde
		μg/ml blood (± *SE*)	
Control			
1 hr	12	1970 ± 280	1.17 ± 0.36
2 hr	12	1930 ± 170	2.65 ± 0.32
Disulfiram			
1 hr	10	2920 ± 200	14.6 ± 1.2
2 hr	10	3430 ± 200	19.6 ± 2.8

TABLE 2

Acetaldehyde and ethanol blood levels in rats treated with acetaldehyde

Acetaldehyde, 300 mg/kg, was injected intraperitoneally at 30-min intervals into rats. Blood levels of ethanol and acetaldehyde were determined by gas chromatography. Protein-free filtrates of whole blood were used for the determinations. Values have been corrected for recovery

Time	Dose of acetaldehyde	Ethanol	Acetaldehyde
min	*mg/kg*	*μg/ml blood* (± *SE*)	
0	300		
15	0	45.0 ± 9.21	10.5 ± 2.2
30	300		
45	0	62.1 ± 6.32	19.7 ± 4.0
60	300		
120	0	8.83 ± 1.78	9.71 ± 0.98

might not only slow the oxidation of ethanol but also result in ethanol generation. This possibility is reasonable, since the equilibrium for this reaction lies far in the direction of ethanol production (23).

DISCUSSION

It has been speculated that the alteration of NE metabolism by ethanol in man might be attributed to a change in hepatic pyridine nucleotide levels as a result of ethanol oxidation (4–7). When ethanol and acetaldehyde are metabolized, there is a decrease of NAD in liver and a net production of NADH. Theoretically this excess of reduced coenzyme would favor the NADH-dependent reduction of the aldehyde intermediates of various amines at the expense of the oxidative pathway, which requires NAD-linked aldehyde dehydrogenase. The increase in NADH:NAD ratio as a result of ethanol oxidation has been shown repeatedly to occur in rat liver (24–31). Despite the known shift in coenzyme ratios in the rat, very large doses of ethanol did not modify the metabolism of ^{14}C-NE in this species. Yet, administration of acetaldehyde, disulfiram, calcium carbimide, or the latter two agents in the presence of ethanol caused a significant fall in ^{14}C-VMA excretion with a simultaneous diversion of the intermediate aldehyde to ^{14}C-MHPG. These findings could explain why other workers have failed to observe an effect of ethanol on neuroamine metabolism in brain either *in vivo* (10) or *in vitro* (11). Since ethanol administration did not affect the metabolism of peripherally administered ^{14}C-NE in the rat, no effect on centrally administered biogenic amines should be expected. In liver homogenates with high alcohol dehydrogenase activity, ethanol can evoke changes in amine metabolism (9, 11). In brain homogenates, however, ethanol would be unable to produce this effect, since only a limited amount of alcohol dehydrogenase is present (32) and the generation of acetaldehyde from ethanol would be minimal.

The current work has shown that acetaldehyde is the quantitatively important mediator of the ethanol-evoked alterations in monoamine metabolism. The lack of an effect of ethanol alone on norepinephrine metabolism in rats is contrary to the results of several studies in humans (2, 7, 8). On the other hand, disulfiram treatment markedly modified catecholamine metabolism in rats, and comparable effects have been reported for man (8). Therefore, drug-induced modifications in norepinephrine metabolic pathways can be produced in the rat *in vivo*, but not by ethanol administration. The ineffectiveness of ethanol in the rat coincides with the relative insensitivity of this species to the

pharmacological effects of alcohol. Our results indicate that it would be preferable to study the effects of alcohol in a species other than the rat, one that would more closely parallel man in his sensitivity to both the intoxicating and addicting effects of alcohol.

The failure of ethanol to shift the metabolism of NE from an oxidative to a reductive route in the intact rat may be related to the fact that the metabolic fate of NE is different in the rat than in man.

The acid metabolite (VMA) is the major excretion product in man, whereas the glycol metabolite (MHPG) is the predominant product in the rat. It is possible that the greater propensity of man to oxidize the aromatic aldehyde derivative to the acid metabolite may make this pathway in man more sensitive to the effects of agents which competitively inhibit the oxidation of the aldehyde. Moreover, the affinity of aldehyde dehydrogenase for acetaldehyde may be markedly different in the two species. Our results are in accordance with these possibilities, since modifications of NE metabolism in the rat were observed when high blood levels of acetaldehyde were achieved either by direct administration or by inhibition of the oxidation of acetaldehyde derived from ethanol.

It is concluded from this study that the genesis of the alteration in amine metabolism by ethanol in man is mediated primarily by the proximal metabolite of ethanol, acetaldehyde. The mechanism of this effect is most probably a competitive interaction between acetaldehyde and the aldehyde intermediate of the amine for the active site on aldehyde dehydrogenase (12, 13). That inhibition of the oxidative pathway could be involved in the shift in amine metabolism was further shown by the altered pattern when disulfiram or calcium carbimide, each a potent aldehyde dehydrogenase inhibitor *in vivo*, was administered to rats.

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