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Effect of alcohol and hexachlorobenzene administration on hepatic alcohol and xenobiotic metabolizing enzymes

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A variety of studies have shown that prolonged alcohol consumption may alter the hepatic activities of alcohol metabolizing enzymes including the microsomal ethanol oxidizing system (MEOS) [1–3]. Chronic treatment with alcohol increases the hepatic activities of other microsomal enzymes metabolizing xenobiotics and the hepatic content of microsomal components [4–7]. It is of particular interest that the formerly widely used fungicide hexachlorobenzene (HCB) exhibits characteristics similar to those of alcohol regarding enzyme-inducing properties of MEOS activity and of other microsomal functions in the liver [8].

In the present investigation the question was studied to what extent the combined treatment with alcohol and HCB may alter the hepatic activities of alcohol and xenobiotic metabolizing enzymes above the level achieved by either treatment alone.

Materials and methods

Materials. The chemicals were obtained from the following sources: ethanol 99.5%, Tris(hydroxymethyl)-aminomethane, potassium thiocyanate, nitric acid, potassium dihydrogen phosphate, disodium hydrogen phosphate 2-hydrate, perhydrol, sodium azide, semicarbazide hydrochloride, isocitrate dehydrogenase from pig heart, sodium dithionite, potassium chloride, magnesium chloride 2-hydrate cryst., from Merck Corp., Darmstadt, F.R.G.; glutathione reductase, α-oxoglutarate, glycylglycine, L-γ-glutamyl-3-carboxy-4-nitro-anilide, lactate dehydrogenase, L-aspartate, malate dehydrogenase, α-ketoglutarate, ADP, L-alanine, triethanolamine, ammonium acetate, *p*-nitrophenyl phosphate, NAD, NADH, NADP (disodium salt 98%), NADPH (tetrasodium salt 98%), isocitric dehydrogenase (grade II), 7-ethoxycoumarin from Boehringer Corp., Mannheim, F.R.G.; DL-isocitric acid Na_2-salt, Na_2-EDTA, cytochrome *c* from horse heart from Serva Corp., Heidelberg, F.R.G.; benzo(α)pyrene from Ferak Corp., Berlin, F.R.G.; 7-hydroxycoumarin from EGA-Chemie Corp., Heidelberg, F.R.G.

Alcohol feeding and treatment with hexachlorobenzene (HCB). Female Wistar rats (N = 64) were obtained from the Zentral-Institut für Versuchstierzucht Hannover (F.R.G.) and fed Altromin chow (Altromin Corp., Lage, F.R.G.) and tap water *ad libitum* until they reached a body weight of about 190–220 g. The animals were then housed in individual cages and group-fed with either of the following diets: (1) control diet, (2) alcohol diet, (3) control diet containing 0.02% HCB, and (4) alcohol diet containing 0.02% HCB. The control and alcohol diets are liquid and nutritionally adequate and were prepared as described by DeCarli and Lieber [9]. In the alcohol formula ethanol replaced carbohydrates to the extent of 36% of total calories. All four diets were given on an isocaloric basis for either 10 or 60 days.

Analytical procedures. After killing of the animals by decapitation the livers were perfused with ice-cold 0.15 mol/l KCl through the portal vein, excised and weighted. A 25% liver homogenate and washed liver microsomes as well as the cytosolic fraction were prepared as described previously [10]. The hepatic activity of catalase [11] and the hepatic content of cytochrome P-450 [12] were determined in the liver homogenate. The microsomal content of cytochrome P-450 and the activities of the microsomal ethanol-oxidizing system (MEOS) [10], NADPH-cytochrome *c* reductase [13], 7-ethoxycoumarin deethylase (7-EOC-D) [14], and arylhydrocarbon hydroxylase (AHH) [15] were assessed in washed liver microsomes. Microsomal losses during the preparative procedures were corrected as described previously [16]. The activity of alcohol dehydrogenase (ADH) was assayed in the cytosolic fraction of the hepatocytes [10]. The determination of protein was performed according to the method of Lowry *et al.* [17], using human albumin as standard.

Statistical analysis. The results are expressed as means ± S.E.M., and the significances of the differences were assessed by the Student *t*-test.

Results

Liver weight and body weight. Liver weight and body weight were not different between the animals fed the control diet and those on the alcohol diet irrespective of whether the diets were administered for 10 or 60 days (Table 1). Conversely, HCB treatment for 10 or 60 days with either control or alcohol diet significantly increased the liver weight compared to the treatment with the corresponding diet in absence of HCB.

Alcohol dehydrogenase (ADH). Compared to animals fed the control diet for 10 days, there were no significant differences of the hepatic ADH activities after treatment with either the alcohol diet or with HCB or with HCB plus alcohol diet (Table 2). Similar results were obtained after 60 days of treatments with the various regimes for hepatic ADH when the activity was expressed per mg cytosolic protein. There was, however, a significant increase of hepatic ADH activity per 100 g of body weight after HCB treatment with either the control or alcohol diet in comparison to animals treated with the corresponding diet in the absence of HCB.

Microsomal ethanol-oxidizing system (MEOS). After treatment with the alcohol diet lasting 10 days, HCB or the combination of both the hepatic activity of MEOS remained unaffected with the exception of a significant increase of the specific activity per mg of microsomal protein after the alcohol diet compared to the control diet (Table 2). The treatment for 60 days resulted in a significant enhancement of MEOS activity with the alcohol diet compared to the control diet. HCB application in animals treated with the control diet resulted in a signficant fall of specific activity of MEOS per mg of microsomal protein and in a striking rise of enzymatic activity per 100 g of body weight when compared to animals on the control diet alone. After treatment with HCB plus the alcohol diet there was a significant decrease of the hepatic activity of MEOS per mg of microsomal protein but not per 100 g of body weight when compared to animals fed the alcohol diet alone.

Catalase. There was little if any change of hepatic catalase activity after the various treatments for 10 days (Table 2). Similar results were obtained for the enzymic activity after prolonged treatment for 60 days compared to the animals receiving the control diet. However, treatment for 60 days with HCB and the control diet resulted in a significant fall of hepatic catalase activity in comparison to animals on the control diet alone. Similarly, in alcohol fed animals treated with HCB for 60 days there was a significant reduction of hepatic catalase activity per g of liver protein but not per 100 g of body weight when compared to animals on the alcohol diet only.

Cytochrome P-450. The hepatic microsomal content of cytochrome P-450 was augmented following 10 and 60 days

Table 1. Effect of alcohol and hexachlorobenzene (HCB) feeding for 10 or 60 days on liver weight and body weight

	Control diet	Alcohol diet	HCB + control diet	HCB + alcohol diet
10 days treatment				
Liver weight (g)	5.8 ± 0.1	6.1 ± 0.3	7.9 ± 0.2*	7.8 ± 0.2*
(g/100 g b.w.)	3.1 ± 0.1	3.3 ± 0.1	4.2 ± 0.1*	4.2 ± 0.1*
Body weight (g)	187.0 ± 2.0	185.0 ± 3.0	188.0 ± 3.0	186.0 ± 2.0
60 days treatment				
Liver weight (g)	6.2 ± 0.2	6.3 ± 0.1	11.9 ± 0.4*	12.7 ± 0.9*
(g/100 g b.w.)	2.9 ± 0.1	2.9 ± 0.1	5.4 ± 0.4*	5.7 ± 1.3*
Body weight (g)	216.0 ± 3.0	219.0 ± 5.0	225.0 ± 11.0	225.0 ± 6.0

The data are derived from 8 animals of each experimental group and are expressed, when indicated, per 100 g of body weight. The results are given as means ($\bar{x}$ ± S.E.M.). The statistical analysis compares the alcohol or the HCB treatment with the control diet and the alcohol/HCB diet with the alcohol treatment.

* $P < 0.001$.

of treatment with the alcohol diet compared to the control diet (Table 3). HCB treatment for 10 days increased the hepatic content of cytochrome P-450 irrespective whether administered together with the control or alcohol diet. Compared to animals on the control diet, the application of HCB for 60 days with the control diet significantly increased the hepatic content of cytochrome P-450 per 100 g of body weight but not per mg of microsomal protein. The hepatic cytochrome P-450 content per mg of microsomal protein was found to be significantly reduced after treatment with HCB and the alcohol diet for 60 days compared to the alcohol treatment alone.

NADPH-cytochrome c *reductase.* The activity of the hepatic microsomal NADPH-cytochrome *c* reductase was not significantly influenced by alcohol feeding for either 10 or 60 days when compared to the treatment with the control diet (Table 3). HCB application caused some increase of NADPH-cytochrome *c* reductase activity with either the control or alcohol diet administered for 10 or 60 days.

7-Ethoxycoumarin deethylase (7-EOC-D). The activity of the hepatic microsomal 7-EOC-D was significantly enhanced following the application of the alcohol diet for either 10 or 60 days compared to the control diet (Table 3). A further increase of enzymic activity was achieved by the combination of HCB treatment and alcohol feeding, whereas the results obtained with HCB treatment plus the control diet were intermediate.

Arylhydrocarbon hydroxylase (AHH). Compared to the control diet, the application of the alcohol diet left the hepatic activity of microsomal AHH virtually unchanged (Table 3). A striking increase of enzymic activity was observed following HCB treatment for either 10 or 60 days, and this HCB mediated enhancement was more pronounced when combined with the alcohol than with the control diet.

Turnover numbers. When microsomal enzyme activities were calculated per unit of cytochrome P-450 under the various experimental conditions, the resulting turnover numbers showed striking differences depending on the employed compound used for the assay and the particular treatment (Table 4).

Discussion

The present study shows that prolonged treatment with alcohol or HCB has variable effects on the hepatic activities of alcohol metabolizing enzymes (Table 2) and on the induction pattern of microsomal parameters (Table 3) without a consistent additive influence by the combination of the two treatments. This variability can be explained to a great extent by the existence of various isoenzymes of cytochrome P-450 exhibiting differences with regard to substrate specificity [18–23] and inducibility by various exogenous compounds [24–29].

A signficant increase of hepatic MEOS activity per mg of microsomal protein was found after 10 and 60 days of alcohol treatment (Table 2), and similar results have been reported for a treatment period in between [1, 2, 16, 30]. There was an associated enhancement of cytochrome P-450 content (Table 3) with similar turnover numbers of MEOS activity/cytochrome P-450 content after alcohol treatment for 10 days and with increased values after 60 days of treatment (Table 4). This enhancement after 60 days can be ascribed to a specific ethanol inducible form of cytochrome P-450 with an increased metabolic capacity towards ethanol [18, 19, 31–34].

The alcohol mediated increase of the specific activity of MEOS per mg of microsomal protein was reduced by the concomitant treatment with HCB for 60 days but was sustained by the combined treatment of only 10 days, whereas HCB alone had no consistent effect on the specific enzymic activity of MEOS (Table 2). These HCB mediated changes can be attributed to the fact that long-term application of HCB causes a so-called mixed type induction of cytochrome P-450 isoenzyme since a simultaneous increase of both the phenobarbital and benzo(α)pyrene type can be observed [25]. This is in line with the present finding that the turnover numbers for MEOS/cytochrome P-450 after HCB or HCB plus alcohol treatment are quite different from the corresponding values obtained with either the control or the alcohol diet alone (Table 4).

The participation of different cytochrome P-450 isoenzymes has also been shown for 7-EOC-D [21] and AHH [21–23] which are both increased in their activities after treatment with HCB alone or combined with alcohol, whereas the alcohol diet alone led to an increased activity of only the former enzyme but not of the latter one (Table 3). It was of particular interest that treatment with alcohol, HCB or the combination of both yielded quite different turnover numbers for 7-EOC-D/cytochrome P-450 and AHH/cytochrome P-450 compared to MEOS/cytochrome P-450 (Table 4), findings in line with the presence of various isoenzymes of cytochrome P-450 [21–23, 32, 34]. This study also demonstrates that the hepatic activity of microsomal NADPH-cytochrome *c* reductase as another component of the microsomal xenobiotic metabolizing enzyme system [8, 21, 23] exhibited no consistent induction pattern in

Table 2. Effect of alcohol and hexachlorobenzene (HCB) feeding for 10 or 60 days on the activities of hepatic alcohol metabolizing enzymes. For experimental details see legend to Table 1

	Control diet	Alcohol diet	HCB + control diet	HCB + alcohol diet
10 days treatment				
Alcohol dehydrogenase				
(nmoles NADH/min/mg cytosolic protein)	3.5 ± 0.4	3.6 ± 0.2	3.5 ± 0.3	3.4 ± 0.1
(nmoles NADH/min/100 g b.w.)	828.6 ± 93.6	876.9 ± 66.6	1055.0 ± 90.4	998.2 ± 33.8
Microsomal ethanol-oxidizing system				
(nmoles acetaldehyde/min/mg microsomal protein)	8.1 ± 0.6	13.3 ± 1.6**	9.3 ± 1.4	13.5 ± 1.6
(nmoles acetaldehyde/min/100 g b.w.)	1052.7 ± 50.5	1371.8 ± 249.2	1266.6 ± 189.3	1761.3 ± 196.9
Catalase				
($U \times 10^3$/g liver protein)	13.5 ± 2.3	20.1 ± 2.2	19.2 ± 1.3*	23.7 ± 2.1
($U \times 10^3$/100 g b.w.)	6.2 ± 0.6	8.1 ± 1.2	7.9 ± 0.3*	9.5 ± 0.8
60 days treatment				
Alcohol dehydrogenase				
(nmoles NADH/min/mg cytosolic protein)	2.8 ± 0.2	2.5 ± 0.2	2.5 ± 0.2	2.6 ± 0.4
(nmoles NADH/min/100 g b.w.)	604.0 ± 33.1	533.7 ± 35.7	1019.5 ± 83.4***	1014.4 ± 84.0***
Microsomal ethanol-oxidizing system				
(nmoles acetaldehyde/min/mg microsomal protein)	8.1 ± 0.4	17.5 ± 0.7***	5.4 ± 0.6**	7.0 ± 0.6***
(nmoles acetaldehyde/min/100 g b.w.)	915.3 ± 61.4	2118.6 ± 210.3***	2958.7 ± 534.9**	2459.3 ± 395.3
Catalase				
($U \times 10^3$/g liver protein)	23.3 ± 2.7	25.0 ± 2.9	8.4 ± 0.9***	9.9 ± 1.5**
($U \times 10^3$/100 g b.w.)	7.3 ± 1.1	7.3 ± 0.7	4.1 ± 0.5*	5.4 ± 1.1

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

Table 3. Effect of alcohol and hexachlorobenzene (HCB) feeding for 10 or 60 days on hepatic microsomal parameters. For experimental details see legend to Table 1

	Control diet	Alcohol diet	HCB + control diet	HCB + alcohol diet
10 days treatment				
Cytochrome P-450				
(nmoles/mg microsomal protein)	0.5 ± 0.05	0.8 ± 0.04***	1.2 ± 0.08***	1.5 ± 0.1***
(nmoles/100 g b.w.)	33.9 ± 3.8	66.9 ± 3.2***	118.8 ± 10.5***	168.0 ± 14.0***
NADPH-cytochrome *c* reductase				
(nmoles/min/mg microsomal protein)	127 ± 12	142 ± 6	208 ± 17*	228 ± 12***
(nmoles/min/100 g b.w.)	16,572 ± 1439	14,499 ± 1968	30,204 ± 4413*	30,186 ± 2513**
7-Ethoxycoumarin deethylase				
(nmoles/min/mg microsomal protein)	0.9 ± 0.1	2.0 ± 0.2***	6.3 ± 0.7***	7.8 ± 0.5***
(nmoles/min/100 g b.w.)	63.3 ± 7.2	155.5 ± 14.6***	620.7 ± 73.5***	856.5 ± 82.7***
Arylhydrocarbon hydroxylase				
(nmoles/20 min/mg microsomal protein)	5.0 ± 0.5	5.6 ± 0.7	60.5 ± 5.9***	63.8 ± 5.4***
(nmoles/20 min/100 g b.w.)	333.7 ± 37.1	436.5 ± 41.4	5862.3 ± 573.8***	6938.1 ± 689.2***
60 days treatment				
Cytochrome P-450				
(nmoles/mg microsomal protein)	0.6 ± 0.03	1.0 ± 0.04***	0.6 ± 0.06	0.7 ± 0.04***
(nmoles/100 g b.w.)	38.7 ± 1.3	82.9 ± 3.7***	82.1 ± 10.3***	106.6 ± 10.8
NADPH-cytochrome *c* reductase				
(nmoles/min/mg microsomal protein)	128 ± 10	139 ± 12	195 ± 49***	198 ± 26***
(nmoles/min/100 g b.w.)	14,581 ± 1519	16,706 ± 1945	106,386 ± 17,615**	73,785 ± 13,963**
7-Ethoxycoumarin deethylase				
(nmoles/min/mg microsomal protein)	0.6 ± 0.04	1.6 ± 0.2***	2.5 ± 0.3***	3.0 ± 0.3**
(nmoles/min/100 g b.w.)	42.3 ± 2.6	128.0 ± 13.4***	374.6 ± 50.0***	475.0 ± 85.0***
Arylhydrocarbon hydroxylase				
(nmoles/20 min/mg microsomal protein)	6.9 ± 0.4	7.3 ± 0.6	35.4 ± 4.1***	47.9 ± 3.5***
(nmoles/20 min/100 g b.w.)	458.4 ± 32.3	589.1 ± 56.8	5362.3 ± 783.8***	6809.4 ± 1380.3***

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

Table 4. Effect of alcohol and hexachlorobenzene (HCB) feeding for 10 or 60 days on turnover numbers for cytochrome P-450

	Control diet	Alcohol diet	HCB + control diet	HCB + alcohol diet
10 days treatment				
MEOS/P-450	18.1 ± 2.9	15.9 ± 1.9	7.8 ± 0.9**	8.8 ± 0.9**
7-EOC-D/P-450	2.2 ± 0.5	2.3 ± 0.2	5.1 ± 0.3***	5.1 ± 0.3***
AHH/P-450	11.0 ± 2.0	6.6 ± 0.6	50.0 ± 3.6***	42.9 ± 4.5***
60 days treatment				
MEOS/P-450	13.9 ± 1.0	16.9 ± 0.5*	9.9 ± 0.5**	10.2 ± 1.2***
7-EOC-D/P-450	1.1 ± 0.1	1.5 ± 0.1*	4.6 ± 0.4***	4.3 ± 0.3***
AHH/P-450	11.8 ± 0.8	7.1 ± 0.6***	65.3 ± 5.5***	62.4 ± 8.7***

The calculations are based on the activity of the microsomal ethanol-oxidizing system (MEOS, nmoles/min/mg microsomal protein), 7-ethoxycoumarin deethylase (7-EOC-D, nmoles/min/mg microsomal protein) and arylhydrocarbon hydroxylase (AHH, nmoles/20 min/mg microsomal protein) each per nmole cytochrome P-450/mg microsomal protein.

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

relation to the other microsomal enzymes (Tables 2 and 3), thereby substantiating that the degree of microsomal metabolism of exogenous compounds depends on the cytochrome P-450 isoenzymes rather than on the reductase.

Alcohol consumption for 10 or 60 days had little if any influence on hepatic ADH and catalase activities (Table 2) in accordance with previous studies [2]. HCB alone or combined with the alcohol diet administered for 60 days increased ADH activities per 100 g of body weight (Table 2) due to the striking rise of the liver weight (Table 1). The hemoprotein catalase was reduced in its activity after HCB and HCB plus alcohol diet for 60 days (Table 2) under conditions of an increased hepatic content of the hemoprotein cytochrome P-450 (Table 3), which may reflect competition of both hemoproteins for heme as an important precursor for their synthesis.

In conclusion, alcohol and HCB show variable effects on the hepatic alcohol metabolizing enzymes and the induction pattern of microsomal parameters which can be ascribed at least in part to the induction of various isoenzymes of cytochrome P-450.

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Hepatic glutathione release upon decreases of extracellular calcium concentration*

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Recently, the hepatic release of GSH across the sinusoidal plasma membrane [1, 2] was found to be substantially stimulated upon the addition of hormones such as vasopressin or phenylephrine [3]. As shown in Table 1, the thiol release is about 23 nmol/min per g wet wt in the control state, and is increased to 33 or 38 nmol/min per g, respectively. Since the mechanism of action of such hormones includes calcium transients, it was of interest to examine the effects of calcium movements themselves. Therefore, the effects of the calcium ionophore, A23187 and the effects of manipulating extracellular calcium were studied; the latter involved variations in the calcium concentration as such or by the titration with EGTA.

Livers from male Wistar rats (200–500 g body wt), fed on stock diet, were perfused as previously [3]. Thiol concentration and GSH concentration as well as pH and pO_2 were followed as previously [3, 4].

Figure 1 demonstrates a significant decrease in hepatic thiol release upon the addition of A23187, a calcium ionophore. It is generally accepted that the ionophore elicits calcium transients, and in the presence of the physiological

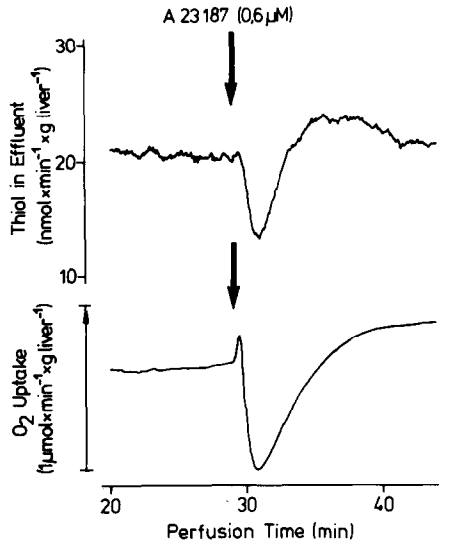

Fig. 1. A23187 induced decrease in thiol release (top) and O_2. A23187 was infused for 20 sec and corresponds to a supply of 7.4 nmol/liver. Standard perfusion condition, i.e. calcium ion concentration was 1.25 mM.

Table 1. Thiol release from perfused rat liver

Addition	Thiol release µM	Thiol release (nmol/min per g liver wet wt)
None (12)	4.9 ± 0.2	23 ± 1
Vasopressin, 12 nM (6)	7.1 ± 0.2	33 ± 1
Phenylephrine, 1.6 µM (3)	7.3 ± 0.4	38 ± 1
plus prazosin, 3 µM (2)	4.1	22
Glucagon, 11 nM (3)	6.0 ± 0.4	27 ± 2
Angiotensin II, 14 nM (3)	7.7 ± 0.4	39 ± 3
PAF, 13 nM (3)	3.6 ± 0.1	14 ± 0.3
A23187, 0.4 µM (4)	3.7 ± 0.2	14 ± 0.7
Calcium omission (4)	12.4 ± 0.5	51 ± 2
EGTA, 1.5 mM (3)	12.0 ± 0.5	54 ± 2

extracellular concentration of 1.2 mM Ca^{2+} there is an influx of Ca^{2+} across the sinusoidal plasma membrane into the cells. Thus, it appears that during the net flux of calcium into the cells there is a restriction of thiol efflux.

The reverse, e.g. an efflux of calcium from the cells, can be achieved by decreasing the calcium concentration in the extracellular space. As shown in Fig. 2, the omission of calcium in the entering perfusate is accompanied by a substantial increase in both thiol and GSH release, the rates being even higher than those obtained with vasopressin or phenylephrine (Table 1). There is no cell damage, as indicated by the lack of a significant leakage of lactate dehydrogenase in the effluent perfusate (1 mU/min per g liver) before calcium omission and until 34 min, the time point when the peak in thiol release has already occurred. The concentration dependence (Fig. 3A) shows that the thiol releasable by the omission of calcium is observed only

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