Table 3. Effects of different acidifiers on the free concentration of bupivacaine in human serum

	Free bupivacaine concentration (μg/ml)					
	Total concentration (μg/ml)					
	1.5	3.5	6.0	17.0	36.0	56.0
Normal	0.032[a]* ± 0.001	0.151[a] ± 0.030	0.635[a] ± 0.011	3.110[a] ± 0.047	10.848[a] ± 0.644	18.107[a] ± 1.156
CO_2	0.051[b] ± 0.007	0.194[b] ± 0.006	0.826[b] ± 0.025	4.172[b] ± 0.058	15.217[b] ± 1.268	24.897[b] ± 1.510
Lac	0.041[c] ± 0.006	0.180[c] ± 0.017	0.657[a] ± 0.098	3.790[c] ± 0.378	13.233[c] ± 0.544	22.372[c] ± 1.116
HCl	0.041[c] ± 0.006	0.135[d] ± 0.025	0.695[a] ± 0.018	5.402[d] ± 0.480	11.400[a] ± 1.047	21.357[c] ± 0.932

Each value is the mean ± S.D.
* Means with the same letter within the same total concentration range are not significantly different ($P < 0.05$).

increase in the free bupivacaine concentration at 1.5, 17 and 56 μg/ml, when compared with control serum. A significant ($P < 0.05$) decrease in the free bupivacaine concentration resulted at a total bupivacaine concentration of 3.5 μg/ml (Table 3).

In this study, the resulting free bupivacaine concentrations for both lactic and hydrochloric acid were significantly lower than those resulting with carbon dioxide. The increase of either of these two ions and/or the ionic concentrations, resulted in an increase or maintenance of the binding capacity for both the Class 1 and 2 binding sites despite the decrease in pH. The alteration in the overall serum binding capacity (net increase) due to the addition of hydrochloric or lactic acid resulted in a reduction in the magnitude of the *in vitro* pH effect. This explanation assumes that the addition of carbon dioxide represents only a pH effect.

In summary, the effect on the *in vitro* human serum bupivacaine binding was not solely dependent on the reduction of the serum pH. The magnitude of the effect was dependent on the acidifier used. Before *in vitro* protein binding data under acidic conditions can be extrapolated to that occurring *in vivo*, the effect of the acidifier used must be considered.

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Effects of γ-glutamyltranspeptidase inhibitor and reduced glutathione on renal acetaldehyde levels in rats

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Much attention has been directed to decreases in tissue-reduced glutatione (GSH) contents following ethanol (EtOH) administration [1–5]. This effect has been discussed in relation to a suppression of GSH synthesis [3] or to an accelerated utilization of GSH detoxifying lipid peroxides [1] or acetaldehyde (AcH), the first metabolite of EtOH [6]. Although Speisky *et al.* [5] concluded that the interaction between GSH and AcH is less important in the liver, the kidney possessing an extremely high γ-glutamyltranspeptidase (γ-GTP) activity [7] may relate to other events since both cysteinylglycine produced from GSH by γ-GTP action and further degradation product, cysteine, have high reactivities to complexes with AcH, non-enzymatically [8]. With a specific γ-GTP inhibitor, L-γ-glutamyl-(*O*-carboxy)-phenylhydrazide (L-OC) [9, 10], and exogenously administered GSH, the present *in vivo* study was carried out to elucidate if the non-enzymatic conjugation reaction between AcH and GSH degradation product(s) has a significant role in AcH and GSH metabolisms after EtOH intake.

Materials and methods

L-OC was obtained through the courtesy of Dr S. Minato, from Fermentation Research Laboratories, Sankyo Co.

Ltd. Male Wistar rats (145–155 g) fasted overnight were orally given 2 g/kg EtOH as a 20% solution in saline or isocaloric amount of glucose, between 9 and 10 a.m. L-OC and GSH dissolved in phosphate buffer pH 7.4 were injected intraperitoneally before and after EtOH ingestion. Blood samples were collected from the tip of the tail, into heparinized glass micropipettes (Clay Adams). One min after the last blood sampling, the animal was killed by a blow on the head and the liver and kidney immediately freeze-clamped [11]. After treatment of the blood and frozen tissue samples according to the methods of Eriksson *et al.* [11], EtOH and AcH were measured by the head-space gas chromatographic procedure [11, 12].

From the same frozen tissue, a 10% homogenate was made with 0.6 N perchloric acid/6 mM EDTA. Non-proteinic free sulfhydryl (NPFSH) content in this supernatant was estimated using 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) [13]. Total glutathione (GSH + oxidized glutathione) was also determined by the glutathione reductase–DTNB method [14]. The changes in the total glutathione levels paralleled the NPFSH levels in this study.

A 10% homogenate was also obtained from the frozen tissue with 20 mM phosphate buffer pH 7.4 containing 1% Triton X-100. The supernatant (5000 *g* × 20 min) was analysed for alcohol and aldehyde dehydrogenase activities, spectrophotomerically, at 30° either sodium phosphate (pH 7.4) or sodium pyrophosphate buffer (pH 9.0) with 2 mM NAD and 2 μM rotenone. Assay medium for alcohol dehydrogenase contained 20 mM EtOH and 1 mM semicarbazide. For aldehyde dehydrogenase assay, two different concentrations of substrate (100 μM or 20 mM AcH), 0.1 mM pyrazole and 1 mM $MgCl_2$ were included. γ-GTP activity was measured spectrophotometrically using the 5000 *g* supernatant, as described by Meister *et al.* [15]. The direct assessment of inhibitory effects, using the L-OC treated animal tissue, yielded no useful information on the degree of inhibition, because of the competitive inhibition nature of L-OC on γ-GTP [9, 10]. However, more than 90% of the γ-GTP activity of both kidney and liver was inhibited by the addition of 0.45 mM L-OC to the *in vitro* assay system.

Results and discussion

The blood EtOH and AcH levels following 2 g/kg EtOH ingestion were examined, with or without pretreatment with 0.45 mmole/kg L-OC (Fig. 1). Although there was no significant difference in EtOH level and EtOH elimination rates, a gradual accumulation of AcH in the L-OC treated group was observed. When EtOH and AcH levels in the liver and kidney were compared 3 hr after EtOH ingestion [Table 1(A)], a significant change was observed only in the renal AcH content with a higher level double that of the control. Under the same conditions, measurements were also made of tissue NPFSH contents. NPFSH level was significantly reduced by EtOH administration alone (2.41 ± 0.09 for control vs 2.27 ± 0.12 μmole/g tissue for EtOH group, $P < 0.05$). More pronounced decreases in renal NPFSH level were observed by the L-OC treatment (1.92 ± 0.18 μmole/g, $P < 0.001$). However, EtOH administration to the L-OC pretreated animals did not lead to a further NPFSH depletion (1.86 ± 0.08 μmol/g). Contrary to the findings with the kidney, alterations in hepatic NPFSH and total glutathione levels were not detected, even with L-OC treatment. As 0.5 mmole/kg L-OC, a dose close to that used in our study, effectively inhibited γ-GTP activity *in vivo* [10, 16], and L-OC did not affect alcohol and aldehyde dehydrogenase activities *in vitro* and *in vivo* (data not shown), it is highly probable that the accelerated accumulation of AcH by L-OC is due to a reduced formation of AcH-reactive sulfhydryls through the γ-GTP inhibition.

Table 1. EtOH and AcH concentrations (mean ± S.D.) in tissues. (A) Animals given (N = 11) or not given (N = 13) L-OC treatment were killed 3 hr after EtOH administration. See Fig. 1 for details. (B) 1 mmole/kg GSH was given i.p. 75 min after 2 g/kg EtOH by intubation. Animals given (N = 7) or not given (N = 8) GSH treatment were killed 90 min after EtOH (i.e. 15 min after GSH).

			AcH (nmole/g)	EtOH (μmole/g)
(A)	Liver	Control	23.15 ± 9.36	23.11 ± 8.70
		L-OC	19.83 ± 10.28	26.02 ± 7.47
	Kidney	Control	12.47 ± 7.88	26.17 ± 8.91
		L-OC	28.55 ± 14.50*	29.07 ± 10.15
(B)	Liver	Control	27.15 ± 8.14	26.86 ± 2.14
		GSH	25.90 ± 5.38	28.77 ± 4.18
	Kidney	Control	15.75 ± 3.70	32.14 ± 3.10
		GSH	10.51 ± 2.87*	31.80 ± 4.71

* $P < 0.02$.

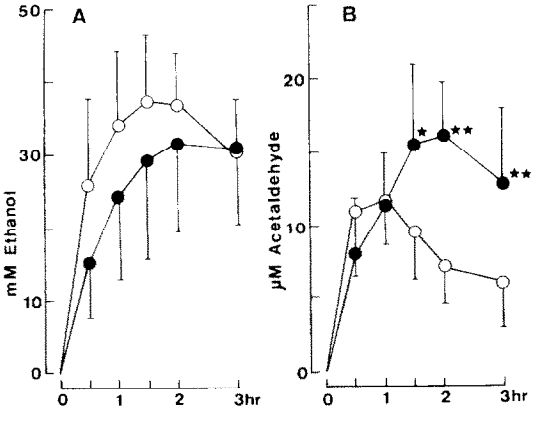

Fig. 1. Effect of L-OC on EtOH (A, mM) and AcH (B, μM) levels (mean ± S.D.) in blood. Rats were given 2 g/kg EtOH with (●, N = 11) or without (○, N = 13) i.p. injection of 0.45 m mole/kg L-OC 30 min before EtOH intubation. *$P < 0.05$, **$P < 0.005$.

To observe the effect of AcH level of GSH at its higher than normal levels, GSH was administered after EtOH ingestion. Since preliminary experiments showed that maximal NPFSH levels in blood were attained 15–45 min after i.p. injection of 1 mmole/kg GSH, and a relatively high concentration of both EtOH and AcH was maintained from 1 to 2 hr after 2 g/kg EtOH ingestion, GSH was administered 75 min after EtOH ingestion and tissue EtOH and AcH contents were measured after 90 min of EtOH. The only significant change was detected in kidney AcH levels [Table 1(B)], the value being 30% lower than the control group, and which was associated with the reduction of renal NPFSH level (Table 2). Although marked elevation of NPFSH content was noted in both liver and kidney 15 min after GSH injection, the reducing effect of EtOH on the NPFSH content was not evident in the liver. These results further support the view that γ-GTP may play some role in AcH clearance, by providing GSH degradation product(s) capable of reacting with AcH. Although tissue levels of cysteinylglycine, cysteine and their possible conjugation products with AcH, thiazolidinecarboxylic acid derivatives, were not determined, these GSH-derived sulfhydryls seem to be present in the kidney in levels sufficient to trap even low concentrations of AcH, because

Table 2. Effects of EtOH and GSH on tissue NPFSH levels (mean ± S.D.). Experimental conditions were the same as in Table 1(B). Number of animals is given in parenthesis

		Liver (μmole/g)	Kidney (μmole/g)
Glucose/buffer	(8)	3.27 ± 0.39	2.57 ± 0.22
EtOH/buffer	(8)	2.97 ± 0.54	2.22 ± 0.30*
Glucose/GSH	(8)	5.04 ± 0.46	4.72 ± 0.37
EtOH/GSH	(8)	4.87 ± 0.59	4.21 ± 0.44*

* $P < 0.05$.

the kidney contained 2–3 mM GSH (Table 2), and an extremely high activity of γ-GTP [7]. Renal L-cysteine and L-cysteinylglycine levels are considered to be in the range 10–100 μM [17], under normal conditions. Furthermore, a 4–7 times higher GSH level in rat aorta plasma than in renal vein plasma (due to the action of renal γ-GTP [16]) suggest the clearance of blood AcH in the kidney, despite the very low γ-GTP activity in the blood [18]. Thus, the accelerated accumulation of AcH in the blood as well as in the kidney by the L-OC treatment may be ascribed to the γ-GTP inhibition.

As already noted [4, 19], a lower dose (2 g/kg) of EtOH used in the present study led to no change in hepatic GSH content, but there were decreases in the renal NPFSH level as early as 90 min after EtOH intake. This reduced level was maintained during the intoxication period without acceleration of lipid peroxidation [4]. With a large dose (5 g/kg) of EtOH, a rapid fall in NPFSH followed by a progressive acceleration of lipid peroxidation in the kidney was distinct from alterations in the liver where these two phenomena gradually proceeded and appeared inversely related [4]. Although Speisky *et al.* [5] concluded that inhibition of GSH synthesis [3, 5] and the increased loss of GSH [5], which probably includes utilization of GSH for detoxifying lipid peroxides [1], are important in the hepatic GSH decrease after EtOH intake, such a rapid fall in renal NPFSH level may result from the conjugation reaction between AcH and GSH-derived sulfhydryls.

Taken together, the present results suggest that the conjugation mechanism between AcH and degradation product(s) of GSH contributes, at least in part, to AcH clearances in the blood and kidney and which also results in renal NPFSH (GSH) depletion during EtOH intoxication.

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